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PRODUCTION OF PHENYLPYRUVIC ACID AND PHYTASE BY MICROBIAL FERMENTATION

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ABSTRACT

Feed and food additives are very popular in industry to increase the quality of diets. They have been used for various applications such as nutritional quality enhancing, coloring, flavor enhancing, antimicrobial effects, thickening, stabilization, increasing shelf-life, and calorie reducing. Phenylpyruvic acid (PPA) and phytase are just two of the important feed and food additives.

PPA and phytase have important applications in the industry. PPA is being experimentally used as a substitute for phenylalanine in feeds to decrease excessive nitrogen accumulation in the manure to prevent negative effects on the environment. PPA is also used for kidney patient's diets to decrease urea accumulation in the body. Additionally, PPA is a very important compound for cheese and wine production to generate a specific taste and aroma. Phytase breaks down phytate, which serves as an anti-nutrient agent by binding divalent ions, amino acids, and proteins in the intestine. Also, phytase is an important additive in monogastric animals' diet to decrease excessive phosphorus accumulation in the manure, which may cause changing flora and fauna.

Therefore, the purpose of this research was to enhance PPA and phytase productions in bioreactors by optimizing growth and fermentation medium parameters, also conducting various fermentation modes. Additionally, the effect of two microparticles (talcum and aluminum oxide) on fungal phytase production was studied.

For PPA fermentation, strain selection was first performed using four microbial strains (*Morganella morganii, Zygosaccharomyces rouxii*, *Proteus vulgaris*, and *Corynebacterium glutamicum*) based on a literature search and *P. vulgaris* was selected as the best microorganism based on PPA production level in shake-flask fermentation, (358 mg/L). Thereafter, growth parameters such as temperature, pH, and aeration were optimized for PPA and biomass production in the bioreactors by using Response Surface Methodology (RSM). Optimum conditions were determined as 34.5°C, 5.12 pH, and 0.5 vvm aeration for PPA production and 36.9°C, pH 6.87, and 0.96 vvm aeration for biomass production. Under optimum conditions, 1054 mg/L PPA and 3.25 g/L biomass productions were obtained. ANOVA results showed that all of growth factors evaluated were significantly ($p<0.05$) effective on biomass production, however aeration was not a significant ($p<0.05$) factor for PPA production.

Additionally, concentration levels of fermentation medium ingredients such as glucose, yeast extract, and phenylalanine were optimized by RSM. The optimum ingredient concentrations for PPA production were determined as 119.4 g/L for glucose, 3.68 g/L for yeast extract, and 14.85 g/L for phenylalanine, whereas 163.8 g/L glucose, 10.75 g/L yeast extract, and 9.84 g/L phenylalanine were the best for biomass production. PPA and biomass concentrations increased to 1349 mg/L and 4.35 g/L, respectively, under these respective optimum conditions.

Furthermore, fed-batch and continuous fermentation were conducted to further enhance PPA production. It was determined that addition 4 g of phenylalanine into the reactor at 30 h of fermentation increased PPA concentration to 2958 mg/L. Also, PPA productivity was increased to 259 mg/L/h in continuous fermentations, which is almost 5 times higher compared to productivity in batch fermentation.

Additionally, PPA and biomass production, glucose and phenylalanine consumption by *P. vulgaris* were predicted with modified Gompertz and modified logistic models. The models over-predicted PPA and biomass productions however lower-predicted phenylalanine and glucose consumption data. The modified Gompertz model predicted the experimental data with lower root mean square error (RMSE) and mean average error (MAE) values compared to modified logistic model for all predictions.

For phytase fermentation, first phytase producer microorganisms were screened by evaluating four different strains (*Lactobacillus plantarum*, *Lactobacillus acidophilus*, *Lactobacillus amylovorus*, and *Aspergillus ficuum*) and *A. ficuum* was selected as the most productive strain among them. The highest phytase activity was measured as 1.02 U/ml in shake-flask fermentation. *A. ficcum* phytase activity increased to 2.27 U/ml in bioreactors by optimization of growth parameters. For phytase production these were determined to be 33°C, pH 4.5, and 0.9 vvm aeration and all the factors were significantly effective on phytase production.

Also, optimum pH and temperature values were determined as 5.5 and 55^{o}C, respectively for *A. ficuum* using a phytase assay. Additionally, the lowest phytase activity loss was seen under 4^{o}C storage conditions for 1 week. However, -20^{o}C storage conditions provided lower activity loss for *A. ficuum* phytase when storage was more than 1 week of storage period.

Thereafter, phytase fermentation medium ingredients such as glucose, Na-phytate, and $CaSO_4$ were optimized in the next study. Maximum phytase activity was measured as 3.45 U/ml with 126 g/L of glucose, 14 g/L of Na-phytate, and 1.1 g/L of $CaSO_4$.

Furthermore, the effect of glucose and phytate additions on phytase activity were evaluated in fed-batch fermentations. Maximum phytase activities were measured as 3.84, 4.63, and 4.83 U/ml, when 45 g of glucose, 5 g of Na-phytate, and 10 g of Na-phytate was added into the bioreactors, respectively, at 96 h of the fermentations. Also, continuous fermentation of phytase increased the productivity almost 7-fold compared to batch fermentation. Additionally, the effects of aluminum oxide and talcum were studied on phytase production with *A. ficuum*. It was reported that phytase activity remarkably increased by addition of 15 g/L of aluminum oxide and talcum to 2.01 and 2.93 U/ml, respectively, compared to the control (1.02 U/ml) in shake-flask fermentations. Moreover, microparticle addition provided small pellets and prevented bulk fungal growth in the fermentation broth. The highest phytase activity was measured as 6.49 U/ml by addition of 15 g/L of talcum in bioreactors at 96 h of fermentation. Also, the effect of talcum addition on fed-batch and continuous fermentations were studied. Phytase activity increased from 4.912 to 9.587 U/ml and from 3.3 to 6.3 U/ml in fed-batch and continuous fermentations, respectively, by addition of 15 g/L talcum. Moreover, maximum phytase productivity was measured as 0.621 U/ml/h at 0.1 h^{-1} dilution rate with 15 g/L of talcum added continuous fermentation.

Finally, phytase production and glucose consumption were predicted with modified Gompertz model and modified logistic model. Modified Gompertz model predicted phytase activity slightly more successfully compared to modified logistic model. Lower MAE and RMSE values were obtained for the prediction of glucose by the modified logistic model compared to the modified Gompertz model, however higher R^2 and slope values were obtained by the modified Gompertz model.

In conclusion, PPA production increased almost 10 fold in fed-batch studies compared to shake-flask fermentations. Similarly, phytase productions increased almost 5 and 10 fold in fed-batch and microparticle added fed-batch fermentations, respectively, compared to shake-flask fermentations. These studies clearly demonstrated that PPA and phytase productions were significantly enhanced by optimization and using novel fermentation methods. Also, mathematical models provided better understanding of microbial kinetics for both fermentations. With these enhancements, industrial productions of PPA and phytase by using submerged fermentation are now closer to reality than before.

TABLE of CONTENTS

LIST of FIGURES

LIST of TABLES

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CHAPTER 1

INTRODUCTION

Recently, feed and food additives have become popular in industry to increase quality of diets. A feed or food additive can be any compound for animals and humans to increase their welfare by adjusting their nutritional levels in their diets and optimize their growth. Food and feed additives are mainly used for controlling factors such as decomposition and deterioration, nutritional losses, and loss of functional properties. Major food and feed additives are being used in the diets can be classified as, vitamins, minerals, aroma substances, flavor enhancers, sugar substitutes, sweeteners, food colors, acids, bases, antimicrobial agents, antioxidants, chelating agents, surface active agents, thickening agents, gel builders, stabilizers, anticaking agent, bleaching agent, humectants, clarifying agents, protective gases, binders, calorie reducing agent, fat substitutes, and amino acids (Branen et al., 2002).

Among the functional groups, amino acids are broken down in metabolic cycles, such as Krebs cycle to their precursors, which are known as “keto acids” or “oxo acids”, which are deaminated amino acids and contain a carboxylic acid group and a ketone group (Lehninger et al., 2000). There are three different kinds of keto acids. Alpha keto acids, or 2-oxo acids such as pyruvic acid have the keto group adjacent to the carboxylic acid. Beta keto acids or 3-oxo acids, such as acetoacetic acid have the ketone group at the second carbon from the carboxylic acid. The last group, gamma keto acids or 4-oxo acids, such as levulinic acid have the ketone group at the third carbon from the carboxylic acid. Alpha keto acids are primarily used as energy for liver cells and in fatty acid synthesis (Reissbrodt et al., 1997). In the food industry, alpha keto acids have significant value, since it has been reported that they are more effective at initiating the special aroma and taste of the food than amino acids (Lehninger et al., 2000). The most important application of alpha keto acids is the supplementing human and animal diets. Alpha ketoglutarate (alpha ketoglutaric acid), which is an alpha keto acid and takes place in Krebs cycle after isocitrate and before succinyl CoA, is experimentally used as a nutrition supplement, especially in poultry. Alpha keto acids are also used as therapeutic agents for many applications. Alpha ketoisovalerate, which is the precursor of L-valine, L-leucine amino acids, serves today as a substitute for L-valine or L-leucine in chronic kidney disease patients (Krause et al., 2010). Also, Takagi et al. (2009) mentioned that L-ascorbic acid (vitamin C) can be produced via conversion of alpha ketoglutarate. Sluis et al. (2001) reported that alpha keto betamethylvalerate, alpha keto

isovalerate and alpha keto isocaprote can be converted into higher alcohols, which are flavor compounds. Ganesan et al. (2004) stated that alpha keto acids are important flavorful compounds in cheese production too. Similarly, Casey et al., (2004) reported that instead of amino acids, alpha keto acids are more effective on development of cheese flavor.

Alpha keto acid production is based on removal of amino groups from amino acids via transaminase or deaminase enzymes. These enzymes can be secreted by several microorganisms. It was shown that D-amino-acid oxidase was purified from the yeast *Rhodotorula gracilis* (Simonetta et al., 1989). Similarly, the same enzyme was produced by a genetically modified *Escherichia coli* (Kim et al., 2008). Moreover, several *Proteus* and *Morganella* species were also identified as deaminase producers in the literature (Massad et al., 1995). To date, alpha keto acid production using microorganisms have been performed by several researchers. However, most of these studies were done in shake-flasks and they are not scalable. As a result, alpha keto acid production cannot keep up with the demand. There is a need that, fermentation methods must be studied to make the alpha keto acids more economical for the industry to use. Therefore, this study is undertaken to improve phenylpyruvic acid (PPA) production, which is the alpha keto acid form of phenylalanine. First, microbial selection was studied to determine the most productive microorganism using selective agar tests and performing shake-flask fermentations. Thereafter, growth parameters such as temperature, pH, and aeration were optimized to increase PPA production in bioreactors (Chapter 3). Later on, important fermentation medium ingredients such as glucose, yeast extract, and phenylalanine concentrations were optimized in the bioreactors as well (Chapter 4). Also, fed-batch and continuous fermentation were run to improve PPA production (Chapter 5). Finally, modeling of batch PPA fermentation was studied, which can be used for monitoring, predicting, and optimizing of PPA production parameters during fermentation (Chapter 6).

Phytase (myo-inositol-hexakisphosphate phosphohydrolase) is an enzyme, which catalyzes the hydrolysis of phytate to inositol and orthophosphoric acid (Liu et al., 1998). Phytate is a major phosphorous source in plants and has a negative influence in animal and human diets by chelating several minor elements in the body (Bucci, 1995). Recently, phytase has been supplied to animal and human diets to improve the availability of several minerals and amino acids. Studies showed that phytase application increased minor nutrients such as Ca^{+2}, Mg^{+2}, Zn^{+2}, Cu^{+2}, and Mn^{+2} utilization and protein digestibility. Phytase application on cereals has become quite popular to decrease iron deficiency in humans by increasing iron absorption. Also,

phytase has been used in the bakery industry to decrease fermentation period and improve bread volume and crumb texture. Phytase is now accepted as an energy metabolism regulator and used in diets of monogastric (simple stomached) farm animals and humans (Haefner et al., 2005).

Phosphorus pollution has become an important environmental issue. Monogastric animals utilize phytate poorly due to low phytase activity in their intestine and this results in high phosphorous excretion in their manure. Excessive phosphorous excretion leads to eutrophication in rivers and water reservoirs. This may result in toxic algal accumulations, fish kills and fauna and flora changes (Selle and Ravindan, 2007). Therefore, phytase has been started used as feed supplement to reduce phosphorous excretion in manure.

Phytase is secreted by several microorganisms in the industry. *Lactobacillus amylovorus, Aspergillus ficuum* and *Aspergillus niger* were reported as the important phytase producer strains by the previous studies in the literature (Vohra et al., 2003). Additionally, there are several studies indicating *Bacillus, Escherichia, Pseudomonas, Klebsiella*, *Mucor* and *Rhizopus* as potential strains for phytase productions (Liu et al., 1998).

To date, phytase production studies were done in shake-flasks and with solid state fermentation. Due to the limitations of those systems, phytase productions cannot keep up with the demand at the commercial scale. Therefore, other fermentation methods must be studied to improve production using novel approaches and make the phytase more economical for the industry to use. Therefore, a second study was performed to improve phytase production. First, microbial selection was studied to determine the most productive microorganism using selective agar tests and performing shake-flask fermentations. Thereafter, growth parameters such as temperature, pH, and aeration were optimized to increase phytase production in bioreactors (Chapter 7). Later, important fermentation medium ingredients such as glucose, phytate, and $CaSO_4$ concentrations were optimized in the bioreactor (Chapter 8). The, effect of fed-batch addition of glucose and phytate on phytase activity was evaluated in Chapter 9. Additionally, the effect of microparticle addition on phytase activity in shake-flask and batch bioreactor scale fermentations were described (Chapter 10). Similarly, the effect of addition of microparticles on fed-batch and continuous phytase production were evaluated (Chapter 11). Finally, modeling of batch phytase fermentation was studied, which can be used for monitoring, predicting, and optimizing production parameters during fermentation (Chapter 12).

CHAPTER 2

LITERATURE REVIEW

Feed and food additives are important compounds, which have multiple applications in the industry. They can increase the safety, shelf life, and nutrition of the foods. Food and feed additives can be divided into six major groups as preservatives, nutritional additives, flavoring agents, coloring agents, texturizing agents, and miscellaneous additives (Branen et al., 2002). Phenylpyruvic acid (PPA) and phytase are two of important additives, which are the focus in this study.

The objective of this literature review is to provide an overview of alpha keto acid and phytase production. Therefore, this chapter includes info about amino acids, conversion to alpha keto acids, production of amino acids, application, determination and importance for living organisms. Also, an overview of alpha keto acids, their applications, chemical and biological production. In addition, microbial enzymes, which convert amino acids to alpha keto acids and their microbial sources are described. Additionally, for the phytase section, phytate, removal of phytate, phytase, and application of phytase was provided. Additionally, microbial phytase sources, characterization, and effect of microparticles on fungal phytase production are described. Also, current alpha keto acid and phytase production in batch, fed-batch, and continuous fermentations in the literature are described as well. Finally, modeling of microbial fermentation are discussed.

2.1. Amino Acids

Amino acids are important compounds, which have two functional groups as amino (-NH_2) and carboxylic acid (-COOH). Amino acids as they are named have an amino and acid group in their structure. Except for glycine, all amino acids have an asymmetric carbon and optical activity. The absolute configuration of amino acids is defined by the glyceraldehydes. Except for proline, all amino acids have the primary amino and carboxyl group linked to α carbon hence they are called α amino acids (Wu, 2009). A general structure of an amino acid is shown in Figure 2.1.

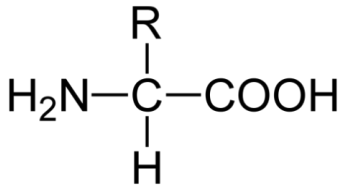


Figure 2.1. General structure of an alpha amino acid (Scheve, 1984).

Amino acids are stable in the solid form at room temperature and they can be autoclaved at 100 to 120ºC for up to 2 h without significant denaturation except for glutamine (Meister, 1957). Solubility of the amino acids in water varies. Cysteine and tyrosine are the least soluble, whereas proline and hydroxyproline are extremely soluble in the water. Several properties of the common amino acids are shown on Table 2.1 (Meister, 1957).

Table 2.1. Some properties of the common amino acids*.

Amino acids	**Denaturation point (ºC)**	**Solubility g of L- amino acid in 100 g water at 25ºC (unless otherwise indicated)**	**pI**
Alanine	297	16.51 (16.72 DL)	6.00
Arginine	238	14.87 (20ºC)	10.76
Asparagine	236	3.11 (28ºC)	5.41
Cysteine	178	28	5.07
Cystine	261	0.011 (0.0326 DL at 19ºC)	4.60
Glutamic acid	249	0.843 (2.054 DL)	3.22
Glutamine	185	3.6 (18ºC)	5.65
Glycine	290	24.99	5.97
Histidine	277	4.29	7.59
Isoleucine	284	4.117 (2.011 DL)	6.02
Leucine	337	2.19 (1.00 DL)	5.98
Lysine	224	150	9.74
Methionine	283	3.35 (DL)	5.74
Phenylalanine	284	2.965 (1.29 DL)	5.48
Proline	222	162.3	6.30
Serine	228	5.023 (DL)	5.68
Threonine	253	20.5 (DL)	6.16
Tryptophan	282	1.14	5.89
Tyrosine	344	0.045 (0.351 DL)	5.66
Valine	315	8.85 (7.09 DL)	5.96

*Meister, 1957.

Although each amino acid has a different catabolic pathway, there are several common ways for catabolism of amino acids in organisms such as transamination, deamination, oxidative deamination, etc. (Table 2.2). Transamination happens in the presence of the amino acid and alpha keto acid. At the end of the transamination process, the initial amino acid is converted to an alpha keto acid and the initial alpha keto acid is converted to amino acids. Deamination occurs by the removal of the amine group by deaminase enzyme. Oxidative deamination takes place via amino acid oxidase enzyme. Table 2.2 indicates the various reactions involved in the catabolism of the amino acids.

Table 2.2. Reactions initiating amino acid catabolism in animals*.

Reactions	Examples
Transamination	Leucine + α-ketoglutarate ↔ α-ketoisocaproate + glutamate
Deamination	Glutamine + H_2O → glutamate +NH_4
Oxidative deamination	Glutamate + NAD ↔ α-ketoglutarate + NH_3 + NADH + H
Decarboxylation	Ornithine → putrescine + CO_2
Hydroxylation	Arginine + O_2 +BH_4 +NADPH +H → NO +BH_4 + citrulline + NADP
Reduction	Lysine + α-ketoglutarate +NADPH +H → saccharopine + NADP
Dehydrogenation	Threonine+ NAD → 2-amino-3-ketobutyrate + NADH + H
Hydrolysis	Arginine + H_2O → ornithine + urea
Dioxygenation	Cysteine + O_2 → cysteinesulfinate
One-carbon unit transfer	Glycine + MTHF ↔ serine + THF
Condensation	Methionine + Mg-ATP → S-adenosylmethionine + Mg-PPi + Pi
Oxidation	Proline + ½O_2 → pyrroline-5-carboxylate + H_2O
Aminotransferation	Glutamine + F6P ↔ glucosamine-6-phosphate + glutamate
Deaminated oxidation	D-amino acid + O_2 + H_2O ↔ α-ketoacid + H_2O_2 + NH_3
Dehydration	Serine → aminoacrylate + H_2O
Cleavage	Glycine + NAD + THF + ↔ MTHF + CO_2 + NH_3 + NADH + H

* Wu, 2009.

2.1.1. Amino Acid Production

The amino acid production and consumption take a large part in the medical and food industry around the world. Ikeda (2003) indicated that amino acid production increased to 1.5 million tons per year in the world. Amino acids are commonly produced by extraction, enzymatic methods, fermentation or chemical synthesis. Worldwide production amounts of amino acids are reported on Table 2.3 (Herrmann and Somerville, 1983).

Table 2.3. The world supply of amino acids in 1996[*].

Amino acid	Method of production	Amount (tons/year)
Alanine	Enzymatic method	500
Arginine	Fermentation	1,200
Aspartate	Enzymatic method	7,000
Cystine	Extraction, enzymatic method	1,500
Glutamate	Fermentation	1,000,000
Glutamine	Fermentation	1,300
Glycine	Chemical synthesis	22,000
Histidine	Fermentation	400
Isoleucine	Fermentation	400
Leucine	Extraction, fermentation	500
Lysine	Fermentation	250,000
D,L-Methionine	Chemical synthesis	350,000
Phenylalanine	Chemical synthesis, fermentation	8,000
Proline	Fermentation	350
Serine	Fermentation	200
Threonine	Fermentation	4,000
Tryptophan	Fermentation, enzymatic method	500
Tyrosine	Extraction	120
Valine	Fermentation	500

[*]Ikeda, 2003.

2.1.2. Microbial Production of Amino Acids

In recent years, microbial fermentation has been in the limelight due to the high potential productivity. In order to produce large amount of amino acids, industrial scale fermentation systems have been developed. Commercial scale fermenters have working volume capacities between 50 to 3 million liters. Additionally, demand for amino acids has increased remarkably and producers are trying to reduce production cost so. Therefore, the volume of fermenters will continue to increase in the future (Ikeda, 2003).

Industrial scale amino acid fermentation is generally done using batch or fed-batch processes. Although batch processes are less labor intensive than fed-batch and an extra tank for feeding nutrients is not required, the industry tends to use fed-batch processes. The main reason for this is that a fed-batch process can provide better productivity, yield, and reduce fermentation time. Also, fed-batch processes are chosen, when critical concentrations of a certain ingredient in the medium can affect the yield or productivity. High sugar concentration may inhibit microbial growth in batch fermentation due to formation of by-products such as acetate and lactate, which

reduce yield significantly. Therefore, not only in amino acid fermentation but in other fermentation, high sugar amounts can be provided by using fed-batch fermentation without substrate inhibition. Moreover, adding these inhibitory nutrients under control will shorten lag time of microorganisms, which will result in less cultivation time and, correspondingly more cost effective productions (Ikeda, 2003).

Several microorganisms are being used as amino acid producers in the industry. The discovery of *Corynebacterium glutamicum*, which is a soil bacterium, capable of producing L-glutamic acid with high productivity was an important improvement for the production of amino acids via fermentation. It was an important finding that a wild strain could be used on an industrial scale. The fermentation methods for L-threonine and L-tryptophan now well established. For these two amino acids, several recombinant *Escherichia coli* strains have been shown to be high level producers. In 2005, demand for L-threonine and L-tryptophan were 70,000 and 3,000 tons, respectively (Leuchtenberger et al., 2005). Moreover, L-phenylalanine and L-cysteine can be also produced cost effectively with *E. coli* strains allowing the market to grow (Leuchtenberger et al., 2005). Almost all proteinogenic amino acids can be produced in industrial scales by mutants of *Corynebacterium glutamicum* or *E. coli*. Performance of these mutants are shown in Table 2.4 indicates that amino acids can be produced by genetically modified microorganisms at up to 100 g/L.

Table 2.4. Selected microbial strains for the production of amino acids [*].

Amino acid	Strain/mutant	Titer (g/l)	Estimated yield (g/100 g sucrose)
L- Lysine HCl	*C. glutamicum* B-6	100	40-50
L-Threonine	*E. coli* KY 10935	100	40-50
L-Tryptophan	*C. glutamicum* KY9218/pIK9960	58	20-25
L-Tryptophan	*E. coli*	45	20-25
L-Phenylalanine	*E. coli* MWPWJ304/pMW16	51	20-25
L-Arginine	*Brevibacterium flavum* AJ12429	36	30-40
L-Histidine	*C. glutamicum* F81/pCH99	23	15-20
L-Isoleucine	*E.coli* H-8461	30	20-30
L-Serine	*Methylobacterium sp.* MN43	65	30-35
L-Valine	*C. glutamicum* VR3	99	30-40

[*]Ikeda, 2003.

2.1.3. Applications of Amino Acids

Amino acids have been used as animal and human feed additives, flavor enhancers and special nutrient supplements in the industry. Especially lysine, methionine, threonine, and tryptophan are significant amino acids in the production of feeds. For animal feed supplements, DL-methionine and lysine monohydrochloride are produced globally at 180,000 and 25,000 tons per year, respectively (Herrmann and Somerville, 1983). Additionally, amino acids are used in the food industry to improve absorption of minerals from the supplements (Leuchtenberger et al., 2005). The worldwide output of glutamate used as a seasoning is 270,000 tons annually (Herrmann and Somerville, 1983). Also, amino acids are under development as components for biodegradable polymer productions. Biodegradable polymers are environmental friendly materials are used in medicine for drug delivery and construction of prosthetic implants. Similarly, biodegradable polymers are used for production of disposable diapers and in agriculture (Gross and Kalra, 2002). Additionally, amino acids are being used in medical applications. Regimes involving single amino acids as therapeutic agents include the use of glutamine and histidine for peptic ulcers, dihydroxyphenylalanne for Parkinson disease, and argine, citrulline, and ornithine to treat hepatic disease (Herrmann and Somerville, 1983).

Taking into consideration all these, it can be understood why the demand is high for amino acids. Poor nutrition constitutes a growing public health problem in today's world. Unhealthy foods cause many acute and life-long diseases such as diarrheal diseases even various forms of cancer. Since amino acids are crucial in the diets of animals and people, economical production of them is a very important issue in today's world.

2.1.4. Determination and Quantification of Amino Acids

Determination of amino acid concentration in fermentation samples is commonly performed with HPLC. In recent years pre-column derivatizations have taken the place in amino acid determination protocols instead of post-column derivatizations by including reverse-phase HPLC columns. Pre-column derivatizations have several advantages comparing to post-column derivatization techniques. For example, pre-column derivatization instrumentation is not only used for amino acid analysis. Also in pre-column derivatizationsensitivity of analysis increases by 250 times, and analysis time is shorter than post-column derivatizations procedures. Also, pre-column derivatizations are more commonly used, because pre-column derivatization avoids

dilution of peaks and increases sensitivity (Irvine and Davidson, 2002). O-phthalaldehyde (OPA) is one of the most widely used one among these derivatization agents. There are two disadvantages of OPA such as, derivatives have short stability and secondary amino acids cannot be detected. In order to overcome these disadvantages, auto samplers, which are capable of mixing reagent and sample prior to injections are used. Also, there are some techniques have been developed for detection of secondary amino acids by oxidation of chloramine T or hypochlorite before derivatization with OPA (Harris, 1988). Characteristics of derivative agents are used for amino acid detections were shown on Table 2.5.

Table 2.5. Characteristics of derivative agents used for amino acid detection in HPLC*.

Derivatization reagent	**O-Phthalaldehyde (OPA)**	**Phenylisothuocyanate**	**9-Fluoroenylmethyl chloromormate (FMOC)**	**Dansyl or dabsyl chloride**
Detection of secondary amino acids	No	Yes	Yes	Yes
Stability of derivatives	Poor (Less than 10 min)	Moderate (1-2 days)	Good	Moderate (1-2 days)
Detection limits	0.5 pmol	1 pmol	0.5 pmol	10 pmol (dansyl) 0.5 pmol (dabsyl)
Detection method	Fluorescence	UV absorption	Fluorescence or UV absorption	Fluorescene or UV absorption
Interference by excess reagent or byproducts	No	Yes	Yes	Yes
Ease of automation	Good	Poor/moderate	Moderate	Poor

*Harris, 1988.

In order to maximize sensitivity all reagents and solvents should be prepared by using highest grade solvents. It has been also observed that there is a reverse relationship between the temperature and the fluorescence signal. It was reported by Chen et al. (1979) that OPA-amino

acid signal increased by 35 and 65%, when analysis were performed at 21 and 42°C, respectively, compared to results were obtained at 5°C. Additionally, it was shown that alkaline pH increases the OPA fluorogenic signal, however, it is not always needed to adjust pH in alkaline levels to measure the signals. Also, studying effect of dimethyl sulfoxide (DMSO) on the fluorescence of OPA derivatives of amino acids showed that DMSO increased the fluorescence signal. Fluorescence intensity of OPA-Histidine derivative was measured 2.45 times more in 20% DMSO than water. In addition, presence of DMSO provided more pronounced shoulder on the peaks at 345 nm. Similarly, sodium dodecyl sulfate (SDS) is very useful to increase fluorescence signal of Leucine-OPA complex. Fluorescence signal was doubled by using 0.04 M SDS in the derivatization procedure (Chen et al., 1979).

2.1.5. Essential and Non-essential Amino Acids

Among the amino acids, and phenylalanine, valine, threonine, tryptophan, methionine, leucine, isoleucine, lysine, and histidine are classified as essential amino acids (Young, 1994). Non-essential amino acids are required for normal health and growth but can be synthesized and derived in the body from essential amino acids. Figure 2.2 shows that non-essential amino acids can be synthesized by transamination or some more complex reactions.

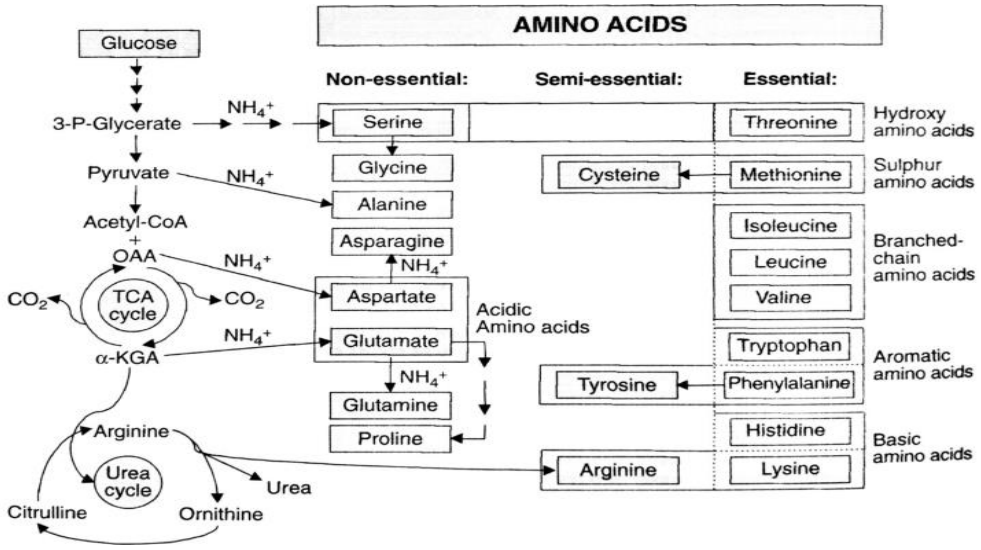


Figure 2.2. Essential amino acids and synthesis of semi and non-essential amino acids (Young, 1994).

Additionally, amino acid requirement varies among the individuals depending on age, body size, and gender. World Health Organization (WHO) reported amino acid requirements for adults in Table 2.6 (WHO, 2007).

Table 2.6. Amino acid requirements for human milligram per kilogram body weight per day*.

Amino acid	Adult
Lysine	12
Leucine	14
Methionine + cysteine	13
Threonine	8
Phenylalanine + tyrosine	21
Tryptophan	3.5
Histidine	0
Isoleucine	10
Valine	10

* WHO, 2007.

2.1.6. Phenylalanine

Phenylalanine is an essential amino acid with $C_6H_5CH_2CH(NH_2)COOH$ molecular formula and 165.19 g/mole molecular weight. Phenylalanine was described for the first time in 1879 by Schulze and Barbieri in yellow lupine seedlings. Then, Erlenmeyer and Lipp synthesized phenylalanine from phenylacetaldehyde, hydrogen cyanide, and ammonia in 1882. Additionally, the genetic codon for phenylalanine was discovered in 1961 (Matthaei and Nirenberg, 1961). It takes role not only in protein synthesis, but also synthesis of non-essential amino acid tyrosine and consequently noradrenaline, adrenaline, and the thyroid hormones. Phenylalanine is naturally found in breast milk. It is biologically converted to tyrosine and to dopamine, noreapinephrine, and epinephrine (David, 2006). Phenylalanine and tyrosine synthesis starts with the isomerization of chorismate to prephenate. This reaction is catalyzed bye chorismate mutase. Then, there are three pathways leading to formation of phenylalanine and tyrosine via phenylpyruvic acid, p-hydroxyphenylpyruvate or arogenate. Metabolic pathways for phenylalanine and tyrosine productions are shown on Figure 2.3.

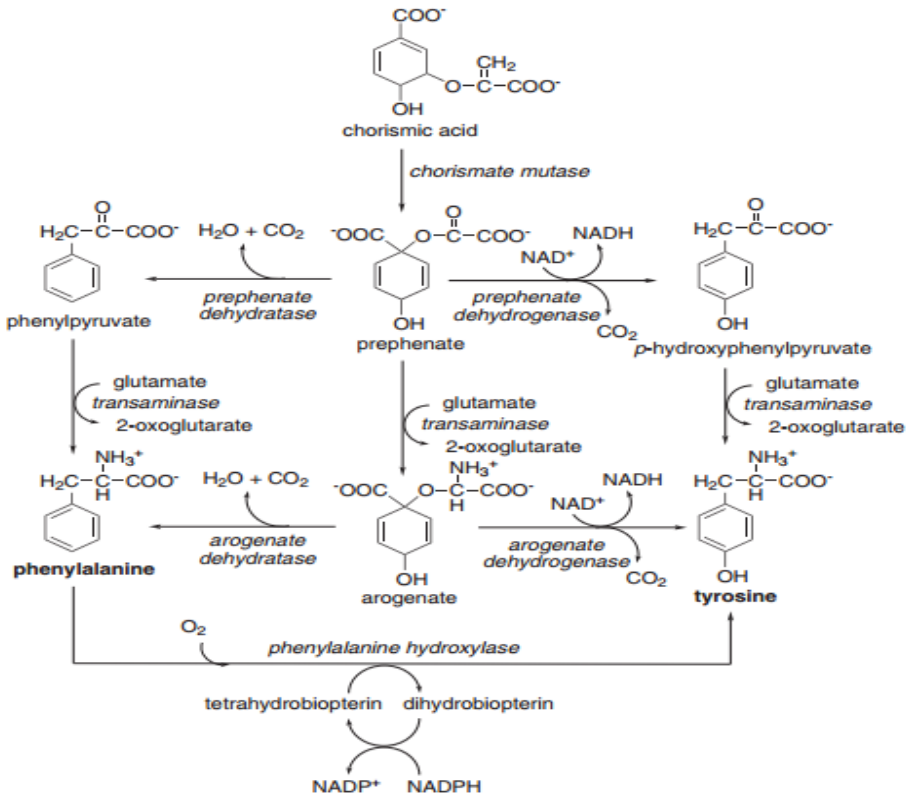


Figure 2.3. Biosynthesis of phenylalanine and tyrosine from chorismate (David, 2006).

Phenylalanine synthesis was studied in *E. coli.* It was reported that the enzyme catalyze the reaction is inhibited by phenylalanine. In case of excessive phenylalanine concentration, the active dimeric enzyme is converted to inactive form. Maximal inhibition is observed, when each units binds to one phenylalanine molecule (Garner and Herrmann, 1983).

Phenylalanine used to be a small portion in industrial amino acid production. However, production of phenylalanine has increased to 150 tons annually in this decade. Phenylalanine is mainly used for nutrient purpose, but also for production of L-aspartylphenylalanine as a sweetener. To date, maximum microbial production of L-phenylalanine was reported as 25 g/L with *Brevibacterium lactofermentum* (Enei and Hirose, 1985). Additionally, microbial production of phenylalanine was studied by using PPA, which is the intermediate precursor. It was reported that *Alcaligenes faecalis* B141-1 converted phenylpyruvic acid to L-phenylalanine with a 78% yield. In this reaction, L-aspartic acid, L-leucine, and L-glutamic acid were used as amino donors (Enei and Hirose, 1985). Also, it was reported that auxotroph of *Micrococcus glutamicus*

produced 6.3 g/L of phenylalanine using tyrosine as substrate (Herrmann and Somerville, 1983). Additionally, Tsuchida et al. (1975) reported that 23.1 g/L of phenylalanine was produced by 5-methyltryptophan resistant mutant *Brevibacterium lactofermentum.* Moreover, some enzymes of phenylalanine synthesis were found in fungi and yeast such as *Claviceps*, *Neurospora*, *Saccharomyces*, *Penicillum,* and *Hansenula* (Herrmann and Somerville, 1983).

2.2. Alpha Keto Acids

Keto acids or oxo acids have a carboxylic acid and a ketone group in their molecular structure (Lehninger et al., 2000). Keto acids are named based on the position of keto group in the structure as shown in Figure 2.4. Alpha keto acids have general formula RCOCOOH, where R is aryl, alkenyl or heterocyclic group. They are important intermediate compound for production of amino acids, medicines, food-feed additives, and agricultural chemicals. Alpha keto acids naturally occur in Krebs cycle as intermediate compounds. Additionally, pyruvic acid and glyoxylic acid were found in eastern Pacific Ocean and concluded that they were excreted by an algae in the photic zones as a result of photorespiration. Since alpha keto acids are intermediate compounds, which regulates protein turnover, external alpha keto acid intake takes an important role in human and animal nourishment.

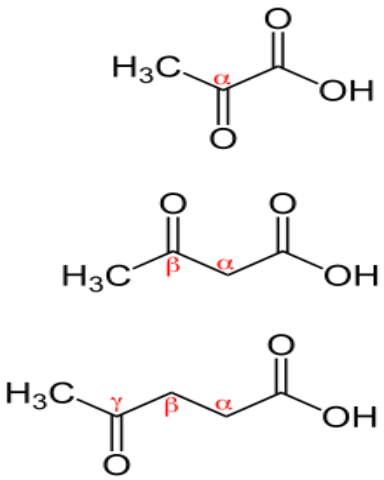


Figure 2.4. Molecular structure of alpha, beta, and gamma keto acids (Lehninger et al., 2000).

Among all alpha keto acids, branched-chain and phenyl-substituted alpha keto acids have different physical characteristics. Generally, the straight chain alpha keto acids are either liquids or low-melting solids (Waters, 1947). Adickes and Andresen (1943) classified the straight chain alpha keto acids into two groups based on their amount of carbon atoms. It was point out that melting point increases as the molecular weight increase and difference between the melting points of two members in either series become smaller with increasing length of carbon chain. For example, it was measured that an increase in the number of carbon atoms from five to seven causes an increase in melting point with 23^{o}C. Also, an increase from six to eight in carbon atoms, increases the melting point by 26^{o}C. However an increase in carbon atoms from seven to nine and eight to ten causes both 14^{o}C increase in the melting point of straight chain alpha keto acids. Additionally, alpha keto acids show different stability characteristics among each other. Some of the alpha keto acids are not stable in the air such as PPA and bezaldehyde, benzoic acid, carbon dioxyde acid and oxalic acid have been detected as the decomposition products. On the other hand, some other alpha keto acids are quite stable, when they are heated with acids or bases (Adickes and Andresen, 1943). Additionally, alpha keto acids can develop characteristic colors, when they react with ferric chloride. They can be titrated with bases and can form calcium, barium and silver salts, which makes them easier to detect. Moreover, alpha keto acids tend to condense with themselves in the presence of strong acid or alkaline. For example, pyruvic acid and PPA can be condensed with benzyl cyanide to form cyanohydro derivative.

Alpha keto acids are important intermediate compounds in biosynthesis of amino acids, carboxylic acids and sugars. Concentration of alpha keto acids may increase highly in sera and urine in patients, who have maple syrup urine disease, phenylketonuria and tyrosinosis. Therefore, measurement of alpha keto acids in urine and sera is important for diagnosis and treatment of these diseases (Hara et al., 1985).

2.2.1. Diseases Identified Related To Alpha Keto Acids

Maple syrup urine disease (MSUD) is a rare inborn error of metabolism that the defect of branched-chain oxo-acid dehydrogenase. It is encountered by 1:185,000 rate in the people (Bender, 1985). As a result of this disease, concentration of branched-chain amino acids and alpha keto acids increased in tissue and urine. Therefore, the name of the disease comes from the odor of the urine, which contains high amount of branched-chain alpha keto acids. Patients, who are not treated may have severe mental disorders, suffer neurological and respiratory issues (Bender, 1985).

Similar to MSUD, phenylketonuria (PKU) is also an inborn error of amino acid metabolism, which is caused by a deficiency of phenylalanine hydroxylase. PKU is caused by mutation of the gene encodes phenylalanine dehydrogenase enzyme, which converts phenylalanine to tyrosine (Palka and Kanska, 2012). PKU is the most common disease about metabolic defects in amino acid metabolism, which can be encountered in one person from 15,000. Simply, it is defined as excessive accumulation of phenylalanine. In normal conditions, phenylalanine is converted to tyrosine and then to tissue proteins, melanin, catecholamines, and fumarate acetoacetate. However, in PKU patients' metabolism, phenylalanine is oxidized to PPA and then phenyllactate and phenylacetate. These metabolites give urine a characteristic mousey odor. The conversion reaction of phenylalanine to PPA and then phenyllactate and phenylacetate in PKU patients was shown in Figure 2.5 (David, 2006).

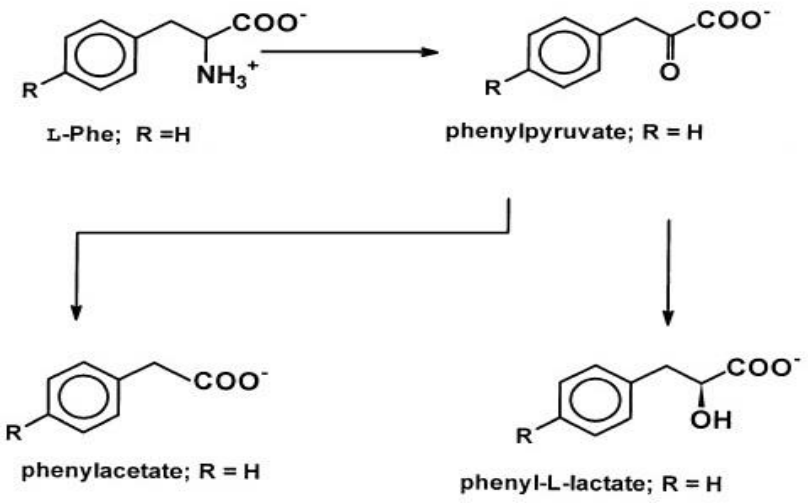


Figure 2.5. Phenylalanine conversion in PKU patients (David, 2006).

This disease shows several symptoms such as mental retardation, hyperactivity, tremor, and failure to walk or talk. Average IQ level was assumed as 20 for PKU patients. Additionally, patients with PKU often show a deficiency of pigmentation (Champe et al., 2008). Also, 50% of the patients die before 20 years old and 75% before 30. Early diagnosis of PKU is important for the treatment of the disease by modifications in diets. Phenylalanine concentration must be between 1.2 and 3.4 mg/100 ml of blood. Therefore, phenylalanine concentration is aimed to

maintain close to normal range by modifying the diets with synthetic supplements at early ages of the infant starting 2-3 weeks after birth. Dietary management is not very important after six years old since brain gets mature and toxic effects of phenylalanine becomes not susceptible (Scheve, 1984). The earlier treatment results in less neurologic damage in the patients. Therefore, determination of PPA concentration in these samples helps diagnosis for this health problem (Hayashi et al., 1976).

2.2.2. Assays for Alpha Keto Acid Quantifications

There are several factors should be taken into considered while development of the alpha keto acid assays. First, these compounds are generally in low concentrations in the biological systems. Also, stability of each alpha keto acids is very different among each other. Moreover, a development of a suitable separation method is very important for the assay accuracy. There are two general approaches have been studied to analyze alpha keto acids. First is the isolation of alpha keto acids by extraction and enzymatic or colorimetric analysis of them. Second is the conversion of alpha keto acid into specific derivatives with suitable derivatization agents and analysis of them by chromatography. Currently, the second method is more widely used comparing to the first one. An appropriate derivative agent should provide higher stability than alpha keto acid structure, increase the detectability sensitivity, stimulate chromatographic separation, and react only with alpha keto acids (Fromke, 1966). Similar to amino acid analyses, alpha keto acid determinations are commonly performed in HPLC. Hydrazines, such as 2,4-dinitrophenylhydrazine (DNPH), can selectively react with aldehydes and ketones to form stable hydrazones. Moreover, aromatic hydrazine reacts with carbonyls under acidic conditions and forms insoluble hydrazone derivatives (Elias et al., 2008). DNPH is commonly used for determination of ketone groups, which allows high specificity and selectivity in HPLC for keto acid detection. It was reported that even 10 ng of alpha keto acid can be detected by DNPH derivatization with a signal:noise ratio 7:1 at 366 nm (Hemming and Gubler, 1979). It was shown that some keto acid-DNPH derivatives gave multiple peaks in HPLC results. For example, there were two peaks observed for pyruvic acid, which the first one was larger than the second one. Furthermore, the sample showed reversal peak proportion after incubation for one week in methanol. Similarly, oxalacetic acid-DNPH sample generated three peaks in HPLC results, which were run by phosphate-methanol gradient flow. DNPH derivatives of aromatic compounds such as phenylpyruvic acid and p-hydroxyphenylpyruvic acid also produced two peaks. These

aromatic keto acids are less soluble comparing to other keto acid-DNPH derivatives in water-methanol solvent. Therefore, optimization of separation procedure of these compounds needs other solvents such as acetonitrile (Hemming and Gubler, 1979).

2.2.3. Phenylpyruvic Acid

Phenylpyruvic acid (PPA) also named as 2-hydroxy-3-phenylpropenoic acid is the alpha keto acid form of phenylalanine, which has $C_9H_8O_3$ molecular formula and 164.16 g/mole molecular weight. An intramolecular hydrogen bond exists between enol and carboxyl O atoms. The molecules are bound together by hydrogen bonds between carboxyl groups. The O-O distances of intramolecular and intermolecular hydrogen bonds are 2.639 and 2.673 Å, respectively (Okabe and Inubushi, 1997). PPA is produced by oxidative deamination of phenylalanine, which is catalyzed by phenylalanine dehydrogenase in vivo (Palka and Kanska, 2012). Norseth and Haavaldsen (1964) reported 5.10^{-4} M and 1.10^{-3} M concentration of PPA induced rat uterus muscle construction. PPA was also found in plants and plant-derived foods. For example, PPA was determined in different wine samples. Ferreira et al. (2007) quantified 0.1-9.6 mg/L PPA in several wines based on wine type and age. It was also shown that white wines include less PPA, comparing to red wines. Recently, interest to natural products has increased by consumers and producing of natural flavor enhancers has become so popular. Groop and Bont (1998) showed that PPA is the intermediate compound in conversion of phenylalanine to benzaldehyde, which is commonly used as a flavor enhancer in the industry. Also, PPA added cheese whey hydrolyzates can be efficiently metabolized by *Lactobacillus plantarum* CECT221 and produce important flavor for the food industry (Pazo et al., 2013). Additionally, it was reported that PPA and phenyllactic can be produced from L-Phenylalanine (Baek et al., 2011).

2.3. Applications of Alpha Keto Acids

Alpha keto acids have several application areas in medicine, agriculture, food and feed industry. It has been reported that alpha keto acid analogues can be substituted for their specific amino acids. Recently, attention has been increased to biological function and metabolism of alpha keto acids. Alpha keto acids are ketogenic or glucogenic precursors, which regulate protein turnover. Therefore, it is important to follow changing in concentrations of these compounds in blood and urine to follow physiological condition of humans, maintain nutrition intake and development of new treatments against some diseases (Pailla et al., 2000). Additionally, alpha

keto acids are especially crucial for kidney patients because they use alpha keto acids instead of amino acids in their diets. Since alpha keto acids do not have amino groups, substitution of amino acids with alpha keto acids in the diet help kidneys and livers to reduce urea accumulation in the body (Krause et al., 2010). Walser et al. (1973) reported that low nitrogen supplemented diet, with five different alpha keto acids, reduced blood urea concentration and improved nitrogen balance in uremic patients. Also, there are a few studies showed that keto acids are utilized in healthy and uremic man. Halliday et al. (1981) labeled alpha keto acids of valine and phenylalanine to feed healthy and uremic subjects. The degree of conversion of these alpha keto acids to their amino acids were between 25-50% regardless of subjects' health. However, a low-protein diet increased conversion rate of alpha keto acid of leucine by about 68-70% (Epstein et al., 1980). Therefore, there studies showed that not only proportions but also amount of the individual alpha keto acids must be carefully defined before using alpha keto acids in the diets.

Another medical and industrial application of alpha keto acid is that, as described earlier by Takagi et al. (2009) that L-ascorbic acid (vitamin C), which is one of the most demanded vitamins in the world, is produced from alpha keto glutarate. Furthermore, antioxidative properties of several alpha keto acids and effect of them on oxidative hemolysis of human erythtocytes was studied by Sokolowska et al. (1999). They showed also alpha ketoglutarate and oxaloacetate have antioxidant characteristics similar to pyruvate.

Alpha keto acids are also experimentally used by poultry as a nutrient supplement. Nitrogen excretion in chickens' manure has an adverse effect on the environment and for producers. To minimize this, producers can start to supply alpha keto acids in chickens' diet instead of amino acids in order to reduce nitrogen in their manure (Summer, 1993). It was also reported that alpha keto acids minimize dietary nitrogen intake and also provides enough amount of nutrient to prevent body loss (Bubl and Butts, 1949). Early studies showed that all alpha keto acids of amino acids except for lysine and threonine can be used as substitutes in the diets of rats as growth supplements. Gaby and Chawla (1976) reported that, there was an increase with 6.5 g/day in the weight of male weanling albino rats, when they were fed with 45.4 μmoles of phenylalanine/g in their diets. No phenylalanine addition caused average weight loss of 1.1 g/day. They also reported that then phenylalanine was replaced with PPA, growth rate increased with more efficiency. Therefore, PPA was reported as more efficient substitute comparing to alpha ketoisovaleric acid and alpha ketoisocaproic acid usage, which are alpha keto acids of valine and leucine, respectively. Furthermore, efficiency of alpha keto acids were also studied by Chow and Walser (1975) by dividing moles of amino acids required for specific growth rate to moles of

alpha keto acids required for same amount of growth rate. It varied between 80% to 30% for alpha ketoisovaleric acid and around 25% for alpha ketoisicaproic acid. This shows that smaller amount of alpha keto acids can provide the same growth rates in rats compared to high amino acid supplement. Also, importance of alpha keto glutarate was reported by Tatara et al. (2005). Alpha ketoglutarate, which is the precursor of glutamate and glutamine is a compound takes in place in citric acid cycle. Alpha ketoglutarate serves as an energy donor and provides glutamine, which stimulates protein synthesis and prevents protein degradation in muscles. Also, it provides metabolic fuel for gastrointestinal cells. Effect of alpha ketoglutarate on bone properties of turkeys was also shown in terms of increasing in bone weight, second moment of inertia, cross sectional area, wall thickness, maximum elasticity, strength and mineral density (Tatara et al., 2005). Moreover, studies done by Kristensen et al. (2002) showed that alpha ketoglutarate also increased proline synthesis, which plays a role in collagen formation. Similarly, it was shown by Escobar et al. (2010) that alpha keto isocaproic acid, which is the alpha keto acid of leucine is significantly effective on synthesis of muscle protein in young pigs. Moreover, positive effect of alpha keto acids on cholesterol content of the foods was also studied by Beyer and Jensen (1992). They reported that chickens were fed with 0.9% alpha ketoisocaproic acid resulted insignificantly reduction in egg cholesterol comparing to the control.

Food flavors have a huge commercial importance in today's world and enriched foods with external nutrients; have become very popular in daily diets. Compounds such as ketones, aldehydes, acids, and esters have been identified in the smoke produced in the pyrolysis process of some woods. However, only aldehydes and ketones are considered as effective in the development of the color, aroma and texture in the smoked foods (Delgado et al., 2008). Alpha keto acids are used as flavor sources and nutrient enrichers by food industries. Ganesan et al. (2004) noted that especially in cheese production, alpha keto acids have a large impact on cheese taste, and flavor. They showed that the catabolism of several amino acids with microorganisms via aminotransferase reactions is one process to produce flavorful compounds in cheese. Also, exogenous addition of pyruvic acid into Gruyere-type cheese increased the aroma significantly. It was also shown that cheese aroma increased by degradation of amino acids especially leucine, methionine, lysine, valine, and phenylalanine to their specific alpha keto acids. However, aroma intensity of the cheese remained the same even amino acid concentration was increased during cheese production. 3-methyl-butanal and 3-methyl-2-butanone, which are aromatic compounds also released by degradation of isovalerate, which is alpha keto acid form of leucine (Casey et al., 2004). Higher alcohols are also used as flavor sources, and they can be produced by conversion of

alpha keto betamethylvalerate, alpha keto isovalerate and alpha keto isocaprote (Sluis et al., 2001). Also, Lee and Richard (1984) reported that phenethly alcohol gives a pleasant rose scented aroma to soft cheeses while L-phenylalanine was converted in the cheese during microbial logarithmic growth stage. Similarly, in Camembert cheese, phenyl acetaldehyde, 2-phenylethanol and derived ester phenethyl acetate, which all occur by the degradation of phenylalanine are important flavor and rose-like odor compounds (Yvon and Rijnen, 2001).

2.4. Production of Alpha Keto Acids

2.4.1. Chemical Synthesis of Alpha Keto Acids

Alpha keto acids can be produced with chemical synthesis. There are four different chemical synthesis methods for the production of the alpha keto acids (Waters, 1947).

2.4.1.1. Hydrolysis of the Acyl Cyanide

In this method, acyl bromide and suprous cyanide are heated in a water batch for 2 h. Acyl cyanide yields out at the end of the incubation. In the case of alpha keto n-butyric acid and pyruvic acid. Many sources have stated that hydrolysis of the acyl cyanides is a general procedure for preparing the alpha keto acids. However, it was also indicated that, this method can work only for short-chain acids with less than 5 carbon atoms (Waters, 1947).

$$\mathrm{RCOX + MCN \rightarrow RCOCN \rightarrow RCOCOOH}$$

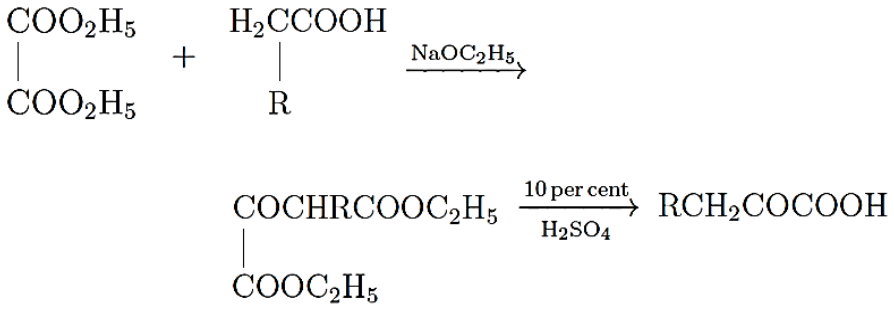
2.4.1.2. The Hydrolysis of the Oxime Ester

This reaction happens by dissolving oxime is in 85% of formic acid and cooled down to 0^{o}C. However, it was found that the yields of the conversion of oxime to alpha keto acids are generally so low. Additionally, PPA cannot be obtained from oxime, so this method is not applicable for all alpha keto acid production (Waters, 1947).

$$\mathrm{RC(=NOH)COOR' \xrightarrow[HOSO_2ONO]{\text{formic acid}} RCOCOOR' \rightarrow RCOCOOH}$$

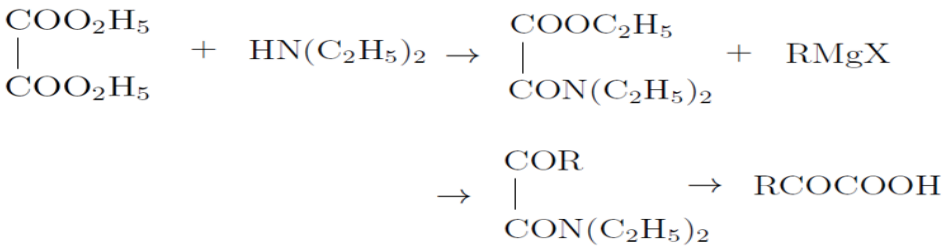
2.4.1.3. Hydrolysis of Ethyl Esters of Oxalo Acids

Refluxing the fatty acid ester, diethyl oxalate and sodium ethoxide in the ether yields in nine alpha keto acid formations. In this technique, the final product alpha keto acid is obtained by boiling alpha oxaloester in the diluted sulfuric acid for 6 h. However, yield of this method varies between 8 to 94% (Waters, 1947).



2.4.1.4. Hydrolysis of Grignard Reagents and Diethyloxamates

This method can be used only a little amount of alpha keto acid production. Also, long refluxing period and necessity of condensation of N, N- diethyloxamate have discouraged the investigators about this method (Waters, 1947).



2.4.2. Biological Production of Alpha Keto Acids

Amino acids can also be converted to their specific alpha keto acids via transamination and deamination reactions in vivo, which is indeed a preferred way for alpha keto acid production if it is going to be used as food and feed additives.

2.4.2.1. Transamination

Studies in the literature have shown that enzymatic transamination between amino acids yields in corresponding alpha keto acids in high purity (Alton, 1952). Transamination reaction starts with the hydrolysis of the alpha carbon amino bond of the amino acid, which results in the release of the alpha keto acid (Bender, 1985). This stage is the half reaction of transamination. The process is completed by reaction of pyridoxamine phosphate (cofactor) with a second alpha keto acid substrate, which forms and intermediate ketamine and then releasing the corresponding amino acid as shown in Figure 2.6.

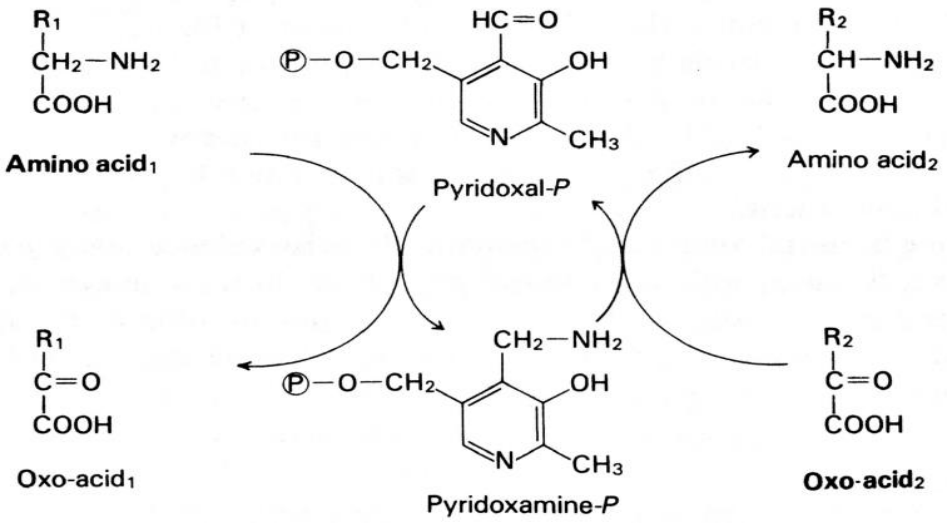


Figure 2.6. Transamination products of amino acids (Bender, 1985).

Transaminase reaction results in ammonia accumulation. Ammonia may be reutilized or converted to urea or uric acid in liver before to extraction via kidneys. Corresponding alpha keto acid products of several amino acids, which are produced by transamination are shown on Table 2.7.

Amino acid transamination is the most important step in the amino acid conversion to flavor compounds by cheese microorganisms. In transamination, reaction is catalyzed by aminotransferases and yields in alpha keto acid and the amino group acceptor alpha keto acid is generally alpha ketoglutarate is transformed to corresponding amino acid, which is glutamate (Yvon and Rijnen, 2001).

Table 2.7. Corresponding alpha keto acid products of amino acids*.

Amino acid	Alpha keto acid
Alanine	Pyruvate
Arginine	Alpha-oxo-gamma-guanidoacetate
Aspartic acid	Oxaloacetate
Cysteine	Beta-mercaptopyruvate
Glutamic acid	Alpha-oxoglutarate
Glycine	Glyoxylate
Histidine	Imidazolepyruvate
Isoleucine	Alpha-oxo-beta-methylvalerate
Leucine	Alpha-oxoisocaproate
Lysine	Alpha-oxo-epsilon-aminocaproate
Methionine	S-methyl-beta-thiol-alpha-oxopropionate
Ornithine	Glutamic-gamma-semialdehyde
Phenylalanine	Phenylpyruvate
Proline	Gamma-hydroxypyruvate
Serine	Hydroxypyruvate
Threonine	Alpha-oxo-beta- hydroxypyruvate
Tryptophan	Indolepyruvate
Tyrosine	p-hydroxyphenylpyruvate
Valine	Alpha-oxoisovalerte

*Bender, 1985.

2.4.2.2. Deamination

Deamination means the removal of the amine group from a molecule. This reaction is catalyzed by deaminase enzymes. In animals, deamination reaction mainly takes place in the liver. However, glutamate is also deaminated in kidneys. There are two types of deamination reactions exist as oxidative and non-oxidative deamination (Nedderman et al., 1996).

2.4.2.2.1. Oxidative Deamination

Deamination reaction occurs in two ways. In oxidative deamination, amino group is removed by amino acid oxidase enzyme as free ammonia as shown in Figure 2.7. Because of the fact that this reaction is oxidative, it requires an electron acceptor such as flavin cofactor (Scheve, 1984). Flavin adenine dinucleotide (FAD) is reduced while amino acid is being oxidized. The reduced FAD is then re-oxidized by reaction with oxygen and this reaction yields hydrogen peroxide. Cytotoxic hydrogen peroxide can be removed by catalase and peroxidase addition to

fermentation medium. It was reported that, L-amino acid oxidase has a wide broad specificity however a low activity compared to D-amino acid oxidase (Bender, 2012).

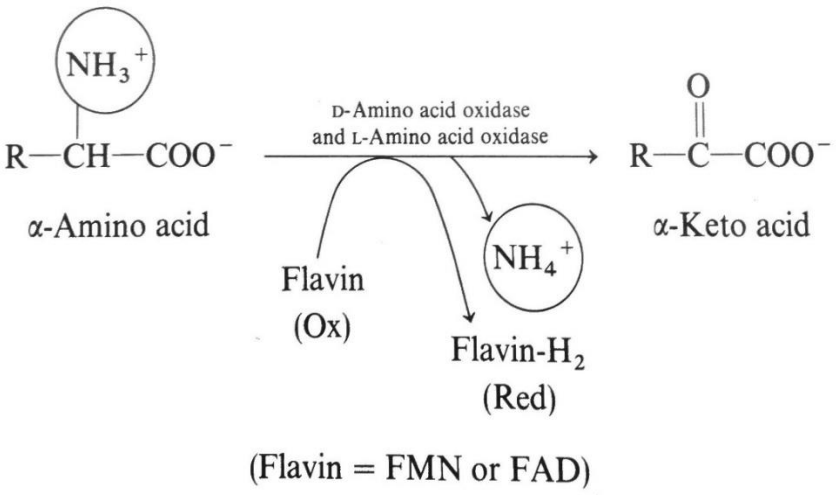


Figure 2.7. Oxidative amino acid deamination (Scheve, 1984).

2.4.2.2.2. Deamination by Dehydration

The other type of deamination reaction is called “deamination by dehydration”. This deamination type has been observed in microorganisms and animal tissues (Meister, 1957). These reactions are catalyzed by enzymes, which are relatively specific for a single amino acid such as serine, threonine, homoserine, cysteine, and homocysteine. These amino acids can be deaminated by removing a water molecule. Figure 2.8 illustrates serine deamination.

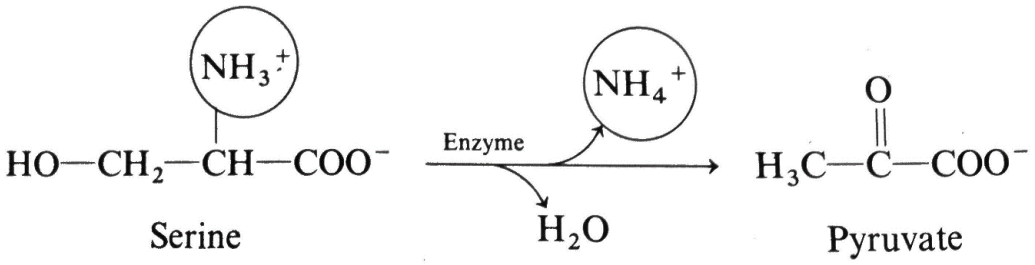


Figure 2.8. Serine deamination by dehydration (Scheve, 1984).

2.5. Microbial Production of Alpha Keto Acids

The enzymes, which convert amino acids to their specific alpha keto acids can be secreted by various microorganisms. Several studies have been done about production of these converter enzymes from microorganisms. Kim (2008) and Simenetta (1989) showed that D-amino acid oxidase enzyme was secreted by *Escherichia coli* and *Rhodotorula gradis* successfully. Similarly, Drechsel et al. (1993) and Massad et al. (1995) reported that several *Proteus, Providencia* and *Morganella* species are producers of these enzymes. Moreover, it has also been reported that the highest L-amino acid oxidase activity was observed near late logarithmic phase of *Providencia* strain PCM 1298 (Szwajcer et al., 1982). Also, Sentheshanmuganathan and Nickerson (1962) reported that the yeast *Trigonopsis variabilis* can utilize many single amino acids as nitrogen source and while D-isomers of amino acids are oxidatively deaminated, L-isomers are transaminated with either alpha ketoglutarate or pyruvate. Moreover, Yang and Lu (2007) studied conversion of the substrates L-arginine and pyruvate into 2-ketoarginine and L-alanine by *Pseudomonas aeruginosa* (PAO1) transaminase enzyme. Also, Soper and Manning (1977) studied the inactivation of transaminase enzymes produced by *Bacillus subtilis* and *Bacillus sphaericus.* In another study, it was shown by Nesbakken et al. (1988) that *Chlorobium spp.* can excrete high amounts of alpha keto acids in the absence or low nitrogen source. Blanco et al. (1990) reported that *Chlamydomonas reinhardtii* can grow under light by using asparagine, glutamine, arginine, lysine, alanine, valine, leucine, isoleucine, serine, methionine, histidine, and phenylalanine as sole nitrogen source. However, in the absence of acetate, cells could only use L-Arginine. It is also mentioned that aspartagine and arginine significantly support algal growth since asparagine provides ammonium after deamination and arginine is used by active transport system during nitrogen starvation (Blanco et al., 1990). Therefore, these studies showed that these microbial enzymes are effective and have high turnover numbers in the reactions of amino acids-alpha keto acids conversion. Additionally, microbial L-amino acid deaminase can be used in the biotransformation processes to convert L or DL-amino acids into D-amino acids, which are commercially important as raw or intermediate materials in the production of pharmaceuticals and food additives (Baek et al., 2011).

Brevibacterium linens growth in low-fat cheddar cheese increased consumer acceptance due to sulphur metabolism. *B. linens* enhances ripening process of the cheese by its proteolytic activity. As a result of proteolysis, volatile sulfur compounds, alpha keto acids and fatty acid occur, which take an important role in flavor development (Ganesan et al., 2004).

Amino acid deaminase enzymes are also used for environmental issues. Ammonifying bacteria were able to utilize peptone and yeast proteins as nitrogen source efficiently and provided high chemical oxygen demand (COD) reduction in waste water treatment processes (Dahiya and Prabhu, 1984). Furthermore, alpha keto acids are produced by bioremediation and removal of hazardous waste. For example, Kunz et al. (1998) showed that alpha ketoglutarate and pyruvate can be produced in the process of non-enzymatic microbial cyanide removal. They demonstrated that not only some alpha keto acids were produced, but also toxicity of cyanide was reduced by *Pseudomonas fluorescens NCIMB 11764.*

2.5.1. *Proteus* Genus

Proteus spp. are Gram-negative and rod shaped bacteria, which can be found in fecal flora of human. *Proteus* spp. include *Proteus vulgaris*, *Proteus zenkeri*, and *Proteus mirabilis.* By the time, several investigations have been performed and new classifications have been proposed for the *Proteus* group of bacteria. Not like *Enterobacteriaceae*, these genus are not considered as pathogens (Ohara et al., 2000). There are a few health problems has been reported, which were caused by *Proteus* genus. For example, the urease enzyme of *P. penneri* is believed to be the reason of kidney stone formation. Indeed, this microorganism has also been isolated from the center of the kidney stone. The indole-negative *P. mirabilis* species are less resistant to antimicrobials than *P. penneri*, *P. vulgaris*, and *P. hauseri* (Ohara et al., 2000). *Proteus* is a common cause of urinary infections in the men. Among several *Proteus* genuses, *Proteus vulgaris* was characterized as gas producer from glucose, urease positive, H_2S producer and able to liquefy the gelatin (Larsson, 1984).

It has been reported that *Proteus* spp. can produce deaminases, which can convert amino acids to their specific alpha keto acids (Singer and Volcani, 1954). Produced alpha keto acids can be detected by formation colorimetric iron complexes. Henriksen (1950) showed that *P. vulgaris* can deaminase phenylalanine to PPA, which reacts with ferric ions and yields a green color. It was reported that *P. vulgaris* and *P. mirabilis* species remarkably higher phenylalanine deaminase activity comparing to *P. morganii*, *P. rettgeri*, and *P. providence* strains. Similarly, Smit (1966) studied 9 *P. vulgaris*, 22 *P. rettgeri*, 74 *P. morganii*, and 24 *Providence* species in terms of their phenylalanine deaminase activities. He showed that all *P. vulgaris* species produced the highest deaminase activity, whereas *P. morganii* and *Providence* species only produced the same or up to 5 times lower activity. The highest deaminase activity of *P. rettgeri* species was half of the lowest

P. vulgaris deaminase activity and the lowest activity of *P. rettgeri* species was measured as 200-fold less that *P. vulgaris* deaminase activity. Additionally, Bernheim et al. (1935) reported that *P. vulgaris* can oxidize practically all L-amino acids. Pelmont et al. (1972) shown that *P. mirabilis*, *P. vulgaris*, and *P. morganii* have rich oxidase activity, when they tested against various L-amino acids. It was reported by Baek et al. (2011) that there are two types of L-amino acid deaminase enzyme have been isolated from *Proteus* species. One can react with high activity toward a wide range of amino acids including monoaminomonocarboxylic, imino, aromatic, sulfur-containing, and β-hydroxy amino acids such as L-Phenylalanine, has limited specificity catalyzing the oxidative deamination of the basic amino acids such as L-Histidine (Baek et al., 2011 and Duerre and Subbas, 1975). Out of two oxidase enzymes, fraction I has measurable activities toward L-valine, L-threonine, L-alanine, L-serine, and L-proline. However, activities were less than 3% towards L-phenylalanine. Moreover, fraction I did not show any measurable activity toward glycine, L-citrulline, basic L-amino acids, L-dicarboxylic amino acid or any D-isomers.

It was also reported that microbial L-amino acid oxidase can be obtained cell-free by using supersonic vibrations. When young bacterial cell suspension was treated with supersonic vibrations, extract can oxidase amino acids successfully. Relative velocities of oxidation of amino acids by cell suspensions and cell-free extracts of *P. vulgaris* are shown on Table 2.8 (Stumpf and Green, 1944).

It was also reported that the cell-free enzyme solution was stable for weeks at 0^{o}C. Stability was decreased as pH was decreased and enzyme rapidly inactivated below pH 4. Moreover, above 50^{o}C, enzyme was unstable. It was reported at incubation at 50^{o}C for 5 minutes caused 78% decrease in the enzyme activity (Stumpf and Green, 1944).

Table 2.8. Relative velocities of oxidation of amino acids by cell suspensions and cell-free extract of *P. vulgaris*[*].

Amino acid	Fresh suspension	Aged suspension	Cell-free extract
DL-Phenylalanine	100	100	100
L-Tyrosine	99	54	62
L-Leucine	93	97	91
DL-Isoleucine	89	31	15
L-Methionine	87	91	65
L-Tryptophane	75	94	88
L-Histidine	73	25	33
DL-Norleucine	80	90	108
DL-Norvaline	90	87	60
DL-Aminobutyric acid	72	19	12
L-Argine	42	23	30
DL-Serine	63	0	0
L-Aspartic acid	57	0	0
L-Glutamic acid	54	0	0
DL-Alanine	45	0	0
DL-Valine	24	0	0
L-Proline	22	0	0
DL-Threonine	22	0	0
L-Ornithine	14	0	0
L-Lysine	14	0	0
Glycine	10	0	0
DL-Phenylglycine	0	0	0

[*]Stumpf and Green, 1944.

2.6. Current Alpha Keto Acid Productions

Several alpha keto acid productions are reported in the literature. Pyruvic acid and alpha keto glutaric acid are the most common studied alpha keto acids, since they take place in Krebs cycle, which enables them to produce in higher concentrations. For example, it was reported by Kunz et al. (1998) that pyruvic acid and alpha keto glutaric acid were produced during cyanide removal and they were further converted into ammonia and carbon dioxide as the final products (Kunz et al., 1998). Production of 2-keto-L-gulonic acid was studied using a mixed culture of *Ketogulonicigenium vulgare DSM 4025* and *Bacillus megaterium* or *Xanthomonas maltophilia* (Takagi et al., 2009). In that study, 90 g/L of 2-keto-L-gulonic acid production was achieved (Takagi et al., 2009). Also, several yeast species including *Yarrowia lipolytica* were utilized to produce alpha keto glutaric acid. It was reported that alpha keto glutaric produced with a

genetically modified *Yarrowia lipolytica* up to 40 g/L. However, the highest alpha keto glutaric acid production was achieved, when 10 g/L of ammonium sulfate and 2-4 µg/L of thiamine was used in the fermentation medium (Chernyavskaya et al., 2000). Additionally, *Zygosaccharomyces rouxii* was used to produce ketoisovaleric acid, which is the alpha keto acid of valine in shake-flask fermentations (Sluis et al., 2001). Production parameters of several alpha keto acids using *Eubacterium* species were also studied by Itoh et al. (1994). They reported that, 5.3 mg/L of PPA, 10.9 mg/L of alpha ketoglutaric acid, 2.4 mg/L of pyruvic acid, and 0.62 mg/L of alpha ketobutyric acid were produced. Black pigmented *Bacteroides* species were studied by Tsuchiya et al. (1990) and they reported that aromatic alpha keto acids such as p-hydroxyphenly-pyruvic acid and phenlypyruvic acid were both produced by *B. vingivalis*, *B. endodontalis* and *B. loescheii*. Also, it was determined that, branched-chain alpha keto acids, alpha keto isovaleric acid, alpha ketoisocaproic acid, and alpha keto-beta-methyvaleric acid were produced in higher levels comparing to other alpha keto acids.

Alpha keto acid productions were generally performed in shake-flask fermentation scale without optimization study. Therefore, scale-up and further optimization studies are needed to improve alpha keto acid fermentations to make the productions commercially feasible.

2.7. Phytate

Substrate of phytase enzyme is named as phytate, phytin or phytic acid, however phytate is the most common one (Selle and Ravindan, 2007). Phytate is the primary storage of phosphate and inositol in plants. It was found out that high amounts of phosphorus was liberated from the seed during germination and incorporated into ATP. It was also mentioned inositol phosphate takes place in the transportation of materials into the cell. Phytate has molecular weight of 659.86 g/mole and $C_6H_{18}O_{24}P_6$ formula. Among these terms, phytate generally states for the mixed salt of phytic acid and phytin refers to deposited complex of inositol hexakisphosphate (IP_6) with potassium, calcium and magnesium acid (Harland and Morris, 1995).

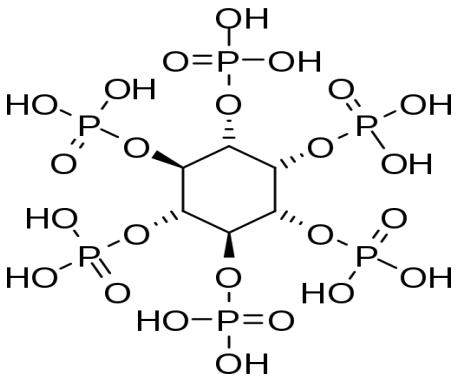


Figure 2.9. Molecular structure of phytate (Haefner et al., 2005).

Six reactive groups of phytate makes it a polyanionic cheating agent (Vohra et al., 2003). Therefore, it acts as an anti-nutrient and it combines with proteins, amino acids and important divalent cations such as Ca^{+2}, Mg^{+2}, Zn^{+2}, Cu^{+2}, Fe^{+2}, and Mn^{+2} in humans and animals (Haefner et al., 2005). The interactions between phytate and various nutrient were shown on Table 2.9.

Table 2.9. Interactions between phytate and nutrients*.

Nutrient	Mode of action
Mineral ions	Formation of insoluble phytate-mineral complex leads to decrease mineral absorption.
Protein	Formation of nonspecific protein-phytate that cannot be hydrolyzed by photolytic enzymes.
Carbohydrate	Formation of phytate-carbohydrate cause less degradable carbohydrates. Also amylase activity is partially inhibited.
Lipid	Formation of lipophytin complex may lead to metallic soap in lumen, which causes low lipid availability.

*Kumar et al., 2010.

Cereals and legume are very rich about phytate content. Therefore, phytate percentage of several cereals is shown in Table 2.10. It can be seen from Table 2.10 that rice and wheat bran have very high phytate content in their structure with more than 70%, whereas sweet potato contents only 24% of phytate. It was estimated that around 14.4 million tons of phytate are produced per year in worldwide production of fruit and seeds. This is equal to 65% of annual sales of phosphorus, which is sold as fertilizer (Kumar et al., 2012).

Table 2.10. Phytate contents of several cereals*.

Cereals	Phytate P (g/ 100 g dry matter)	Phytate P (% of total P)
Corn	0.240	72.0
Wheat	0.270	69.0
Barley	0.270	64.0
Oats	0.290	67.0
Sorghum	0.240	66.0
Rice unpolished	0.270	77.0
Sweet potato	0.050	24.0
Maize	0.180	71.6
Canola meal	0.645	66.4
Cotton seed meal	0.772	77.1
Soya bean meal	0.388	55.9
Rice bran	1.417	79.5
Wheat bran	0.836	76.3

*Haefner et al., 2005; Selle and Ravindran, 2007.

2.7.1. Health Benefits of Phytate

There are some benefits reported regarding phytate on human health. It was shown that phytate has some anti-carcinogenic properties (Harland and Morris, 1995). Formation of Fe-phytate complexes may decrease the Fe-catalysed production of free radicals in the colon (Selle et al., 2000). Phytate can also serve as an antioxidant to reduce free radical formation (Porres et al., 1999). Similarly, it was reported that dietary phytate helps to prevent kidney stone formation (Grases et al., 2000) and protect against coronary heart disease (Jariwalla et al., 1990) and cancer (Vucenik et al., 2003). Additionally, negatively charged myo-inositol polyphosphates can penetrate into plasma and can be internalized by the cells. For example, D-myo-inositol triphosphate (1, 2, 6) was studied and shown to serve an antitumor effect on cancer cells and prevents diabetes complications.

2.7.2. Phytate Removal Strategies

One of the methods to decrease phytate amount in the seeds is soaking in water. Cereals and seeds can be soaked in water for 12 to 16 h at room temperature (Greiner and Konietzny, 1999). Since phytate is water soluble, high amount of phytate removal can be achieved by

soaking in the water. It was also reported that between 26 and 100% of phytate removal from several seeds was achieved at 45-65ºC and 5-6 pH (Greiner and Konietzny, 1999).

Using the natural microflora exogenous microorganisms for fermentation of phytate is also an effective way to remove phytate (Greiner and Konietzny, 1999). Today, defined cultures and processing conditions are used to reduce phytate by fermentation in food industry. In this type of processes, type of starter culture, operating conditions and phytate concentration in the raw materials are important factors on the yield.

Also, addition of external phytase during food processing is a common way for phytate removal. This method is especially used for cereal and legume-derived food products. Heat-stable phytase isolated from *Pichia anomala, Schwanniomyces castellii,* and *Lactobacillus sanfranciscensis* were used in these types of processes. Using phytase isolated from *P. anomala* at 70ºC for 10 minutes resulted in high phytate removal in the legumes (Greiner and Konietzny, 1999).

Moreover, phytase-expressing transgenic plants are also under research to obtain higher phytase concentrations in the seeds comparing to wild type plants. High phytase concentration will save money by not adding external phytase into the process and will improve overall yield of the production (Greiner and Konietzny, 1999).

2.8. Phytase

Phytase, (myo-inositol-hexakisphosphate phosphohydrolase) is an enzyme, which catalyzes the hydrolysis of phytate to inositol and orthophosphoric acid as shown in Figure 2.10 (Liu et al., 1998).

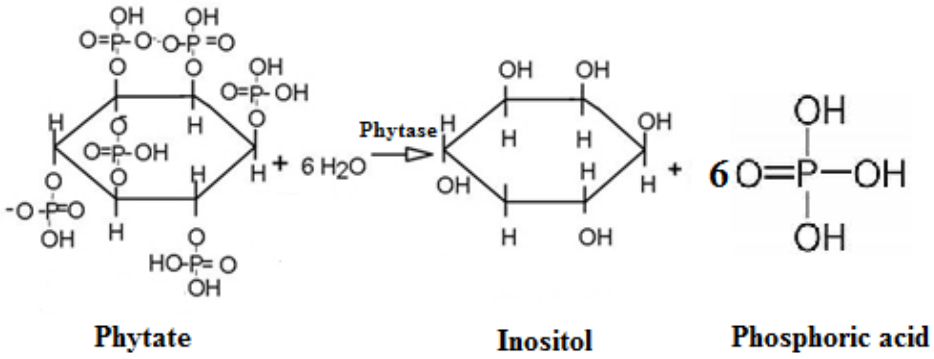


Figure 2.10. Phytate hydrolysis reaction catalyzed by phytase enzyme (Liu et al., 1998).

Phytase can be divided into two categories depending on their hydrolysis activity. 3-phytase (EC 3.1.3.8) liberates phosphorus at the C_3 position, whereas 6-phytase (EC 3.1.3.26) liberates at C_6. It is reported that *A. niger* produces 3-phytase, whereas *E. coli* and *Peniophra lycii* secrete 6-phytase (Wodzinski and Ullah, 1996). Theoretically, phytate hydrolysis occurs in a series from IP_6 till IP_1 and yields in six inorganic phosphorus moieties. However, it should be taken into consideration that the phosphorus residue at the C_2 position is refractory to hydrolysis, compared to other phosphorus. Therefore, hydrolysis of dietary phytate results in five inorganic phosphorus moieties and a myo-inositol monophosphate (IP) (Wodzinski and Ullah, 1996).

2.8.1. Applications of Phytase and Its Importance

Phytase is used mainly in two areas. The first and the most widely used one is phytate elimination in the feed and food industries. The second is myo-inositol phosphate preparation to use for several biochemical investigations.

2.8.1.1. Feed Industry

Plant feedstuffs are commonly used as major constituents in poultry diets. Majority of phosphorus of plant originated feed stuffs is present as phytate a form of phosphorus that is largely unavailable to poultry. Additionally, phytate phosphorus makes problem by chelating several important minerals and reduces their availability.

While ruminant animals sustain the microflora, which enzymatically releases inorganic phosphorus from phytate, monogastric animals such as humans, chickens, and pigs produce little or no phytase in their intestine. Monogastric animals are generally supplied with soybean and other meals to provide necessary nutrients. However, excessive phosphorus accumulation in monogastritic animal manure causes environmental problems such as water pollution, algal blooms, fish kills and changing of fauna and flora (Mullaney et al., 2000).

To solve this problem, microbial phytase can be added into feeds to increase phosphorus availability. However, phytase addition is limited by cost and inactivation of enzyme during pelleting and storage of the feed. In order to overcome these limitations, thermostable phytases with high activity can be produced by microorganisms.

Since phytase is used primarily in the digestion system, it must be optimally active in the pH range prevalent in the digestive tract. Because of this reason, *Aspergillus fumigatus* takes and important role in industrial phytase production. The wild-type *A. fumigatus* phytase gave rise to a phytase with a pH optimum at 2.8 to 3.4 (Tomschy et al., 2002). Also some of the bacterial phytases, especially from the genera *Bacillus* and *Enterobacter*, exhibit a pH optimum in the range from 6.0 to 8.0. Therefore, they are more appropriate to use as feed additives for poultry since their pH optimum is close to the physiological pH of the poultry digestion system (Konietzny and Greiner, 2004).

Phytate removal by phytase was studied by Nelson et al. (1968). They pretreated a corn-soya diet with phytase of *A. niger*. When microbial phytase was fed to low phosphorus diets for broilers, the availability of phosphorus increased to 60%, and the amount of phosphorus in the chicken manure decreased by 50%. Moreover, after 21 days phytase supplementation body weight of male and female chicks increased by 13.2 and 5.8%, respectively. Supplementation of the low phytate diet with phytase also increased the relative retention of total P^{-3}, Ca^{+2}, Cu^{+2}, and Zn^{+2} by 12.5, 12.2., 19.3, and 62.3%, respectively. It has been also reported that phytase usage can reduce phosphorus excretion problem about the environment by up to 50%. Similarly, Simons et al. (1990) reported that addition 1500 FTU/kg phytase in dietary phosphorus (7.5-4.5 g/kg) and calcium (9-6 g/kg) reduced phosphorus by 61% in the manure. Also, Zyla et al. (2001) also showed that phosphorus release decreased by 45% in the broilers manure by addition of microbial phytase and numerical increase in body weight. Moreover, there are several studies have been performed to show the effect of microbial phytase on the growth. Cabahug et al. (1999) showed that addition of 400-800 FTU/kg phytase into the diets increased weight gain by 18.8%, feed intake 9% and feed efficiency 7.9% of the broilers. Also, Cowieson et al. (2004) reported that microbial phytase application into the diets decreased amino acid excretion in the broilers by around 30%. Additionally, amino acid digestibility was increased by 5.1% by phytase addition into the diets. In another study, Kumar et al. (2012) evaluated the effect of phytase on the composition of animal feed. They applied 1000 U of phytase per kg of feed. They reported 61% phosphorus release from commercial feed by application of phytase.

2.8.1.2. Food Additives

Phytase saves feed industry around $2 billion per year by reducing nutritional inputs in the diets. Microbial phytase can be used in the production of phytate free soybean milk. In

legume dephosphorylation processes, generally extracellular phytase from *A. ficuum* was used. A 78% of phytate was lost, when the phytase from *A. niger* was mixed with soybean meal and incubated for 15 h. Moreover, high phytate percentage of commercial whole wheat breads (0.29 to 1.05 % (w/w)) can be decreased by treatment with mold phytase during making dough (Han, 1988). Similarly, Haros et al. (2001) studied the possible usage of phytase in bread making too. They add different levels of fungal phytase in whole wheat breads and noticeable improvements were observed in baking process. The most important one was, fermentation period was shorten without effecting the pH of the dough. Moreover, bread volume increased and crumb texture improved. Additionally, Caransa et al. (1988) reported that steeping required in the wet milling of corn can be accelerated by using microbial phytase. In this way, the properties of corn step liquor can be improved.

Phytase application on complementary foods such as cereals and legume became quite popular to decrease iron deficiency in humans by increasing iron availability (Haefner et al., 2005). Iron deficiency is the most general single micronutrient deficiency around the world. Micronutrient deficiency especially weakens immune system in infants, which results in diarrhea and lower respiratory tract infections. These micronutrient deficiencies are more common in under-developed countries than others because people mainly consume grains and vegetables in these countries. Sandberg et al. (1999) reported that 10 mg phosphorus addition as IP5 to white wheat rolls, resulted in 39% reduction in iron absorption. However, when phytate was dephosphorylated by phytase addition further to IP4 or IP3, iron absorption level became neglectable in vivo.

2.8.2. Microbial Phytase Production

There are several microorganisms, which can secrete phytase and used in industrial productions. Table 2.11 shows common phytase producer microorganisms and productivities.

Table 2.11. Various published phytase productivities*.

Phytase Source	Production Strain**	Phytase Activity (U/ml)	Phytase Productivity (U/l/h)	Phytase Productivity (mg/l/h)
Bacillus sp.		<1	2	
Bacillus amyloliquefaciens	*Bacillus subtilis*		167	
Bacillus licheniformis	*Bacillus subtilis*	28		
Bacillus subtilis		<1	5	
Bacillus subtilis		35		
Citrobacter braakii		1		
Escherichia coli		105	5830	
Escherichia coli		650	7930	
Escherichia coli	*Streptomyces lividans*	950	19792	
Escherichia coli	*Pichia pastoris*	114		
Escherichia coli	*Pichia pastoris*	117	2438	
Escherichia coli	*Pichia pastoris*	4946	25760	
Klebsiella sp.		<1	9	
Klebsiella sp.		2	62	
Lactobacillus amylovorus		146	4562	
Lactobacillus fructivorans		<1	148	
Lactobacillus sanfranciscensis		<1	210	
Megasphaera elsdenii		<1	1	
Mitsuokella jalaludinii		13	1078	
Prevotella ruminicola		<1	4	
Pseudomonas mendocina		<1		
P. putida		<1		
Selenomonas ruminatum		<1	59	
Weissela confusa		<1	139	
Aspergillus sp.		17	177	
Aspergillus awamori		200	1190	
Aspergillus ficuum		15	159	
Aspergillus fumigatus	*Pichia pastoris*	55		
Aspergillus fumigatus	*Aspergillus awamori*	62	369	
Aspergillus fumigatus	*Hansenula polymorpha*			30
Aspergillus niger		7	37	
Aspergillus niger		8	32	
Aspergillus niger		108	643	
Aspergillus niger		1008	4667	
Aspergillus niger	*Escherichia coli*			
Aspergillus niger	*Saccharomyces cerevisiae*	3	186	
Aspergillus niger	*Pichia pastoris*	39	279	30
Aspergillus niger	*Pichia pastoris*	64	593	
Aspergillus oryzae		<1	4	
Aspergillus terreus	*Hansenula polymorpha*			15

Mucor hiemalis		12	160	
Mucor racemosus		26	361	
Rhizopus microsporus		1	18	
Rhizopus oligosporus		5	75	
Rhizopus oligosporus		14	149	
Rhizopus oryzae		6	76	
Rhizopus thailandensis		3	38	
Consensus	*Hansenula polymorpha*			46
Arxula adininivorans		3	63	
Fellomyces fuzhouensis		<1	1	
Pichia anomala		3	63	
Pichia farinose		<1	1	
Rhodotorula gracilis		<1	27	
Schwanniomyces occidentalis		<1	8	
Schwanniomyces occidentalis		<1	9	
Sporidiobolus johnsonii		<1	1	
Sporobolimyces sp.		<1	3	
Sterigmatosporus polymorphum		<1	2	

* Haefner et al., 2005.

** In cases where no production strain is listed, phytase source and production system are identical

2.8.2.1. Bacterial Phytases

Many of phytase producer strains are from *Bacillus, Escherichia, Pseudomonas,* and *Klebsiella* genuses. General characteristics of a bacterial phytase are: The enzyme is active at pH 6.0–6.5 and 60°C, and it is greatly inhibited by addition of EDTA, Zn^{+2}, Ba^{+2}, Cu^{+2}, Cd^{+2}, Fe^{+2}, and Al^{+3} ions. Bacterial phytases have 40-55 kDa molecular weight, which are smaller comparing to fungal phytases because of glycosylation differences (Lei and Porres, 2003). A bacterial strain of *Enterobacter*, which produces extracellular phytase, has been isolated from soil near the root of leguminous plant. When localization of phytase enzyme was studied it was found that the majority of the enzyme activity (82%) was located in the extracellular fraction. A 5% of the activity was detected in the periplasmatic space, and the remaining activity was in the intracellular and cell bound fraction. The enzyme was found to be inhibited by addition of 1 mM Zn^{+2}, Ba^{+2}, Cu^{+2}, Al^{+2}, and EDTA (Liu et al., 1998). Sreeramulu et al. (1996) also investigated various *Lactobacillus* species for phytase production. Among those, *Lactobacillus amylovorus*

produced the highest activity of phytase with 125-146 U/ml. In some studies it was reported that, whereas some microbial phytases have high affinity to phytic acid, plant and some other fungal phytases have broader substrate specificity. These specific phytases can degrade lower inositol phosphates (Wyss et al., 1999). However, phytases from *Bacillus* species, hydrolyze every other phosphate and degrade phytic acid to inositol triphospahte (IP_3) (Kerovuo and Tynkkynen, 2000).

2.8.2.2. Fungal and Yeast Phytases

Isolates have been tested for extracellular phytase production belong to genera *Mucor, Penicillium, Aspergillus*, and *Rhizopus*. Among all the species, *A. niger* was described as the best active fungal phytase producer. All these enzymes exhibited optimum pH at 2.5, 5.0, and 6.0 depending upon the substrate used. Based on a survey of 58 different fungi for the production of extracellular phytase was reported, phytase from *A. ficuum* exhibited the most efficient production. It was reported by *A. niger* PhyA is encoded by a 1.4 kb DNA fragment and has 80kDa molecular weight with 10 N-glycosylation sites (Lei and Porres, 2003). *A. ficuum* can secrete several phosphomonoesterase under phosphate limited medium. One of these acid optimum phosphomonoesterase is known as phytase A (phyA), which shows two pH optima; 5 and 2.5. On the other hand, phytase B (phyB) has only one pH optimum at 2.5. The kinetic parameters of these two enzymes are different, however their active site sequence were very similar. Moreover, it was determined that phyB has a high turnover number and phyA has a low K_m for its preferred substrate, which is myo-inositol hexaphosphate (phytate) (Ullah and Sethumadhavan, 1998). Phytase production from *A. ficuum* has been performed by using three different methods, namely solid state, semisolid, and submerged fermentations. In submerged batch culture, high initial glucose concentration and low aeration rate decrease yield. This problem can be overcome by using fed-batch fermentations. Several studies have shown that wheat bran, soybean meal, corn meal, and rapeseed meal are appropriate phytase production substrates for solid state fermentation systems. The phytase production yield on solid and semisolid medium was higher than submerged conditions. *Aspergillus* species such as *A. amstelodami, A. candidus, A. flavus,* and *A. repens* were also classified as phytase producers. Only a few yeast species such as *Saccharomyces cerevisiae* and *Schwanniomyces castellii* was shown as phytase producers (Liu et al., 1998). Table 2.12 shows several phytase activity values of various *Aspergillus* species. In the literature, one phytase unit (FTU) was described as the amount

of enzyme, which liberates 1 μmole inorganic orthophosphate per minute from 0.0051 M sodium phytate at pH 5.5 and at 37ºC temperature (Selle and Ravindan, 2007).

Table 2.12. Various *Aspergillus* extracellular phytase producer species and activities*.

Strains	Phytase activity (U/ml)
A. niger ATCC 9142	2.1
A. niger ATCC 10864	0.8
A. niger van Tieghem	3.5
A. niger var. cinnamoneum NRRL 348	1.4
A. niger japonicus saito ATCC 1034	0.6
A. niger NRRL 372	1
A. niger NRRL 326	0.7
A. niger NRRL 330	0.8
A. niger NRRL 4361	0.7
A. niger NRRL 337	0.5
A. awamori ATCC 11382	2.3
A. awamori ATCC 11358	3.3
A. saitoi ATCC 11362	1.8
A. carbonarius NRRL 368	1.9
A. carbonarius PCC 104	1.5
A. tubingensis NRRL 4875	2.4
A.ficuum WB 4016	1.1
A. ficuum WB 320	1
A. ficuum WB 364	1.5
A. ficuum WB 4541	1
A. ficuum WB 4781	1.2
A. ficuum NRRL 3135 (soil isolate)	10.5
A. niger X (soil isolate)	5
A. niger K (soil isolate)	4

*Shieh and Ware, 1968.

2.8.3. Characterization of Phytase

2.8.3.1. Catalytic Characterization

Phytase uses phytate as the substrate. This enzyme has been found in plants, animals and microorganisms and releases free inositol, orthophosphate, and intermediary products including the mono-, di-, tri-, tetra-, and pentaphosphate esters of inositol. Therefore, phytase activity can be calculated by measuring amount of inorganic phosphorus liberated in a certain time interval. This measurement is based on a colorimetric assay. Calculation of the phytase activity by

colorimetric assay is based on the measurement of released free phosphorus in the reaction mixture by a colorimetric reagent (Kim et al., 1998). In the literature, there are several studies reported the measurement of phytase activity by colorimetric assay (Soni and Khire, 2007; Papagianni et al., 2001). However, differences in the assay method such as blank composition, reaction pH and temperature, substrate concentration in the reaction mixture, and presence of metal ions in the reaction significantly affect the phytase activity calculations. Therefore, assay conditions should be reported in details for all phytase production studies. Recently, reverse phase C18 HPLC has been used for the determination of phytate and lower inositol phosphates (Hara et al., 1985).

2.8.3.2. Kinetic and Substrate Selectivity

Kinetic parameters of dephosphorylation of phytate have been studied widely. In these studies, the rate of enzymatic hydrolysis generally showed classical Michaelis-Menten kinetics. The liberation of phosphate from phytate by phytase is dependent on substrate concentration. Moreover, inorganic phosphates generally cause product inhibition during the hydrolysis reaction (competitive inhibition). The Michaelis constant (K_m) of mung bean phytase was found to be 0.65 mM, when sodium phytate was used as substrate (Majerus, 1992). Phytases, which were isolated from several other sources have also given similar values. Phytase from *B. subtilis,* canola seed, cotton seed, maize and spelt resulted in 0.5, 0.36, 0.37, 0.117, and 0.4 mM K_m values, respectively. Relatively, lower K_m values have been determined for phytases from *A. ficuum*, maize, wheat bran, *Typha latifolia*, and *S. castellii* as 40, 91, 22, 17, and 38 µM, respectively (Segueilha, 1992).

Cations may serve as inhibitor for phytase activity depending upon the nature of the ion. Table 2.13 shows the effects of cations on phytase activity considering Michaelis constant. Effect of various cations on the activity of phytase purified from *A. ficuum* was studied. No effects were observed for Ca^{+2} and Fe^{+2}; however, enzyme activity increased by 13 and 30% with addition of Co^{+2} and Mn^{+2}, respectively (Ullah, 1988). Also, 20-80 µM Zn^{+2} increased the activity of rat phytase by 40% (Bitar and Reinhold, 1972).

Table 2.13. Effects of several metal ions on phytase activity[*].

Cations	*A. ficuum*	*B. subtilis*	*Enterobacter*	*Maize*	*Pollen (Typha latifolia)*	*S. castellii*	*Soybean*
Al^{3+}	-	I	I	-	-	-	N
Ba^{2+}	-	I	I	-	-	-	-
Ca^{2+}	N[**]	S	-	S	S	M	N
Co^{2+}	S[**]	I	-	-	I	-	N
Cu^{2+}	I[**]	I	I	-	M	I	I
Fe^{2+}	N	I	-	I	-	I	N
Hg^{2+}	-	I	-	-	-	-	I
Mn^{2+}	S	I	I	N	I	I	M
Mg^{2+}	-	I	M[**]	N	N	M	M
Sr^{2+}	-	I	-	-	-	-	-
Zn^{2+}	I	I	I	I	-	I	I

[*]Liu et al., 1998.

[**]Stimulatory: S, Inhibitory: I, Moderately Inhibitory: M, No effect: N, Not determined.

Similarly, Salmon et al. (2012), studied the effect of various metal ions on phytase excreted from *Schizophyllum commune.* Results were shown on Table 2.14 that $MnSO_4$ served as the best activator. When 1 mM ammonium molybdate was used, activity of enzyme was observed as zero.

Table 2.14. Effect of metal ions on *Schizophyllum commune* phytase[*].

Ion concentration (1 mM)	Relative phytase activity (%)
Control	100
$MnSO_4$	369.7
K_2SO_4	356.5
$CaSO_4$	342.9
$MgSO_4$	315.9
$Fe_2(SO_4)_3$	308.4
Na_2SO_4	294.8
$FeSO_4$	293.1
$NiSO_4$	285.4
$CuSO_4$	274.4
$ZnSO_4$	211.6
$CoSO_4$	120.4
NaCl	102.8

[*]Salmon et al., 2012.

2.8.3.3. Optimum Temperature and pH Value for Phytase Activity

Since phytases are also used as animal feed supplements, they should be able to withstand temperatures of 60 to 90°C, which may be reached during the feed pelleting process. Table 2.15 shows optimum temperature and pH values of phytase purified from various microorganisms.

Table 2.15. Physico-chemical properties of several purified phytases[*].

Microorganism	Optimum temperature (ºC)	Optimum pH	Km (mM)
Aerobacter aerogenes	25	4-5	0.135
Bacillus sp. DS 11	70	7	0.55
Bacillus subtilis	55	7	0.04
Enterobacter spp. 4	50	7-7.5	-
Escherichia coli	55	4.5	0.13
Klebsiella aerogenes	60	4.5-5.2	0.11
Klebsiella oxytoca	55	5-6	-
Lactobacillus amylovorus	45	4.4	-
Pseudomonas spp.	40	5.5	0.016
Selenomonas ruminantium	50-55	4-5.5	-
Aspergillus carneus	40	5.6	-
Aspergillus carbonarius	53	4.7	-
Aspergillus niger 92	55	5.0	0.44
Aspergillus terreus	70	4.5	-
Aspergillus niger NRLL 3135	58	2.2	0.04
Neurospora spp.	60	5-6	-
Penicilium caseoicolum	45	3	-
Rhizopus oligosporus	55	4.5	0.15
Arxula adeninivorans	75-80	4.5	0.25
Candida intermedia	65	4.5	-
Candida tropicalis	65	4.5	-
Clavispora lusitaniae	70	4.0	-
Hanseniaspora valbyensis	60-65	4-5	-
Kluyveromyces thermotolerans	60-65	4-5	-
Pichia anomala	60	4	0.2
Pichia rhodanensis	70-75	4-4.5	0.25
Pichia spartinae	75-80	4.5-5.5	0.33
Schwanniomycess occidentalis	75-80	4-5	0.038

[*]Vohra et al., 2003.

2.9. Effect of Microparticles on Fungal Fermentation

Fungal morphology is hard to be controlled during the process. Many factors such as rheology, strain properties, and process variables are effective on morphology chancing. The cultivation of filamentous microorganisms is commonly accompanied by problems, such as clumpy growth and insufficient mass transfer, which result in reduced productivity (Kaup et al., 2008). It was found out by Kaup et al. (2008), talcum and alumina microparticles can strongly influence morphological development of *A. niger*. In another study, effect of titanate microparticle on fructofuranosidase and glucoamylase production with *A. niger* in shake-flasks. It was reported that, fructofuranosidase and glucoamylase production increased by 3.7 (150 U/ml) and 9.5 fold (190 U/ml), respectively, compared to the control. Also, *A. niger* was grown in the presence of 0-50 g/L $TiSiO_4$ and the highest glucoamylase and fructofuranosiade productions were performed under 25 g/L concentration. Pellet diameter was also measured as 1.7 mm, when $TiSiO_4$ was not added into the fermentation medium, whereas diameter of pellet decreased to 0.3 mm, when 25 g/L concentration of $TiSiO_4$ was used (Driouch et al., 2012). In another study with *Caldariomyces fumago* showed that particles around 500 µm diameter did not result in any difference in growth morphology and production in chloroperoxidase production. However, particles smaller than 42 µm diameters dispersed *C. fumago* to single hypae and enzyme production enhanced 5 folds to 1000 U/ml. Additionally, *C. fumago* mycelia diameter was measured as 0.1-0.5 mm in the presence of aluminum oxide or talcum, whereas ~4 mm in the control group. Furthermore, it was reported that presence of 0.5 to 10 g/L talcum enhanced enzyme production, however higher than this concentration, enzyme formation decreased, but produced still in higher levels comparing to control group. In contrast to talcum, no significant decrease was observed, when aluminum oxide was used in 10-15 g/L concentrations (Kaup et al., 2008). Similar to talcum, increase in the concentration of aluminum oxide also decreased mycelium size. However, higher concentrations were needed to achieve the same level with talcum. This might be attributed to the particle size since talcum particles has ~6 µm diameter size, whereas alumina ~14 µm (Driouch et al., 2011). Further microscopic studies showed that the main effect of microparticles is related to the interference with the aggregation of conidia during the initial phase of fermentation. Microparticles prevent the formation of large aggregates in the initial times and also reduced the formation of new ones. It was also determined that rather than chemical properties, physical properties (particle size and shape) are more in morphology development (Driouch et al., 2010). Amoung the various microparticles, several characteristics of aluminum oxide and talcum are shown on Table 2.16.

Table 2.16. Physical characteristics of talcum and aluminum oxide microparticles*.

Characteristic	Talcum*	Aluminum oxide**
Color	White	White
Density	2.7-2.8 g/cm^3	3.98 g/cm^3
MW	379.27 g/mol	101.96
Particle size	< 10 µm	63-200 µm
Molecular formula	$3MgO.4SiO_2.H_2O$	Al_2O_3
Hardness	47 kg/mm^2	1440 kg/mm^2

*Sigma Aldrich

** Baker Analyzed

Both microparticles are white and have higher densities compared to water. Therefore, when agitation is stopped, they tend to settle down in the water-based fermentation broths, which decrease the work during separation and purification steps. Also, talcum size is smaller than 10 µm, whereas aluminum oxide has a ranged particle size between 63 to200 µm (Table 2.16).

2.10. Fermentation Modes

2.10.1. Batch Fermentation

The batch reactor is a generic term for a type of vessel widely used in the industrial processes. The advantage of the batch reactor lies with its versatility. A single vessel can carry out a sequence of different operations without the need to break containment. "Batch fermentation'' term indicates a closed system without any inlet or outlet streams, as nutrients are prepared in a fixed volume of liquid media. In a batch fermentation, the inoculum is transferred into the reactor and then the microorganisms gradually grow and replicate (Najafpour, 2007). Figure 2.11 shows a typical batch fermentation scheme.

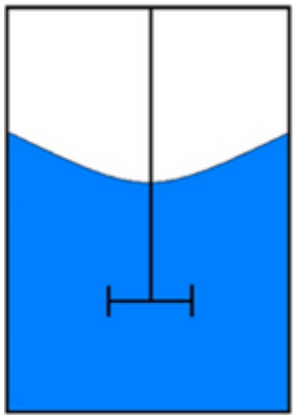


Figure 2.11. Batch fermentation scheme (Montano, 2013).

Advantages of batch fermentation:

- Easy to operate.
- The reactor can be used for various applications.

Disadvantages of a batch fermentation:

- Moderate yield and productivity.
- Risk of substrate of product inhibition.
- Problems with catabolite repression.

Production of alpha keto acids are commonly performed in shake-flask fermentations. However, there are a few studies that alpha keto acids were performed in batch bioreactors. As an example, Takagi et al. (2009) studied production of 2-keto-L-gulonic acid with a mixed culture of *Ketogulonicigenium vulgare DSM 4025* and *Bacillus megaterium* or *Xanthomonas maltophilia.* In that study, 90 g/L of 2-keto-L-gulonic acid production was reported in the bioreactors. Also, Chernyavskaya et al. (2000) produce alpha keto glutaric acid by *Yarrowia lipolytica* reported production as 40 g/L. Additionally, Sawai et al. (2011) studied the pyruvic acid production with *Torulopsis glabrata* NBRC 0005 in batch fermentations and reported 41 g/L concentration at 49 h of fermentation.

There are several phytase productions performed in batch fermentations in the literature. For example, Kumar et al. (2012) produced 8.84 U/ml phytase activity *Achromobacter* spp. PB-

01 in batch fermentations. Also, Shah et al. (2009) reported 80 IU/ml phytase activity in submerged batch fermentations with mutated *Aspergillus niger* NCIM 563 after optimization of the fermentation medium. In another batch phytase fermentation with *A. niger* NCIM 56 was performed by Bhavsar et al. (2013) and they reported 407,200 U/l phytase activity after performing modifications on the fermentation medium. Additionally, Liu et al. (2011) increased *Pichia pastoris* phytase activity from 73.31 U/ml to 161.64 U/ml by optimization of the submerged fermentation medium.

2.10.2. Fed-Batch Fermentation

In fed-batch fermentation, fermentation medium is fed periodically but there is no outflow in the bioreactor. Fed-batch fermentation is used to overcome the substrate limitations, which is especially important for the production of antibiotics. Additionally, fed-batch fermentations are needed to avoid substrate inhibition, which can allow a periodic shift of the growth rate (Najafpour, 2007). Figure 2.12 shows a fed-batch fermentation scheme.

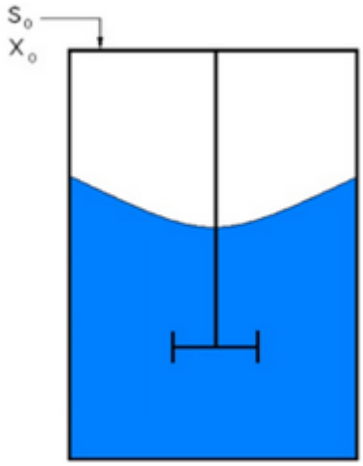


Figure 2.12. Fed-batch fermentation scheme, where S_o and X_o are substrate and biomass concentrations in the feed flow, respectively (Montano, 2013).

Advantages of fed-batch fermentation:

- High cell concentration.
- High yield and productivity.

Disadvantages of a fed-batch fermentation:

- Requires high skills in production.
- Requires accessory instruments such as pump and sensor.

Studies for fed-batch alpha keto acid productions are very limited. Lee et al. (2013) studied fed-batch production of alpha ketoglutaric acid with *Corynebacterium glutamicum* in a 5-L jar fermenter. They maintained the glucose concentration in the fermenter between 1-8% and produced 19.5 g/L alpha ketoglutaric acid by, whereas concentration was only 12.4 g/L in batch fermentations.

A few fed-batch studies were performed about microbial phytase production. For example, Jin et al. (2007) set an artificial neural network pattern recognition (ANNPR) model for fed-batch production with recombinant *Pichia pastoris* phytase. In this study, they maintained the methanol concentration in the reactor between 0-20 g/L to induce cell growth and phytase production. They reported that, phytase activity reached to 226 U/ml at 105 h of fermentation, which is more than four-fold higher compared to their previous studies. Another fed-batch phytase production was studied by Kleist et al. (2003). In this study, phytase production with *Escherichia coli* was enhanced by keeping the glucose concentration constant at low oxygen levels. They reported 120 U/ml phytase in a short fermentation time (14 h) under 5-10% oxygen concentrations.

2.10.3. Continuous Fermentation

In continuous fermentation, an open system is set up. Sterile nutrient solution is added to the bioreactor continuously with a dilution rate (D) flow and an equivalent amount of converted nutrient solution with microorganisms is simultaneously removed from the system (Najafpour, 2007). Figure 2.13 shows a continuous fermentation scheme.

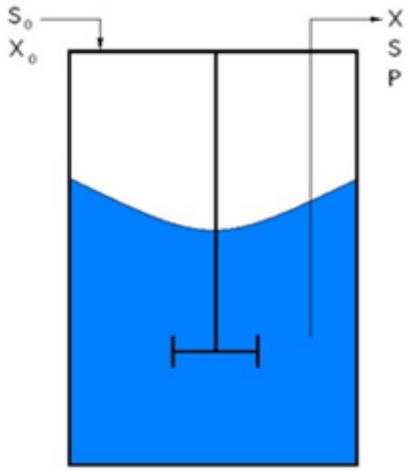


Figure 2.13. Continuous fermentation scheme, where S_o and X_o are substrate and biomass concentrations in the feed flow, X, S, and P are biomass, substrate, and product concentrations in the outflow, respectively (Montano, 2013).

Advantages of continuous fermentation:

- Continuous process.
- Highest productivity.

Disadvantages of continuous fermentation:

- Requires high skills in production.
- Drift in process parameters.

Sawai et al. (2011) evaluated a membrane-integrated continuous system to produce pyruvic acid with *Torulopsis glabrata* in a 2-L bioreactor. They reported that the volumetric productivity of pyruvic acid increased to 4.2 g/l/h, which was four times higher than batch fermentations. Also, the highest pyruvic acid concentration was reported as 36 g/L.

Hidayat et al. (2006), studied continuous acid phosphatase production by *Aspergillus niger* N402A. They showed that *A. niger* produced the highest specific acid phosphatase activity at 0.04 h^{-1} dilution rate as 150 U/g. However, the highest specific acid phosphatase productivity was measured as 7.5 U/g/h at 0.13 h^{-1} dilution rate. They also reported that acid phosphatase production was growth related for specific growth rates between 0.07 and 0.13 h^{-1}.

2.11. Modelling of Fermentation Processes

Mathematical models allow the prediction of microbial growth in the fermentation and food safety issues (Teleken et al., 2010). They are used to describe the behavior of microorganisms and product formation under different physical or chemical conditions such as temperature, pH, and water activity (Zwietering et al., 1990). Primary models predict the microbial growth under constant environmental conditions and secondary models describe the effect of environmental conditions on parameters estimated from the primary model. Primary models such as Gompertz and logistic models are the most common prediction models, which are used for microbial growth and product formation in fermentation. Additionally, Baranyi model is getting popular among the researchers for the prediction of microbial growth (Zhao et al., 2014). Among the several models, modified Gompertz model and modified logistic model are the most commonly used mathematical models are shown below.

Modified Gompertz model: $X_t = X_m \exp\left\{-\exp\left[\frac{\mu_m e}{X_m}(\lambda - t) + 1\right]\right\}$

Modified logistic model: $X_t = \frac{X_m}{\left\{1+\exp\left[\frac{4\mu_m}{X_m}(\lambda - t)+2\right]\right\}}$

where X_t is the concentration of biomass at time "t", X_m is maximum biomass concentration, μ_m is the maximum specific growth rate, λ is the duration of lag phase, and t is the sampling time.

Zhao et al. (2014) modelled the growth of *Proteus mirabilis* under various isothermal conditions by primary models. They reported that Baranyi model performed the best fitting the growth date and followed by the logistic and modified Gompertz model. Mean error square (MSE) values were calculated as 0.038, 0.032, and 0.010 for Gompertz, logistic, and Baranyi models, respectively. Additionally, the maximum growth rate estimated from the each primary model was fitted as a function of temperature by using modified Ratkowsky model. It was reported that the secondary models derived from the modified Gompertz and Baranyi models predicted the microbial growth more successfully from the logistic model.

Additionally, Dufosse et al. (2001) used Gompertz model to evaluate the effect of nitrogenous substrates on microbial growth. They used fish peptones form tuna, cod, salmon and unspecified fish to compare with casein to see the effect on six bacteria species, yeasts, and fungi.

They resulted that fish peptones are significantly effective on microbial growth. Similarly, Gompertz model was used by Pongtharangkul et al. (2006) to model nisin production in the bioreactors. Lv et al. (2005) used logistic model to describe *Lactococcus lactis* growth for fed-batch nisin production. Currently, there is no study in the literature available for the modeling of alpha keto acid production.

There are also several modeling studies were conducted in the literature about fungal fermentations. Marin et al. (2012) studied growth of *Aspergillus flavus* and aflatoxin production on pistachio under different moisture and temperature conditions. Baranyi model was used to predict maximum radial growth rate and lag phase. They reported that among the assayed models, cardinal models gave a good quality of fit for radial growth rate data.

Also, Gougouli and Koutsoumanis (2010) studied modeling growth of *Penicillium expansum* and *Aspergillus niger* at constant and fluctuating temperature conditions. They reported Cardinal model with inflection satisfactorily described the effect of storage temperature on the mycelium growth of *P. expansum* and *A. niger*.

Modeling of potato starch saccharification in batch reactor by an *Aspergillus niger* glucoamylase was studied by Polakovi and Bryjak (2004). They studied six different models for saccharification process. They reported the best fitted prediction was obtained with a six-parameter model, which contains also product and substrate inhibition terms.

2.12. Summary of Literature Review

Amino acids are important compounds for the organisms, which are necessary to produce proteins, enzymes and to maintain the overall metabolism. Amino acids and broken down in metabolic pathways, such as Krebs cycle to their precursors, which are called alpha keto acids. Alpha keto acids are the deaminated form of amino acids and contain carboxylic acid and ketone functional group (Lehninger et al., 2000).

Alpha keto acids are also important compounds and have several application areas in medicine, agriculture, food and feed industry. Alpha keto acids can be replaced for amino acids in the diets to reduce nitrogen accumulation in the body, especially for the kidney patients (Krause et al., 2010). Also, alpha keto acids are used in the diagnosis of several diseases such as maple syrup urine and phenylketonuria. Additionally, they can be used in poultry as nutrient supplement

to prevent excessive nitrogen accumulation in the manure, which has a negative effect on the environment. Furthermore, alpha keto acids have commercial importance in food industry too. Alpha keto acids serve as powerful flavor enhancers especially in cheese, wine, and smoked food production (Delgado et al., 2008). PPA is alpha keto acid of phenylalanine. Similar to other alpha keto acids, PPA is also used for food, medicine, and agricultural applications. However, it was reported that PPA is more effective on food industry as a flavor enhancer compared to other alpha keto acids (Ganesan et al., 2004).

Alpha keto acids can be produced either by chemical or biological reactions. Biological reactions are currently under attention because of environmental properties. Amino acids can be converted to their specific alpha keto acids by enzymes such as deaminase, transaminase, and oxidase. These enzymes can be secreted by several microorganisms, and alpha keto acids can be produced in the bioreactors (Massad et al., 1995). Among several microorganisms, *Proteus* genus is under attention for deaminase production ability. It was reported that *P. vulgaris* and *P. mirabilis* species have remarkably higher phenylalanine deaminase activity comparing to *P. morganii*, *P. rettgeri* and *P. providence* strains (Henriksen, 1950).

To date, PPA production studies were performed using microbial deaminase enzymes. However, most of these studies were done in shake-flasks and they are not scalable for the commercial production. As a result of this, PPA production cannot keep up with the demand. Therefore, this study was undertaken to enhance production of PPA in submerged fermentation. Several objectives are set to improve PPA production in bioreactors. In Chapter 3 microbial selection was performed among several species to identify the best yielded PPA producer. Also, growth parameters such as temperature, pH, and aeration were optimized to increase PPA production in bioreactors. Thereafter, important fermentation medium components such as glucose, yeast extract, and phenylalanine concentrations were optimized in the bioreactors as well (Chapter 4). Also, effects of fed-batch addition of phenylalanine and continuous fermentations on PPA production were evaluated in Chapter 5. Finally, optimum batch PPA fermentation process was modeled in Chapter 6. These models can be used for monitoring, predicting, and optimizing of PPA production parameters during fermentation.

There are several applications were performed for decomposition of phytate, which serves as an antinutrient for monogastric animals. Phytase is the enzyme, which catalyzes the hydrolysis of phytate to inositol and orthophosphoric acid (Liu et al., 1998). Phytase has being used commercially in animal and human diets recently to improve the availability of several

minerals and amino acids. Additionally, phytase has been started to use in bakery to decrease fermentation period and improve bread volume and crumb texture. Another important application of phytase is the elimination of phosphorous pollution that caused because of low utilization of phytate by monogastric animals (Selle and Ravindan, 2007).

There are several studies showed that phytase can be secreted by several microorganisms. Bacteria, yeast, and fungi were studied for microbial phytase production in the literature. Among these microorganisms, fungal phytase is under attention. Especially, *Aspergillus* species were mentioned as the important phytase producers in several studies.

In the literature, fungal phytase productions have been commonly performed in solid state fermentation with small productions and lack of optimization. Therefore, this study was undertaken to enhance submerged fungal phytase fermentation by optimizations of fermentation conditions and using various fermentation methods.

The first objective of this study was to perform a microbial selection to determine the most productive microorganism by using selective agar tests and performing shake-flask fermentations. Later, growth parameters such as temperature, pH, and aeration were optimized in the bioreactors to increase phytase production (Chapter 7). Thereafter, glucose, phytate, and $CaSO_4$ concentrations were optimized in the bioreactors to further enhancement of phytase production (Chapter 8). Additionally, fed-batch fermentations were implemented to improve phytase production (Chapter 9). Moreover, as a novel technique, effect of microparticle on fungal phytase production in shake-flask and batch fermentations were described (Chapter 10). Also, effect of microparticle on phytase productions in fed-batch and continuous fermentations were studied (Chapter 11). Finally, batch phytase fermentation data, which were obtained under the optimum conditions, were modeled (Chapter 12). These models can be used for monitoring, predicting, and optimizing of production parameters during fermentation.

CHAPTER 3

SCREENING OF PHENYLPYRUVIC ACID PRODUCERS AND OPTIMIZATION OF CULTURE CONDITIONS IN BENCH SCALE BIOREACTORS

3.1. Abstract

Alpha keto acids are deaminated forms of amino acids that have received significant attention as feed and food additives in the agriculture and medical industries. To date, their production has been commonly performed at shake-flask scale with low product concentrations. In this study, production of phenylpyruvic acid (PPA), which is the alpha keto acid of phenylalanine was investigated. First, various microorganisms were screened to select the most efficient producer. Thereafter, growth parameters (temperature, pH, and aeration) were optimized in bench scale bioreactors to maximize both PPA and biomass concentration in bench scale bioreactors, using response surface methodology (RSM). Among the four different microorganisms evaluated, *Proteus vulgaris* was the most productive strain for PPA production. Optimum temperature, pH, and aeration conditions were determined as 34.5^{o}C, 5.12, and 0.5 vvm for PPA production, whereas 36.9^{o}C, pH 6.87, and 0.96 vvm for the biomass production. Under these optimum conditions, PPA concentration was enhanced to 1054 mg/L, which was almost three times higher than shake-flask fermentation concentrations. Moreover, *P. vulgaris* biomass was produced at 3.25 g/L under optimum conditions. Overall, this study demonstrated that optimization of growth parameters improved PPA production in bench scale bioreactors compared to previous studies in the literature and was a first step to scale up to industrial production.

3.2. Introduction

Alpha keto acids have general formula as RCOCOOH, where R can be aryl, alkenyl or heterocyclic group. They are important intermediate compounds for the production of amino acids, food additives, pharmaceuticals, and agricultural chemicals (Masato et al., 1986). Recently, greater attention has been given to the biological function and metabolism of alpha keto acids, due to the fact that these compounds can regulate protein turnover. The concentrations of these

compounds in blood and urine allow one to follow the physiological condition of human, maintain their nutritional intake, and develop new treatments against some diseases (Pialla et al., 2000).

Phenylpyruvic acid (PPA) is the alpha keto acid form of phenylalanine and used in several areas. In medicine, it has been used for diagnosis of phenylketonuria, which is a genetic disease involving phenylalanine metabolism (Folling, 1994). It is also used in diets of kidney patients instead of amino acids to reduce urea accumulation in their body without changing nutritional intake (Krause et al., 2010). Interest in natural products such as flavor enhancers has increased by consumers in recent years. Like other alpha keto acids, PPA is becoming popular as a flavor enhancer in food formulations. It was also reported that rather than phenylalanine, PPA is more important in development of specific smell, taste, and texture in cheese and wine manufacturing (Casey et al., 2004). Moreover, PPA has greater advantages compared to other alpha keto acids such as alpha ketoisovaleric acid and alpha ketoisocaproic acid usage, which are the alpha keto acids of valine and leucine, respectively (Gaby and Chawla, 1976). Moreover, PPA is also used in poultry feed as a supplement to prevent excessive nitrogen accumulation in the manure. Excessive nitrogen excretion in manure can have an adverse effect on the environment and it is costly for farmers to dispose. In response, poultry growers could add alpha keto acids to diets of chickens instead of amino acids in order to reduce nitrogen accumulation in the manure (Summer, 1993).

Like all alpha keto acid production, the first step in PPA production is the removal of amino groups, in this case from phenylalanine via deaminase enzymes. These enzymes are secreted by several microorganisms including *Proteus*, *Providencia*, and *Morganella* species. *Proteus vulgaris*, *Proteus rettgeri*, *Proteus morganii*, and *Providence* strains were studied in terms of their phenylalanine deaminase activities (Smit, 1966). It was shown that all *P. vulgaris* strains produced the highest deaminase activity, whereas *P. morganii* and *Providence* strains produced lower activities.

Several studies have been conducted to produce alpha keto acids by using microbial deaminases. Production of 2-keto-L-gulonic acid was studied using a mixed culture of *Ketogulonicigenium vulgare* DSM 4025 and *Bacillus megaterium* or *Xanthomonas maltophilia* (Takagi et al., 2009). In this study, fermentation medium consisted of 120 g of L-sorbose as carbon source and 2 g of yeast extract as nitrogen source and 10% inoculum. Under these conditions 90 g/L 2-keto-L-gulonic acid production was reported (Takagi et al., 2009). Also,

several yeast species including *Yarrowia lipolytica* were utilized to produce alpha keto glutaric acid. It was reported that alpha keto glutaric produced with a genetically modified *Yarrowia lipolytica* up to 40 g/L, when ethanol was used as carbon source, ammonium sulfate as nitrogen source and thiamine as growth supplement. It was reported that the highest alpha keto glutaric acid concentration was achieved, when 10 g/L of ammonium sulfate and 2-4 µg/L of thiamine was used in the fermentation medium (Chernyavskaya et al., 2000). Additionally, *Zygosaccharomyces rouxii* was used in a medium, which includes 20 g of glucose and 6.7 g of yeast nitrogen base, and 5 g of ammonium sulfate to produce ketoisovaleric acid (35 mg/L), which is the alpha keto acid of valine in shake-flasks (Sluis et al., 2001). Production parameters of several alpha keto acids using oral *Eubacterium* species were also studied (Itoh et al., 1994). *Eubacterium* species were precultured in brain heart infusion broth, which was supplemented with 5 mg/L of haemin, 0.5 mg/L of vitamin K, and 5 g/L of yeas extract. Thereafter, *Eubacterium* species were cultured in 3 ml tubes, which includes 2 µmole/ml of amino acids. In this study, 5.3 mg/L of PPA, 10.9 mg/L of alpha ketoglutaric acid, 2.4 mg/L of pyruvic acid, and 0.62 mg/L of alpha ketobutyric acid were produced by using *Eubacterium nodatum* (Itoh et al., 1994). Black pigmented oral species of *Bacteroides* were also evaluated in Brucella broth, which was supplemented with 5 mg/L of haemin and 10 mg/L of vitamin K and found to produce alpha keto acids as well. Several amino acids were added in to the fermentation medium and the corresponding alpha keto acids were produced in the late logarithmic phase as 14.7 mg/L of alpha ketoglutaric acid, 9.2 mg/L of pyruvic acid, 10 mg/L of alpha ketobutyric acid, 22.3 mg/L of alpha ketovaleric acid, 9.8 mg/L of alpha ketoisovaleric acid, 12.9 mg/L of alpha ketoisocaproic acid, and 15.8 mg/L of PPA (Tsuchiya et al., 1990). They also reported that aromatic alpha keto acids such as p-hydroxyphenly-pyruvic acid and PPA were both produced by *B. vingivalis*, *B. endodontalis* and *B. loescheii*. Also, it was determined that, the branched-chain alpha keto acids, alpha ketoisovaleric acid, alpha ketoisocaproic acid, and alpha keto beta methyvaleric acid were produced at higher levels compared to other alpha keto acids (Tsuchiya et al., 1990).

To date, alpha keto acid production including PPA have been mostly performed in shake-flask fermentation with low yields (Sluis et al., 2001; Itoh et al., 1994; Tsuchiya et al., 1990). As it is well known that among shake-flasks, working volume is always limited and growth parameters such as pH, aeration, and mechanical stirring cannot be effectively controlled, which makes shake-flask fermentations difficult to scale up to industrial levels (Shuler and Kargi, 1992). Therefore, there is a need to scale-up and optimize the fermentation conditions for enhancing PPA production in order to make it more economical for industrial production. Therefore, the aim

of this study was undertaken to select the most productive PPA producer strain among several microorganisms, which have been reported as PPA producer in the previous studies in the literature and to enhance PPA concentration in the fermentation medium in bench-top bioreactors by the optimization of the growth parameters including temperature, pH, and aeration using the Box-Behnken RSM.

3.3. Materials and Methods

3.3.1. Microorganisms and Media

Zygosaccharomyces rouxii (Y-12622), *Proteus vulgaris* (B-123), *Morganella morganii* (B-1663), and *Croynebacterium glutamicum* (B-2784) were obtained from the USDA Agricultural Research Service Culture Collection (Peoria, IL). *Proteus vulgaris,* was grown in a test tube containing10 ml of trypticase soy broth (TSB) at 37^{o}C for 24 h. *Morganella morganii*, and *Croynebacterium glutamicum* were grown in 10 ml of TSB at 28^{o}C for 24 h and *Zygosaccharomyces rouxii* was grown in 10 ml of a medium containing 3 g/L of yeast extract, 3 g/L of malt extract, 5 g/L of peptone, and 10 g/L of glucose in deionized water at 24^{o}C for 24 h. After incubation, they were stored at 4^{o}C as working cultures. All cultures were regularly transferred to sterile fresh media every 2 weeks to maintain viability. Stock cultures for future studies were kept frozen in 20% glycerol at -80^{o}C.

Phenylalanine deaminase selective agar slants were prepared as described earlier to perform microorganism screening (Ederer et al., 1971). This medium includes 3 g of yeast extract, 2 g of DL-phenylalanine, 1 g of Na_2HPO_4, 5 g of NaCl, and 12 g of agar per litter of DI water. The pH was adjusted to 7.3 and the medium was autoclaved for 15 min at 121^{o}C.

3.3.2. Screening of Strains for PPA Production

Z. rouxii, *P. vulgaris*, *M. morganii*, and *C. glutamicum* were selected for microbial screening. One ml of 24-h grown culture, which has ~10^6 cell/ml was used to inoculate on the phenylalanine deaminase selective agar slants. Inoculated slants were incubated at their specific growth temperature for 24 h. After incubation, five drops of 10% ferric chloride solution was added to the slants and mixed gently. The occurrence and the darkness of the green color was observed as an indication of microbial deaminase activity (Ederer et al., 1971).

In order to validate selective agar results, shake-flask PPA fermentations were also performed for each strain. The fermentation medium consisted of 80 g of glucose, 8 g of yeast extract, 5 g of NaCl, 1 g of K_2HPO_4, 0.2 g of $MgSO_4$, and 5 g of L-phenylalanine per liter of deionized water. pH was adjusted to 7 and flasks were autoclaved for 15 min. Flasks containing 100 ml of the medium were inoculated with 1% (v/v) 30 h grown inoculum, which had ~10^6 cells/ml and incubated at 37ºC for *P. vulgaris*, 28ºC for *M. morganii*, and *C. glutamicum* and 24ºC for *Z. rouxii* for 96 h. Samples were taken every 5 h until 30 h of fermentation and then every 10 h until 96 h of the fermentation. Then samples were analyzed for PPA concentrations.

3.3.3. Batch Fermentation in Bioreactors and Optimization of Growth Parameters

Selected microorganism strain (*P. vulgaris*) was used for bench-scale fermentations and growth conditions were optimized to obtain maximum PPA production. Sartorious Biostat B Plus bioreactors (Allentown, PA) were used in the experiments equipped with a 2-L vessel and 1-L working volume. The same fermentation medium was also used in bench-top bioreactors. Batch fermentations were conducted to optimize the fermentation medium according to three factors of temperature, pH, and aeration using a Box-Behnken RSM (Table 3.3) and MINITAB statistical software (Version 15, State College, PA). Temperature (28-40°C), pH (4-7), and aeration rate (0-1.5 volume of air/volume of broth/minute (vvm) were evaluated as growth factors. Temperature and pH intervals were based on a review of literature while aeration interval was determined from preliminary experiments (Chernyavskaya et al., 2000; Takahashi et al., 1999). pH values, which are mentioned on Table 3.3, were maintained as the same during the fermentations with 4 N NaOH and 2 N H_2SO_4. Also, agitation speed was maintained to 300 rpm for all fermentation runs. *P. vulgaris*, which was grown in 100 ml TBS at 37ºC and 200 rpm for 24 h was used as the inoculum. Fermentations were started by adding 1% (v/v) inoculum, which had 10^6 cells/ml, then samples were taken every 5 h until 30 h of fermentation and thereafter every 10 h until 96 h of fermentation. Samples were analyzes for PPA, phenylalanine, and glucose concentrations.

3.3.4. Validation of the Model

Fermentations with three replicates were carried out to validate the recommended optimum conditions of the RSM. Results, from fermentations, were compared with predicted

values from the model. If the values were close to each other with a minor standard deviation and coefficient of variation, the model will be accepted as representative.

3.3.5. Analysis

Collected samples were centrifuged at 5,200 x *g* for 15 min to remove the biomass. Then, the supernatant was used for analysis of PPA, and phenylalanine concentrations and samples without centrifugation were used to determine biomass concentrations as explained below.

3.3.5.1. Biomass

Absorbance of the samples was measured at 620 nm using a spectrophotometer (Beckman Coulter, Fullerton, CA) to determine microbial concentration in the broth. Sterile fermentation medium was used as the blank for the measurement and absorbance was converted to g biomass/L using the standard curve.

3.3.5.2. Phenylpyruvic Acid

Cell-free samples were derivatized with 2,4-Dinitrophenylhydrazine (DNPH) as described in the literature (Elias et al., 2008). Derivatization solution was prepared as follows: 0.2 g of DNPH was dissolved in 100 ml acetonitrile and 4 ml of 70 wt % perchloric acid was added to the solution. The sample (0.1 ml) was mixed with 0.24 ml of DNPH solution and 0.04 ml of 25% (w/v) H_2SO_4 solution. The mixture was incubated for 1 h at 60^{o}C. After derivatization, 0.48 ml of a 60:40 acetonitrile:water mixture was added to the mixture and filtered into high performance liquid chromatography (HPLC) vials using 0.2 μm polytetrafluoroethylene (PTFE) filters (VWR, Radnor, PA). Concentrations of PPA were measured using HPLC with an UV detector at 365 nm (Waters, Milford, MA). Separation was achieved using a RP-C18 column (Sigma-Aldrich, St. Louis, MO) with a 1 ml/min gradient flow of solvent A: 25 mM phosphate buffer (pH 2.2) and B: acetonitrile as described in Table 3.1.

Table 3.1. HPLC gradient flow table for phenylpyruvic acid determination.

Time (min)	Flow (ml/min)	%A	%B	Curve
0.01	1.00	90.0	10.0	6
1.50	1.00	90.0	10.0	6
20.00	1.00	10.0	90.0	6
25.00	1.00	90.0	10.0	1
30.00	1.00	90.0	10.0	6

3.3.5.3. Phenylalanine

Samples were derivatized with o-phthalaldehyde (OPA) to determine phenylalanine concentrations. The cell-free samples (0.1 ml) were mixed with 1 ml of OPA and filtered into HPLC vials using 0.2 μm PTFE filters. Phenylalanine concentrations were measured using HPLC with a UV detector at 230 nm (Waters). Measurement was performed by using RP-C18 column with 1 ml/min gradient flow of A: 50 mM sodium acetate (pH 7.2) and solvent B: 25% 50 mM sodium acetate (pH 7.2) and 75% methanol as described in Table 3.2.

Table 3.2. HPLC gradient flow table for phenylalanine determination.

Time (min)	Flow (ml/min)	%A	%B	Curve
0.01	1.00	90.0	10.0	6
1.50	1.00	90.0	10.0	6
22.00	1.00	10.0	90.0	6
23.00	1.00	90.0	10.0	1
26.00	1.00	90.0	10.0	6

3.3.5.4. Statistical Analysis

MINITAB Statistical Software package was used for statistical analyses. Analysis of variance (ANOVA) was performed and R^2, R^2(adj), R^2(pred), RMSE and MAE values were calculated for PPA and biomass concentrations at different growth parameters. Parameters have *p*-value lower than or equal to 0.05 were considered as significant.

3.4. Results and Discussion

3.4.1. Microorganism Screening

There are several microorganisms mentioned in the literature, which can produce deaminase to convert phenylalanine to PPA, however it is important to identify the best yielding strain for economic benefits. Four potential microorganisms including *Z. rouxii, P. vulgaris, M. morganii*, and *C. glutamicum* were evaluated in this study for microbial screening based a review of literature (Smit et al., 1966; Sluis et al., 2001; Takahashi et al., 1999). Microorganisms, which converted phenylalanine to PPA, generated green colors on phenylalanine deaminase selective agar plates after addition of 10% ferric chloride solution (Figure 3.1).

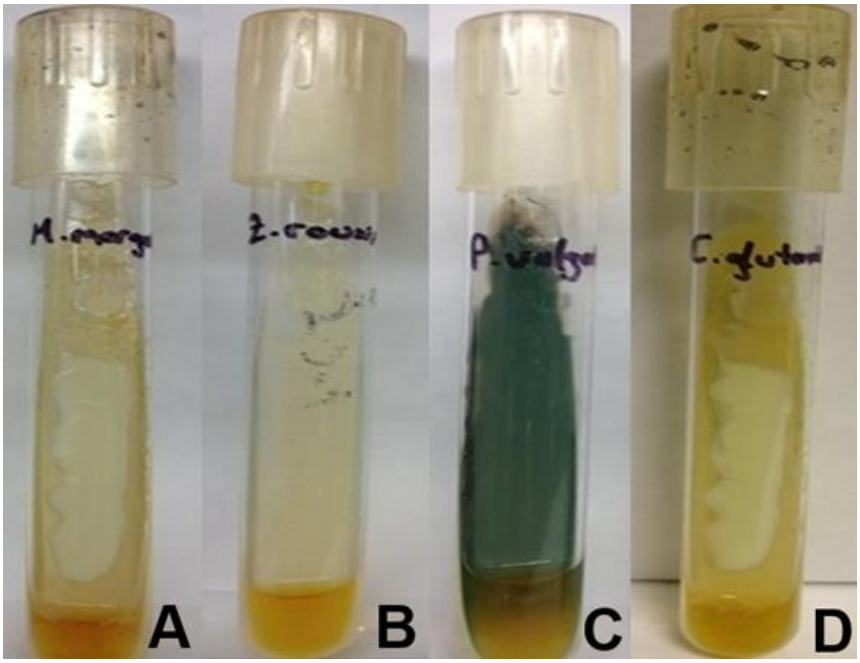


Figure 3.1. PPA selection medium results. A: *M. morganii*, B: *Z. rouxii*, C: *P. vulgaris*, D: *C. glutamicum.*

Among four microorganisms evaluated, *P. vulgaris* demonstrated the darkest green color on the selective agar, which suggests the highest PPA production. Light green was observed in *M. morganii*, and *C. glutamicum* slants, which suggested lower PPA production than the *P. vulgaris* slants. There was no color change on the *Z. rouxii* slant, which suggests phenylalanine was not

converted to PPA. Furthermore, the phenylalanine deaminase agar slants validated the findings of the shake-flask fermentations, when screening these microorganisms. Like the selective agar results, *P. vulgaris* yielded the highest PPA production by 358 mg/L, whereas *M. morganii* and *C. glutamicum* produced 56 and 98 mg/L PPA, respectively. There was no PPA detected in the flasks, with *Z. rouxii* (Figure 3.2).

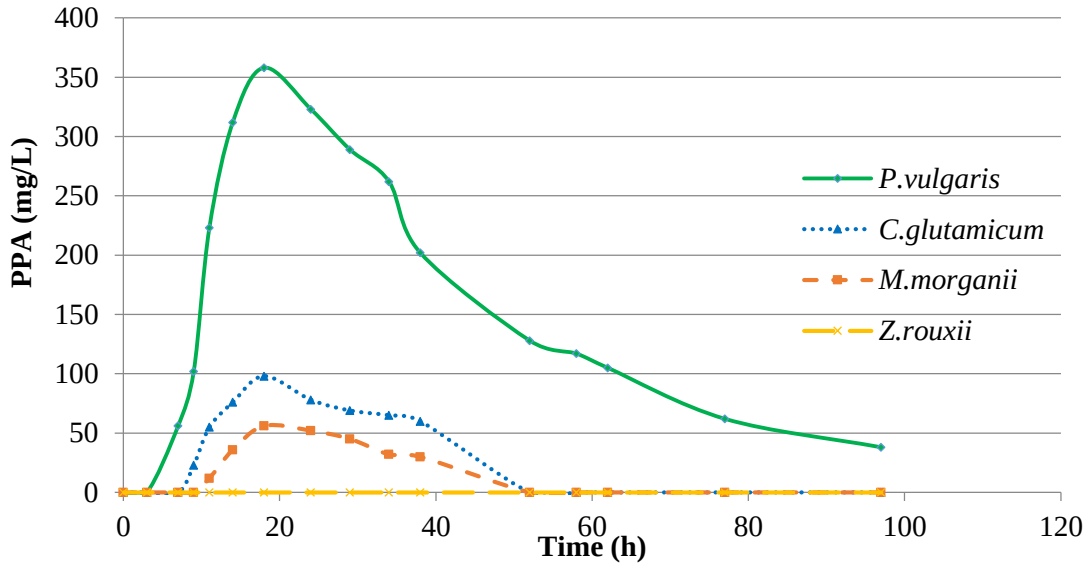


Figure 3.2. Shake-flask PPA production.

Therefore, *P. vulgaris* was selected to study optimization the growth parameters in the bench-top bioreactors. These results were also supported by the study that, 9 *P. vulgaris*, 22 *P. rettgeri*, 74 *P. morganii*, and 24 *Providence* strains were evaluated for their phenylalanine deaminase activities (Smit, 1966). It was showed that all *P. vulgaris* strains produced the highest deaminase activity, whereas *P. morganii* and *Providence* species were similar but with lower activity. The highest deaminase activity of *P. rettgeri* species was only half of the lowest *P. vulgaris* deaminase activity and *P. rettgeri* yielded the lowest deaminase activity among the strains evaluated (Smit, 1966). PPA production increased with microbial growth until 18-25 h, however thereafter PPA concentration decreased after 25 h of fermentation. It is quite possible that PPA may be further consumed by the microorganism or deamination may result in hydrogen peroxide formation, which may broke down PPA or deactivate deaminase enzyme. It was also reported that deamination reaction by product, which is hydrogen peroxide can deactivate enzyme

and/or substrate very fast and causes low yield at the end of the process (Lafuente et al., 1988). In order to overcome this problem, catalase can be used in the future studies, which decomposes hydrogen peroxide to water and oxygen (Xu et al., 2008). Additionally, pH values was measured as 5.8, when maximum PPA concentration was obtained in the shake-flasks, which shows PPA production did not decreased pH value remarkably in the shake-flasks.

3.4.2. Optimization of Growth Parameters

Growth parameters for *P. vulgaris* including temperature, pH, and aeration were optimized in a 2-L batch bioreactor, with 1-L working volume by using a Box-Behnken RSM. Table 3.3 shows the overall design, experimental, predicted PPA concentration and biomass concentration for different fermentation conditions.

PPA was produced at higher concentrations, when the fermentation was run at 34^{o}C compared to runs at 28 and 40^{o}C. The highest PPA concentrations were obtained as 826 and 884 mg/L, at 28 and 40^{o}C, respectively (Run #13 and #11), versus 1050 mg/L at 34^{o}C (run #3) (Table 3.3). It was also observed that PPA was produced at higher concentrations, when medium pH was adjusted to slightly acidic conditions. The highest PPA concentration was obtained at pH 5.5 and 4 as 1050 and 972 mg/L, respectively in runs #3 and #12, compared to 868 mg/L, at pH 7 (run #8). Aeration did not significantly affect PPA production (Table 3.4). Even though, four runs were performed with no aeration during the fermentation (runs #2, #9, #10, and #12), PPA was still produced at least 749 mg/L (run #9). Moreover, when 0.75 vvm aeration was supplied into the fermentation medium, PPA concentration was lowest at 628 mg/L (run #15) and highest at 1050 mg/L (run #3). On the other hand, the evaluated growth parameters were also significantly effective on biomass concentration. Overall, biomass concentration was measured as lowest 2.93 g/L and the highest as 3.23 g/L in the runs (Table 3.3). Higher biomass concentrations were obtained at 34^{o}C and 40^{o}C, comparing to runs, which were run at 28^{o}C. The highest biomass concentration was obtained as 3.02 g/L at 28^{o}C. However, 3.22 and 3.23 g/L concentrations were obtained, when the fermentations were run at 34 and 40^{o}C (runs #2, #3, and #8). Biomass concentration was always higher than 3 g/L, when fermentation was run at pH 7. However, lower biomass concentrations were obtained, when pH was maintained at 4 or 5.5 during the fermentation. Aeration was not that effective on biomass comparing to temperature and pH. The highest biomass concentration was measured as 3.23 g/L (run #8) at 1.5 vvm aeration, whereas the highest biomass concentration was obtained as 3.22 g/L (run #2) at no aeration. Overall, it

was observed that 34ºC and higher temperature values stimulated PPA and biomass productions more comparing to the lower temperatures. Moreover, pH values higher than 5.5 favors biomass concentration, whereas it negatively affects PPA production. Additionally, aeration did not make remarkably differences in both PPA and biomass formations.

Table 3.3. Box-Behnken response surface design for phenylpyruvic acid and biomass concentrations.

Run Order	Temperature (ºC)	pH	Aeration (vvm)	Measured PPA (mg/L)	Predicted PPA (mg/L)	Measured biomass (g/L)	Predicted biomass (g/L)
1	34	4.0	1.50	816±12	844	2.99±0.098	3.00
2	40	5.5	0.00	842±09	860	3.22±0.051	3.21
3	34	5.5	0.75	1050±24	1047	3.22±0.055	3.21
4	28	4.0	0.75	711±05	701	2.92±0.047	2.89
5	34	5.5	0.75	1045±18	1047	3.20±0.015	3.21
6	28	7.0	0.75	601±11	624	3.02±0.101	3.02
7	40	4.0	0.75	823±14	800	3.11±0.089	3.11
8	34	7.0	1.50	868±11	863	3.23±0.028	3.22
9	34	7.0	0.00	749±8	721	3.19±0.088	3.17
10	28	5.5	0.00	814±10	819	2.97±0.045	2.99
11	40	5.5	1.50	884±07	879	3.17±0.102	3.15
12	34	4.0	0.00	972±15	977	3.14±0.078	3.15
13	28	5.5	1.50	826±13	808	2.93±0.059	2.94
14	34	5.5	0.75	1046±19	1047	3.20±0.068	3.21
15	40	7.0	0.75	628±14	638	3.20±0.061	3.23

3.4.3. Response Surface Model

A second order polynomial equation (Equation 3.1) and ANOVA table (Table 3.4) were created using MINITAB software to generate the predicted values and effects of temperature, pH, and aeration on PPA productions.

PPA (mg/L) = $1047.00 + 28.13 \times (T) - 59.50 \times (pH) + 2.13 \times (A) - 183.00 \times (T \times T) - 173.25 \times (pH \times pH) - 22.50 \times (A \times A) - 21.25 \times (T \times pH) + 7.50 \times (T \times A) + 68.75 \times (pH \times A)$ where "T" is temperature and "A" is aeration. ……..**Equation 3.1**

The model has a high R^2, R^2(pred) and R^2(adj) values as 0.9868, 0.7902, and 0.9632, respectively indicating the model fits the experimental data well. Also, RMSE (root mean square error) and MAE (mean absolute error) were calculated as 15.47 and 12.27, respectively. Low RMSE and MAE values proved that, PPA concentration prediction model represents the process successfully. To show the fit, predicted values against the experimental results were plotted (not shown) and the slope of the best fit line was determined as 0.9943, which is very close to 1. ANOVA showed that temperature and pH and two way interactions by themselves were significantly effective ($p< 0.05$) on PPA formation, whereas aeration and its two way interaction by itself were not ($p> 0.05$) (Table 3.4). Among the main effects, pH was the most effective growth parameter on PPA production with the lowest *p*-value (0.05). Also, temperature-temperature and pH-pH interactions had *p*-value as 0, which indicates these interactions are highly effective on PPA formation. Similarly, pH-aeration interaction gave 0.004 *p*-value, which also showed the significance of the interaction. However, aeration was not significantly effective on PPA formation, which gave a *p*-value 0.833 (Table 3.4).

Table 3.4. ANOVA table for phenylpyruvic acid production in bioreactors.

Terms	***p*-values**
Constant	0.000
Temperature	0.032
pH	0.002
Aeration	0.833
Temperature x Temperature	0.000
pH x pH	0.000
Aeration x Aeration	0.170
Temperature x pH	0.176
Temperature x Aeration	0.602
pH x Aeration	0.004

Furthermore, application of optimization in MINITAB suggested that the maximum PPA concentration can be obtained as 1054 mg/L at 34.5^{o}C, pH 5.12 and 0.5 vvm aeration conditions (Figure 3.3). Optimum temperature for PPA production was also supported by the study that *P. vulgaris* deaminase had showed the highest activity at 35^{o}C (Takahashi et al., 1999). Also, it was reported in the literature that the highest alpha ketoglutaric acid formation achieved, when pH

was maintained at 4.5, which is slightly more acidic compared to the optimum microbial growth pH value determined in this study (Chernyavskaya et al., 2000).

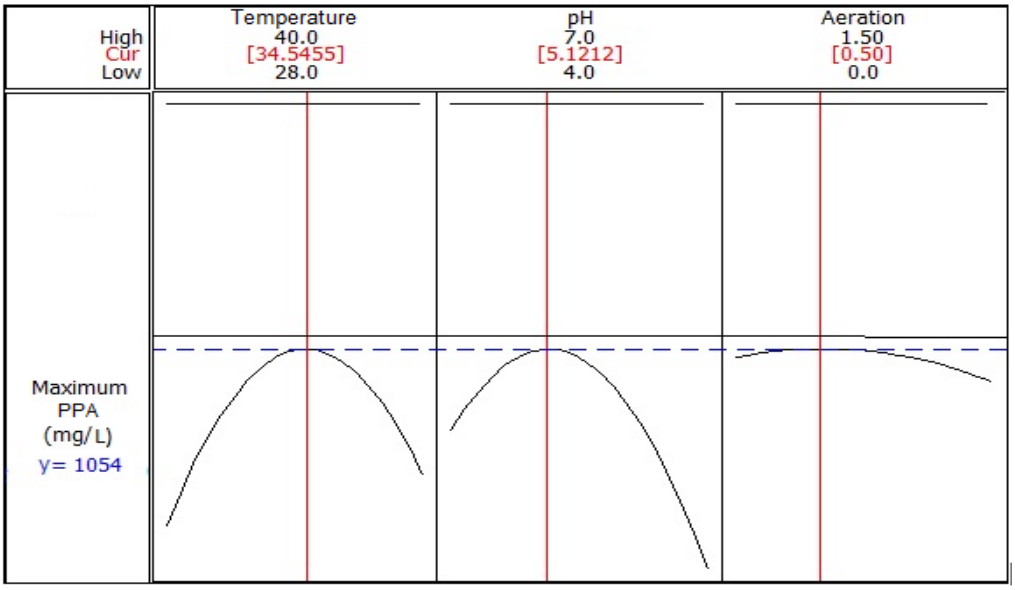


Figure 3.3. Optimum growth conditions for phenylpyruvic acid production with *P. vulgaris.*

PPA concentration trends with changing growth parameters were shown on Figure 3.4. Temperature showed a bell shape-like effect on PPA formation (Figure 3.4A). PPA production was maximized, when the fermentation was run around 35°C. Below and above this temperature, PPA formation decreased. Figure 3.4B shows the effect of pH on PPA formation. It can be seen that PPA was produced at higher concentrations in acidic conditions compared to higher pH values. PPA production is decreased, when pH was maintained at higher than 5 in the fermentation medium. PPA concentration could be measured maximum around 850 mg/L, when fermentation was run at pH 7. As it was explained previously, aeration was not significantly effective on PPA formation (Table 3.4). Therefore, PPA concentration remained very constant within the range of 0 to 1.5 vvm, however the product concentration decreased slightly at higher aeration conditions, yet maximized at the 0.5 vvm level (Figure 3.4C).

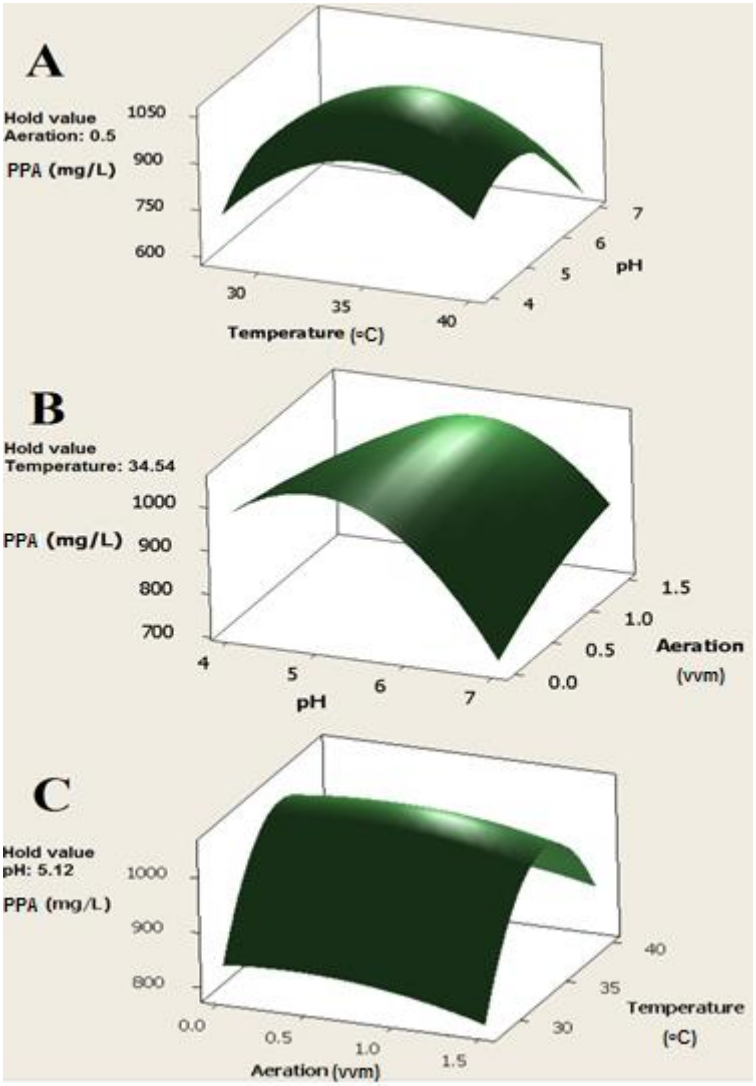


Figure 3.4. Phenylpyruvic acid concentration trends under different growth conditions.

Another second order polynomial equation (Equation 3.2) and ANOVA table (Table 3.5) were created by MINITAB for biomass production in the fermentation runs.

Biomass (g/L) = $3.20667 + 0.10750 \times (T) + 0.06 \times (pH) - 0.025 \times (A) - 0.10458 \times (T \times T) - 0.03958 \times (pH \times pH) - 0.02958 \times (A \times A) - 0.0025 \times (T \times pH) - 0.0025 \times (T \times A) + 0.0475 \times (pH \times A)$ where "T" is temperature and "A" is aeration. ……**Equation 3.2**

The biomass prediction model has also a high R^2 value (0.9817), which represents the process successfully. The kitted slope between measured and predicted biomass values was determined as 0.9758 (not shown). Also, the model has a good R^2 adjusted value (0.9488), which also shows the model can predict biomass concentrations successfully out of the studied growth parameter ranges. Also, R^2(pred), RMSE, and MAE values were calculated as 0.7274, 0.016, and 0.013, respectively. Similar to RMSE and MAE values obtained for PPA concentration prediction model, low RMSE and MAE values. ANOVA showed that all main factors of growth parameters significantly effected *P. vulgaris* biomass production ($p< 0.05$) (Table 3.5). Among the main factors, temperature was the most effective parameter on biomass production (p-value<0.05). Also temperature-temperature interaction gave the lowest p-value among the two way interactions. pH and, aeration and their two was interactions were also significantly effective on biomass formation (Table 3.5).

Table 3.5. ANOVA table for *P. vulgaris* biomass production in bioreactors.

Terms	***p*-values**
Constant	0.000
Temperature	0.000
pH	0.001
Aeration	0.042
Temperature x Temperature	0.001
pH x pH	0.033
Aeration x Aeration	0.080
Temperature x pH	0.855
Temperature x Aeration	0.855
pH x Aeration	0.015

Optimization of growth conditions in MINITAB showed that maximum biomass concentration can be obtained as 3.25 g/L at 36.97ºC, pH 6.88 and 0.97 vvm aeration conditions (Figure 3.5). It is reasonable that the optimum biomass growth temperature measured in this study was almost 37ºC, which is also recommended for *P. vulgaris* in the literature (Ware, 2000). Additionally, pH 6.87, which is very close to neutral, is also generally used in the literature (Tsuchiya et al., 1990; Szwajcer et al., 1982).

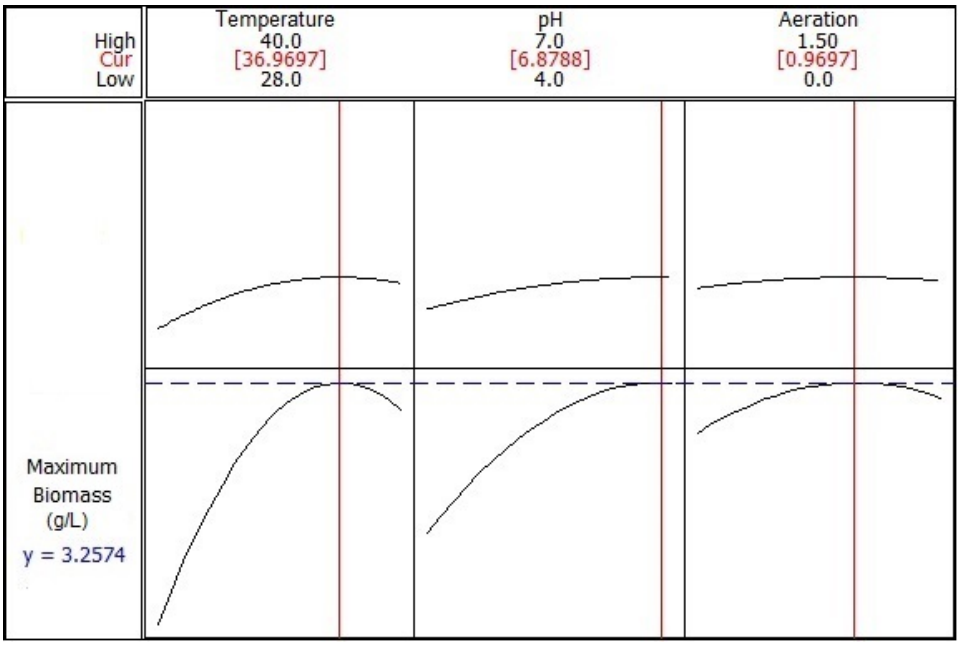


Figure 3.5. Optimum growth conditions for *P. vulgaris* biomass production.

It is important for biomass growth to keep pH level near neutral levels in order to neutralize acidic by-products produced in the fermentation broth such as pyruvic acid, CO_2, etc. Also, compared to optimum PPA production conditions, higher aeration levels are needed for optimum biomass production. Aeration does not only provides oxygen, which serves as an electron carrier in the bacterial energy metabolism, but it is also important for increasing the homogeneity and consequently the mass transfer in the fermentation medium (Sultana et al., 2010).

P. vulgaris biomass concentration trends under different growth parameters were shown on Figure 3.6. Temperature was significantly effective (p-value<0.05) on biomass production (Table 3.5). It can be seen from Figure 3.6A that biomass was produced in very low levels, when the fermentations were run at 30^{o}C. Biomass concentration increased with the increase of temperature until 37^{o}C and remains almost the same at 40^{o}C. Different than PPA production condition, higher *P. vulgaris* biomass concentrations obtained under neutral pH values than acidic conditions (Figure 3.6B). The highest biomass concentrations were obtained at pH 6.8. Aeration was also significantly effective on biomass production (p-value<0.05). Biomass concentration

increased by the aeration till around 1 vvm and slightly decreased, when it reached to 1.5 vvm (Figure 3.6C).

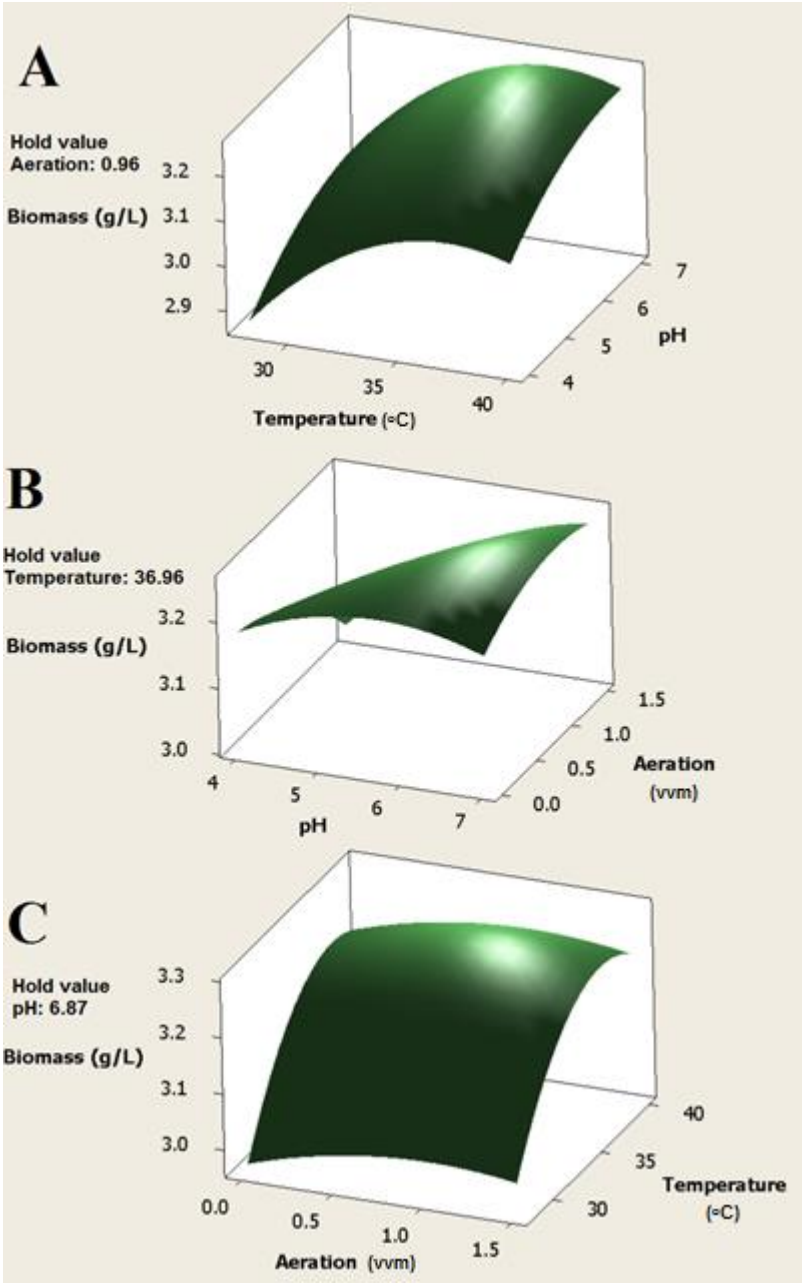


Figure 3.6. *P. vulgaris* biomass concentration trends under different growth conditions.

3.4.4. Validation of the Optimum Conditions

Batch fermentations were conducted in triplicate at the optimum conditions identified for both PPA (34.5ºC, 5.12, and 0.5 vvm) and biomass production (36.9ºC, pH 6.87, and 0.96 vvm). The maximum PPA and biomass concentrations were measured as 1058±24 mg/L (Figure 3.7) and 3.27±0.05 g/L (Figure 3.8), respectively under their specific optimum conditions. Coefficient of variation values was calculated as 0.023 and 0.015 for PPA and biomass productions, respectively. These results were very close to the estimated values, which were predicted by the RSM for optimum conditions. It was also determined that, when the highest PPA concentration was obtained at the 25 h of fermentation, phenylalanine concentration was measured as 925 mg/L in the fermentation medium. Therefore, yield for PPA production based on consumed phenylalanine ($Y_{p/s}$) was calculated as 26%. Under the specific optimum fermentation conditions for maximum PPA and biomass productions, the yield rates were calculated as 58.1 mg PPA/L/h, and 99.8 mg biomass/L/h, respectively (Figure 3.7).

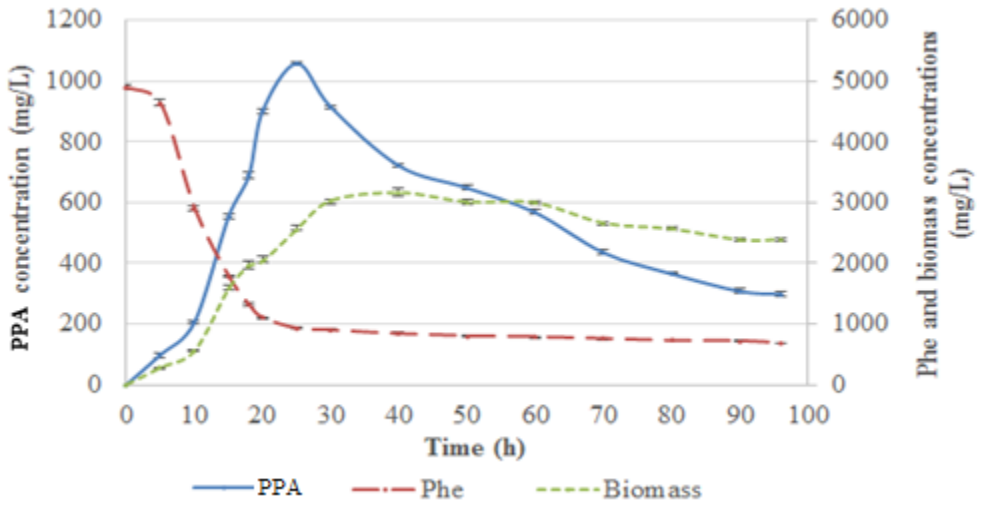


Figure 3.7. Phenylalanine consumption, PPA and biomass accumulation under specific optimum conditions for PPA production (34.5ºC, 5.12, and 0.5 vvm).

Whereas, under specific optimum fermentation conditions for biomass production, maximum PPA and biomass production rates were calculated as 33.7 mg PPA/L/h and 116.4 mg biomass/L/h, respectively (Figure 3.8). PPA and biomass concentration increased in the

fermentation medium with a same pattern. However, similar to the shake-flask productions, PPA concentration decreased remarkably after it reach the maximum whereas, biomass concentration decreased only slightly. It was calculated that almost 72% of produced PPA was lost in the broth at the 120 h of fermentation.

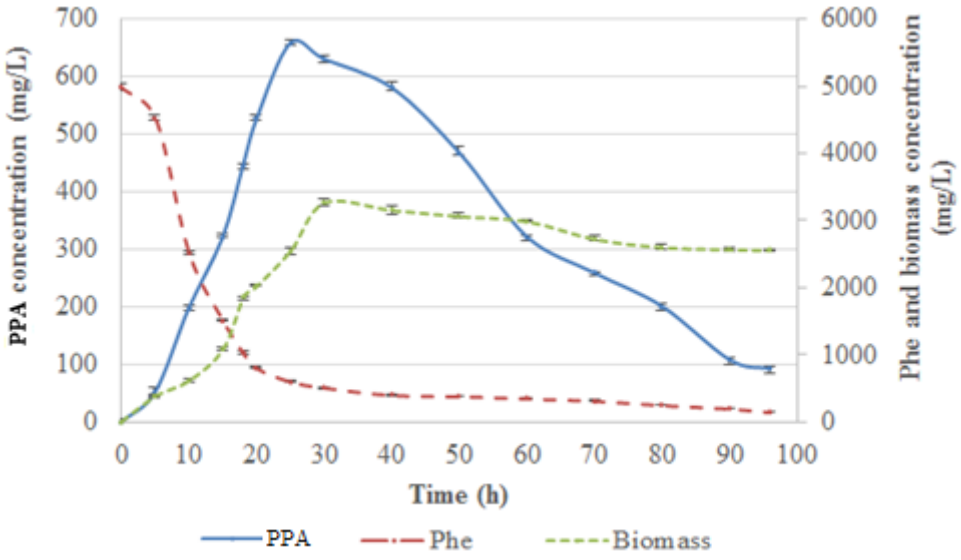


Figure 3.8. Phenylalanine consumption, PPA and biomass accumulation under specific optimum conditions for biomass production (36.9ºC, pH 6.87, and 0.96 vvm).

3.5. Conclusions

In conclusion, the most productive microbial PPA producer strain selection and optimization of growth parameters in 2-L batch reactors were performed. *P. vulgaris* was identified as the most productive strain for PPA production among. *Z. rouxii, P. vulgaris, M. morganii*, and *C. glutamicum*. Utilizing the Box-Behnken RSM, optimum growth conditions for PPA and biomass productions were determined. PPA production was optimized at 34.5°C, 5.12 pH, and 0.5 vvm aeration, whereas 36.9ºC, pH 6.87, and 0.96 vvm aeration were determined as optimum for biomass production. Under these specific optimum conditions maximum PPA and biomass concentrations were measured as 1054 mg/L and 3.25 g/L, respectively. Overall, almost 3 times more PPA concentration was obtained in the bioreactors after optimization of the growth parameters compared to shake-flask fermentations. This study clearly demonstrated that PPA

production can be enhanced by improvements in the upstream processes to reach the ultimate projected goal of 1% PPA level or more.

3.6. References

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CHAPTER 4

PHENYLPYRUVIC ACID FROM *PROTEUS VULGARIS*: ENHANCED PRODUCTION BY OPTIMIZING THE FERMENTATION MEDIUM

4.1. Abstract

Alpha keto acids are important food additives, which have application in agriculture, medicine and as feed additives and dietary supplements. Alpha keto acids are commonly produced by microbial deamination of amino acids. However, fermentation has been commonly performed at the shake-flask scale with low product concentrations. In this study, production of phenylpyruvic acid (PPA), which is the alpha keto acid of phenylalanine was enhanced in 2-L bench scale bioreactors by optimizing of the fermentation medium. Important nutrients in the fermentation medium, specifically glucose, yeast extract, and phenylalanine concentrations were optimized by using the Box-Behnken Response Surface Methodology (RSM) to maximize PPA and biomass production. As a result, the optimum glucose, yeast extract, and phenylalanine concentrations were determined to be 119.4 g/L, 3.7 g/L, and 14.8 g/L, respectively for PPA production, and 163.8 g/L, 10.8 g/L, and 9.8 g/L, respectively, for biomass production. Under these conditions, PPA concentration was enhanced to 1349 mg/L, which was 28 and 276% higher than the unoptimized bioreactor and shake-flask fermentations, respectively. Moreover, *P. vulgaris* biomass concentration was optimized at 4.36 g/L, which was 34% higher than the unoptimized bioreactor conditions. Overall, this study demonstrated that optimization of the fermentation media improved PPA concentration and biomass production in bench-top bioreactors compared to previous studies in the literature and sets the stage for scale up to industrial production.

4.2. Introduction

Alpha keto acids are important intermediate compounds for production of amino acids, food additives, medicines, and agricultural chemicals. They have the general formula R-COCOOH, where R can be aryl, alkenyl or heterocyclic group (Masato et al., 1986). Since alpha keto acids can regulate protein turnover in animal and man, attention has been increased recently

to biological function and metabolism of alpha keto acids. Therefore, following these compounds are very important for physiological conditions of animal and human (Pailla et al., 2000).

Phenylpyruvic acid (PPA) is the alpha keto acid form of phenylalanine and generally used in medicine, the food industry, and agriculture. In medicine, PPA is an important compound for the diagnosis of phenylketonuria, a genetic disease involving phenylalanine metabolism. Under normal conditions, phenylalanine is metabolized to tyrosine by phenylalanine hydroxylase. In phenylketonuria patients phenylalanine accumulates in the plasma and tissues at up to 100 times higher compared to normal concentration. This high concentration results in excessive production of phenylpyruvic acid, phenyllactate, and phanylacetate in the urine, which are known as phenylketones. Furthermore, high plasma concentration of phenylalanine causes disruption of brain development and mental retardation (Folling, 1994). Another important issue about PPA usage is that, since PPA is a deaminated form of the amino acid phenylalanine, it is used in kidney patients' diets instead of amino acids to reduce urea accumulation in their body without changing the nutritional intake (Krause et al., 2010).

Natural products of fermentation are gaining the interest of consumers for flavor enhancement of foods. Like other alpha-keto acids, PPA is becoming popular to increase flavor in food preparations. As an example, it was also reported that compared with phenylalanine, PPA has important attributes in the development of aroma, taste, and texture in cheese and wine manufacturing (Casey et al., 2004). Additionally, it was reported that lesser amount of PPA is more effective to improve nutrition intake and animal growth than comparing to other alpha keto acids such as alpha ketoisovaleric acid and alpha ketoisocaproic acid, which are alpha keto acids of valine and leucine, respectively (Gaby and Chawla, 1976). Another application of PPA and other keto acids is in poultry feed as supplements to reduce nitrogen intake and output. Excessive nitrogen excretion in chicken manure has an adverse effect on the environment and results in extra cost for the farmers to properly manage. In order to prevent this problem, poultry growers have started to add alpha keto acids in poultry diet instead of amino acids to reduce nitrogen accumulation in the manure and to enhance poultry growth (Summer, 1993).

The commercial production of PPA is based on the removal of amino groups from phenylalanine via a deaminase enzyme. These enzymes can be derived from several microorganisms such as *Proteus*, *Morganella*, and *Provedincia* spp. It has been reported that *Proteus*, species can secrete a deaminase with high activity, which can convert amino acids to their respective alpha keto acids (Chapter 3; Smit, 1966).

There are several studies in the literature conducted to produce alpha keto acids using microbial deaminases in shake-flask fermentations. It was reported that pyruvic acid was produced by using *Yarrowia lipolytica* at up to 1.6 g/L (Chernyavskay et al., 2000). Also, ketoisovaleric acid (35 mg/L) was produced by *Zygosaccharomyces rouxii* in shake-flasks, which is alpha keto acid of valine (Sluis et al., 2001). Moreover, *Eubacterium* species were used for production of several alpha keto acids. In that study, 5.3 mg/L of PPA, 10.9 mg/L of alpha ketoglutaric acid, 2.4 mg/L of pyruvic acid, and 0.62 mg/L of alpha ketobutyric acid was produced by using *Eubacterium nodatum* (Itoh et al., 1994). Also, black pigmented oral *Bacteroides* species were evaluated and reported to produce 14.7 mg/L of alpha ketoglutaric acid, 9.2 mg/L of pyruvic acid, 10 mg/L of alpha ketobutyric acid, 22.3 mg/L of alpha ketovaleric acid, 9.8 mg/L of alpha ketoisovaleric acid, 12.9 mg/L of alpha ketoisocaproic acid, and 15.8 mg/L of PPA was produced in the shake-flasks (Tsuchiya et al., 1990).

To date, production of PPA has been studied by various researchers, but mostly in shake-flask fermentations without any optimization of process conditions. In shake-flask systems, working volume is always limited and growth parameters such as pH, mechanical stirring and aeration cannot be controlled effectively, which makes it difficult to scale up to industrial levels. Because of these limiting factors there is an opportunity to better control growth parameters and optimize the fermentation medium for enhanced PPA production. More optimized and economical PPA production approaches have been undertaken to improve industrial production. In our recent work, growth conditions including temperature, pH, and aeration were optimized to increase PPA concentration in bioreactors. In this study, maximum PPA concentration increased from 358 mg/L in shake-flasks to 1054 mg/L in bioreactor scale fermentations after optimization of growth parameters (Chapter 3). Therefore, the aim of this study was to further enhance PPA production by optimizing the fermentation medium to determine the ideal glucose, yeast extract, and phenylalanine concentrations to maximize PPA production in bench-top bioreactors using a Box-Behnken design.

4.3. Materials and Method

4.3.1. Microorganisms and Inoculum Preparation

Proteus vulgaris (B-123), which was obtained from the USDA Agricultural Research Service Culture Collection (Peoria, IL) was grown in 10 ml of trypticase soy broth (TSB) at 37^{o}C for 24 h. After incubation, cultures were stored at 4^{o}C as working cultures and regularly

transferred to sterile fresh media every 2 weeks to maintain viability. Stock cultures were kept frozen in 20% glycerol at -80^{o}C for future studies. Flasks containing 100 ml of TSB were inoculated with 1% (v/v) *P. vulgaris* and incubated at 37^{o}C with 150 rpm agitation for 30 h until late log phase. Inoculums, which had ~10^{6} CFU/ml was used to inoculate the reactors.

4.3.2. Batch Fermentation in Bioreactors and Optimization of the Fermentation Medium

Sartorious Biostat B Plus bioreactors (Sartorious, Allentown, PA) equipped with 2-L vessel with 1-L working volume were used in the experiments. The base fermentation medium consisted of 80 g of glucose, 8 g of yeast extract, 5 g of NaCl, 1 g of K_2HPO_4, 0.2 g of $MgSO_4$, and 5 g of L-phenylalanine per liter of deionized water. Temperature, pH, and aeration values were maintained at 34.5^{o}C, 5.12, and 0.5 vvm, as indicated by our previous optimizing study (Chapter 3). Additionally, agitation was maintained at 300 rpm for all fermentation runs. Fermentations were initiated by adding 1% (v/v) inoculum and samples were taken every 5 h until the 75 h then analyzed for biomass, PPA, and phenylalanine concentrations. Three medium nutrients including glucose (80-180 g/L), yeast extract (1-15 g/L), and phenylalanine (5-20 g/L were evaluated by Box-Behnken Response Surface Methodology (RSM) (Table 4.2). Biomass and PPA concentrations were the dependent variables of response and analyzed to optimize the fermentation medium composition to maximize the concentrations of each outcome using MINITAB statistical software (Version 15, Minitab Inc., State College, PA).

4.3.3. Validation of the Model

Once the optimum nutritional concentrations were determined, fermentations were run under the conditions identified as optimum with three replication to validate the RSM. Results from these fermentations, were compared with the predicted values from the model.

4.3.4. Analysis

Sample replicates collected during the fermentations were first subsampled to determine biomass content then centrifuged at 5,200 x *g* for 15 min to remove the biomass. The supernatant was used for analysis of PPA and phenylalanine concentrations. Samples without centrifugation was used to determine biomass concentrations. All procedural details are indicated below.

4.3.4.1. Biomass

Absorbance of sample was measured at 620 nm by using a spectrophotometer (Beckman Coulter, Fullerton, CA) to determine microbial concentration in the broth. Sterile fermentation medium was used as the blank for the measurement. Absorbencies were converted to g/L by using the standard curve.

4.3.4.2. Phenylpyruvic Acid

Cell-free samples were derivatized with 2,4-Dinitrophenylhydrazine (DNPH) as described in the literature (Elias et al., 2008). Derivatization solution was prepared as follows: 0.2 g of DNPH was dissolved in 100 ml acetonitrile and 4 ml of 70 wt % perchloric acid was added to the solution. A samples (0.1 ml) was mixed with 0.24 ml of DNPH solution and 0.04 ml of 25% (w/v) H_2SO_4 solution. The mixture was incubated at 60^{o}C for 1 h. After derivatization, 0.48 ml a of 60:40 acetonitrile:water mixture was added to the mixture and filtered into high pressure liquid chromatography (HPLC) vials using 0.2 μm polytetrafluoroethylene (PTFE) filters (VWR, Radnor, PA). Phenylpyruvic acid concentrations were measured using Waters HPLC with a RP-C18 column (Sigma-Aldrich, St. Louis, MO) and UV detector (Waters, Milford, MA) at 365 nm. Flow was adjusted at 1 ml/min gradient flow of A: 25 mM phosphate buffer (pH 2.2) and solvent B: acetonitrile as described in Table 4.1. The PPA standard was purchased from Sigma-Aldrich (St. Louis, MO).

4.3.4.3. Phenylalanine

Samples were derivatized with o-phthalaldehyde (OPA) as described (Knauer. 2012). The cell-free sample (0.1 ml) was mixed with 1 ml OPA and filtered into HPLC vials using 0.2 μm PTFE filters. Phenylalanine concentrations were measured at 230 nm by using the same HPLC system with 1 ml/min gradient flow of A: 50 mM sodium acetate (pH 7.2) and solvent B: 25% 50 mM sodium acetate (pH 7.2) and 75% methanol as described in Table 4.1.

Table 4.1. HPLC gradient flow table for phenylpyruvic acid and phenylalanine analysis (solvent A: 25 mM phosphate buffer (pH 2.2) and solvent B: acetonitrile for phenylpyruvic acid analysis. Solvent A: 50 mM sodium acetate (pH 7.2) and solvent B: 25% 50 mM sodium acetate (pH 7.2) and 75% methanol for phenylalanine analysis).

Time (min)	Flow (ml/min)	%A	%B	Curve
0.01	1.00	90.0	10.0	6
1.50	1.00	90.0	10.0	6
20.00	1.00	10.0	90.0	6
25.00	1.00	90.0	10.0	1
30.00	1.00	90.0	10.0	6

4.3.4.4. Statistical Analysis

MINITAB Statistical Software package was used for statistical analyses. Analysis of variance (ANOVA) was performed for investigating statistically significant differences between PPA and biomass concentrations at different growth parameters and terms, which have *p*-value lower than 0.05 were considered as significant. Additionally, Tukey's method was used to determine the significant difference level in PPA productions among shake-flask and bioreactor fermentations.

4.4. Results and Discussion

In this study, optimum medium compositions were determined to enhance PPA production in submerged fermentations using bench-top bioreactors. Additionally, effectiveness of each studied medium parameter on PPA and biomass productions were determined by ANOVA results and second order polynomial equations were created to predict and optimize the production of both PPA and biomass.

4.4.1. Optimization of Medium Parameters

Concentrations of glucose, yeast extract, and phenylalanine were optimized in a 2-L batch bioreactor by using a Box-Behnken design. Table 4.2 shows the overall design, the

experimental and predicted PPA concentrations, and biomass levels for different fermentation conditions.

Table 4.2. Box-Behnken response surface design for phenylpyruvic acid and biomass concentrations.

Run Order	Glucose (g/L)	Yeast extract (g/L)	Phenylalanine (g/L)	Measured PPA (mg/L)	Predicted PPA (mg/L)	Measured biomass (g/L)	Predicted biomass (g/L)
1	180	1	12.5	1013	1018	3.12	2.97
2	130	8	12.5	1312	1308	4.05	4.07
3	130	1	20.0	1290	1258	2.39	2.59
4	80	8	5.0	911	884	2.98	3.03
5	80	1	12.5	1165	1199	2.19	2.15
6	180	15	12.5	991	957	3.99	4.02
7	130	15	20.0	1039	1046	3.26	3.27
8	130	15	5.0	917	949	3.99	3.79
9	130	8	12.5	1304	1308	4.09	4.07
10	130	1	5.0	1098	1091	2.75	2.74
11	180	8	20.0	922	949	3.75	3.70
12	80	8	20.0	1123	1121	2.88	2.71
13	130	8	12.5	1308	1308	4.08	4.07
14	180	8	5.0	921	923	3.88	4.05
15	80	15	12.5	912	907	2.68	2.83

4.4.1.1. Phenylpyruvic Acid Production

PPA was mostly produced in higher concentrations, when the fermentations were run with either 80 or 130 g/L glucose concentrations compared to 180 g/L glucose runs. Maximum PPA concentration was measured as 1123 mg/L (run #12) and 1312 mg/L (run #2), when fermentations were run with 80 or 130 g/L glucose concentrations, respectively (Table 4.2). However, maximum PPA concentration was obtained as 1013 g/L (run #1), when fermentation was run with 180 g/L. It was also determined that PPA production increased, when fermentations were run with low yeast extract concentrations. PPA concentration was always higher than 1000 mg/L and measured the highest as 1290 mg/L (run #3), when 1 g/L yeast extract was used in the

fermentations. However, maximum PPA concentration was determined as 1039 mg/L (run #7), when 15 g/L yeast extract was added in the fermentation medium. Moreover, the highest PPA concentration was measured as 1312 mg/L, when the fermentation was run with 12.5 g/L phenylalanine (run #2). However, PPA concentration were measured as 1098 mg /L (run #10) and 1290 g/L (run #3), when the medium included 5 g/L and 20 g/L phenylalanine concentrations, respectively.

4.4.1.2. Biomass Production

On the other hand, biomass concentration was also affected by the different concentrations of evaluated medium parameters. Overall, biomass concentration was measured the lowest as 2.19 g/L and the highest as 4.09 g/L in the runs (Table 4.2). Higher biomass concentrations were obtained, when high glucose concentrations were used in the fermentation medium. The highest biomass concentrations were measured as 4.09 g/L (run #9) and 3.99 g/L (run #6), when 130 g/L and 180 g/L glucose was used in the runs, respectively. However, maximum biomass concentration was measured as 2.98 g/L, when 80 g/L glucose was used in the bioreactors (run #4). Similar to glucose concentration, high yeast extract concentrations increased biomass concentrations in the productions. The biomass concentration was measured as 3.12 g/L (run #1), when 1 g/L yeast extract was used in the productions. However, maximum biomass concentration was increased to 3.99 g/L (run #6 and #8), and 4.09 g/L (run #9), when 15 g/L and 8 g/L yeast extract used in the fermentations, respectively. Different phenylalanine concentrations were not significantly effective on the biomass production (Table 4.2). For example, biomass concentration was determined as 3.99 g/L (run #8), 4.09 g/L (run #9), and 3.75 g/L (run #11), when 5, 12.5, and 20 g/L phenylalanine was used in the fermentations, respectively.

4.4.2. Response Surface Model

4.4.2.1. Phenylpyruvic Acid Production

A second order polynomial equation (Equation 4.1) and ANOVA table (Table 4.3) were created by MINITAB software to show the predicted values, effects of glucose, yeast extract and phenylalanine concentration on PPA productions.

$$\text{PPA (mg/L)} = -563.358 + 20.8123 \times (\text{G}) - 1.98997 \times (\text{YE}) + 90.4033 \times (\text{P}) - 0.0809 \times (\text{G} \times \text{G}) - 1.7449 \times (\text{YE} \times \text{YE}) - 2.42667 \times (\text{P} \times \text{P}) - 0.165 \times (\text{G} \times \text{YE}) - 0.140667 \times (\text{G} \times \text{P}) - 0.333333 \times (\text{YE} \times \text{P})$$ where "G" is glucose, "YE" is yeast extract, and "P" is phenylalanine concentration. ……...**Equation 4.1**

The model has a high R^2 value (0.9828) indicates the model fits the experimental data well. To show the good fit, experimental and predicted values were plotted (not shown) and the slope of the best fitted line was determined as 0.9832, which is very close to "1". ANOVA showed that glucose, yeast extract, and phenylalanine concentrations are significantly effective ($p < 0.05$) on PPA production (Table 4.3).

Table 4.3. Significance of medium ingredient factors on the phenylpyruvic acid prediction model.

Terms	***p*-values**
Constant	0.000
Glucose (G)	0.045
Yeast extract (YE)	0.001
Phenylalanine (Phe)	0.003
G x G	0.000
YE x YE	0.005
Phe x Phe	0.001
G x YE	0.021
G x Phe	0.030
YE x Phe	0.363

Among the main effects, yeast extract concentration was the most effective medium parameter on PPA production with the lowest *p*-value. Moreover, application of optimization in MINITAB suggested that the maximum PPA concentration can be obtained as 1349 mg/L with 119.4 g/L glucose, 3.68 g/L yeast extract, and 14.85 g/L phenylalanine concentrations (Figure 4.1).

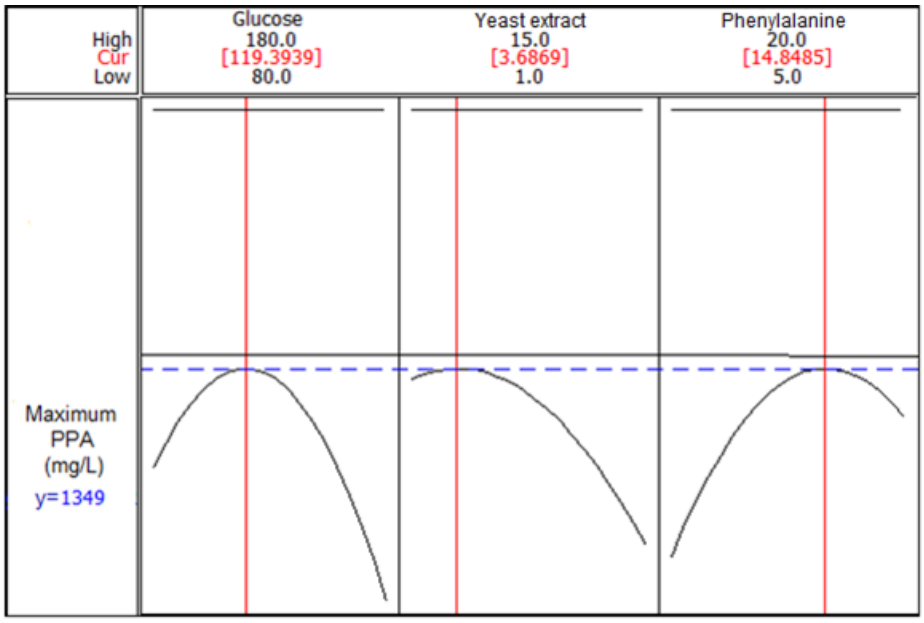

Figure 4.1. Optimum medium parameters for phenylpyruvic acid production with *P. vulgaris*.

PPA concentration trends with changing medium parameters were shown on Figure 4.2. PPA production was increased, when glucose concentration increased until 120 g/L. However, production decreased remarkably in higher glucose concentrations (Figure 4.2A). Figure 4.2B clearly shows that PPA was produced in higher concentrations, when low yeast extract concentrations were used in the fermentations. When yeast extract concentration was increased more than 5 g/L, PPA production decreased significantly. Also, there is a direct relationship with PPA concentration and phenylalanine concentration in the fermentations. As it was shown on Figure 4.2C, PPA production increased remarkably by increasing phenylalanine concentration till 15 g/L and remained almost constant in higher phenylalanine concentration values.

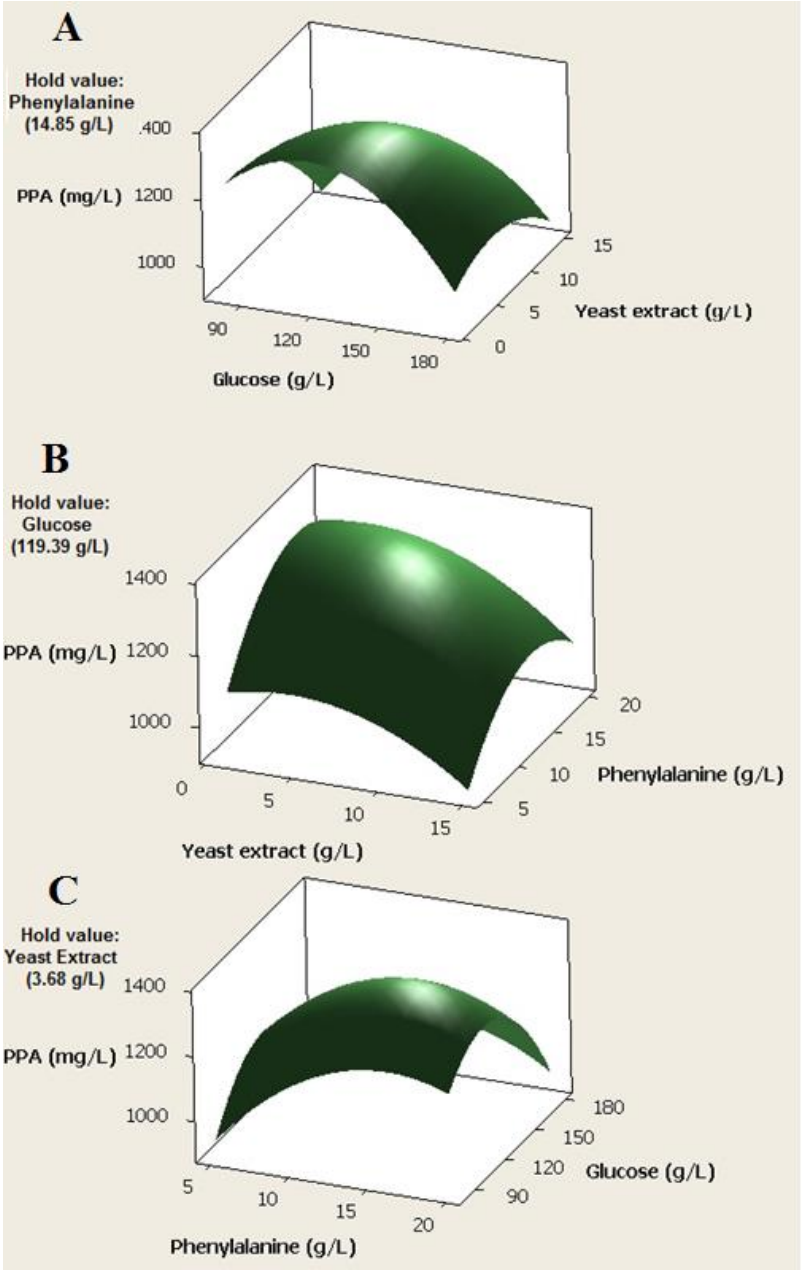

Figure 4.2. Phenylpyruvic acid production profile under various medium conditions.

4.4.2.2. Biomass Production

Another second order polynomial equation (Equation 4.2) and ANOVA table (Table 4.4) were created by MINITAB for biomass production in the fermentation runs.

Biomass (g/L) = $-1.80713 + 0.0498769 \times (G) + 0.269655 \times (YE) + 0.127658 \times (P) - 0.000160667 \times (G \times G) - 0.0138095 \times (YE \times YE) - 0.00531852 \times (P \times P) - 0.000271429 \times (G \times YE) - 0.00002 \times (G \times P) - 0.0017619 \times (YE \times P)$ where "G" is glucose, "YE" is yeast extract, and "P" is phenylalanine concentration. ……..**....Equation 4.2**

The biomass prediction model has also a high R^2 value (0.9698), which shows that the process was represented successfully. Fitted slope between measured and predicted biomass values was determined as 0.9697 (plot not shown). Moreover, the model has a good R^2 adjusted value (0.9153), which also shows the model can also predict biomass concentrations successfully out of the studied growth parameter ranges. ANOVA showed that all main factors expect for phenylalanine concentration are significant on *P. vulgaris* biomass production (*p*-value< 0.05) (Table 4.4).

Table 4.4. Significance of medium ingredient factors on the biomass prediction model.

Terms	***p*-values**
Constant	0.000
Glucose (G)	0.001
Yeast extract (YE)	0.002
Phenylalanine (Phe)	0.063
G x G	0.011
YE x YE	0.001
Phe x Phe	0.032
G x YE	0.376
G x Phe	0.942
YE x Phe	0.388

Optimization of medium parameters in MINITAB showed that maximum biomass concentration can be obtained as 4.35 g/L at 163.8 g/L glucose, 10.75 g/L yeast extract, and 9.84 g/L phenylalanine concentration conditions (Figure 4.3).

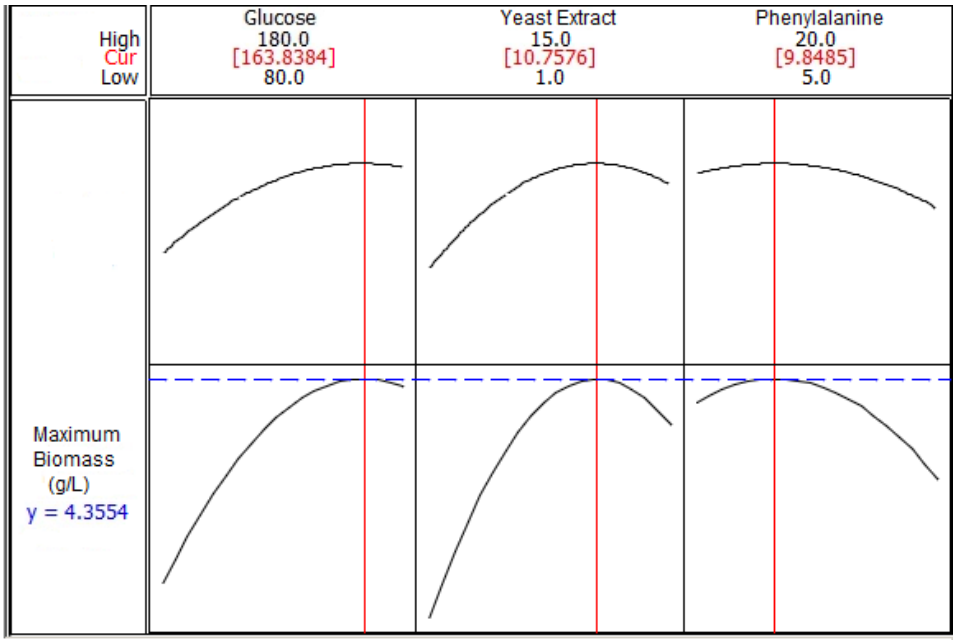

Figure 4.3. Optimum medium parameters for *P. vulgaris* biomass production.

It is reasonable that higher glucose concentrations are needed for the biomass growth comparing to PPA production, since carbon source directly affects the microbial growth. Similarly, optimum yeast extract concentration for biomass growth is higher than for PPA production. It was also shown that alpha keto acids are produced in higher concentrations, under low initial nitrogen concentrations in the fermentation medium (Chernyavskaya et al., 2000; Nesbakken et al., 1988; Raunio, 1966). It was shown that *E. coli* produced 4 times higher alpha keto acid, when fermentations were run with 50 µg/ml ammonium sulfate concentrations, comparing to 1000 µg/ml ammonium sulphate containing medium (Raunio, 1966). Similarly, alpha ketoglutaric acid production was 40 g/L, when 10 g/L ammonium sulfate was used in the fermentation medium. However, production decreased to 20 g/L, when initial ammonium sulfate concentration was increased to 12 g/L (Chernyavskaya et al., 2000). It can be explained that microorganisms use more amino acid in the fermentation medium to maintain nitrogen intake in the low yeast extract concentration conditions. This can be explained by that, high PPA production needs low yeast extract and high phenylalanine concentration, whereas opposite conditions are needed for biomass production.

Effect of medium ingredient concentrations on *P. vulgaris* biomass concentration were shown on Figure 4.4. Biomass concentration increased by glucose concentration until around 160 g/L (Figure 4.4A). Higher values did not make significant difference on biomass concentration. Yeast extract concentration remarkably increased biomass concentration until 10 g/L. Further increasing the yeast extract concentration slightly decreased biomass formation in the fermentations (Figure 4.4B). Figure 4.4C showed that phenylalanine concentration was not significantly effective on biomass formation. There was not a significant difference between the biomass concentrations, when the fermentations were run with 5 g/L or 10 g/L of phenylalanine. However, higher than 10 g/L phenylalanine concentrations negatively affected the biomass productions.

4.4.3. Validation of the Optimum Conditions

Batch fermentations were conducted in triplicate at the conditions identified as optimum for both PPA (119.4 g/L glucose, 3.68 g/L yeast extract, and 14.85 g/L phenylalanine concentration) and biomass productions (163.8 g/L glucose, 10.75 g/L yeast extract, and 9.84 g/L phenylalanine concentration). The maximum PPA and biomass concentrations were measured as 1350 mg/L (Figure 4.5) and 4.35 g/L (Figure 4.6), respectively under their specific optimum conditions. These results were very close to the estimated values, which were predicted by RSM under optimum conditions. Moreover, it was also determined that, when the highest PPA concentration was obtained at 30 h, phenylalanine concentration was measured as 1350 mg/L in the fermentation medium. However, almost 54% of produced PPA was lost in the broth at the 75 h of fermentation. PPA and biomass concentration increased in the fermentation medium with a same pattern. However, PPA concentration decreased remarkably after it reach the maximum whereas, biomass concentration decreased slightly. This may be explained by usage of PPA by microorganisms as a nutrient, further degradation of PPA to aromatic compounds, breaking down of produced PPA by hydrogen peroxide effect, which was produced during microbial deamination reaction. It was reported that, amino acid deamination reaction by product, which is hydrogen peroxide can deactivate enzyme and/or substrate and causes low yields at the end of the process (Lafuente et al., 1988). Similarly, in another study it was reported that, hydrogen peroxide can break down pyruvic acid to acetate, CO_2, and water and decrease production yield (Xu et al., 2008). In order to solve this problem, they used a genetically modified strain of

Hansenula polymorph and *Pichia pastoris,* which express both glycolate oxidase and catalase to be used in the deamination reaction.

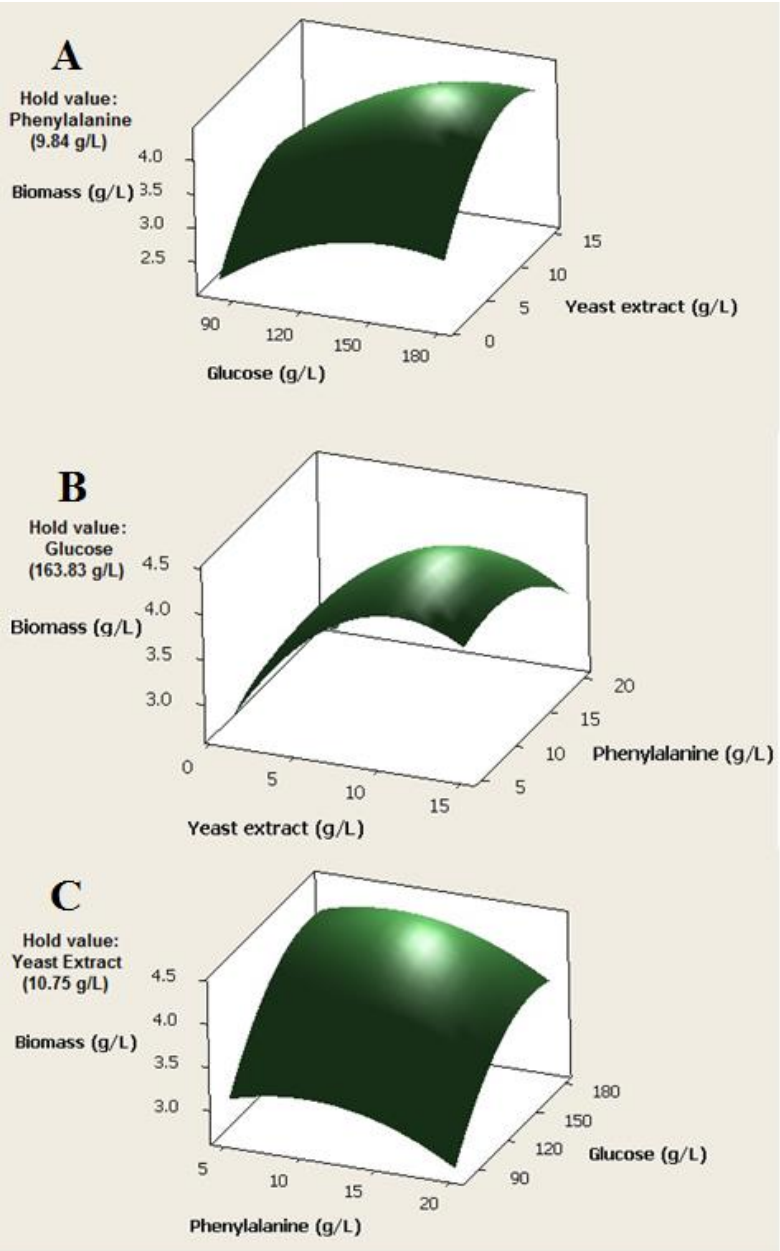

Figure 4.4. *P. vulgaris* biomass production profiles under various medium conditions.

Under the determined optimum fermentation medium for PPA production, maximum PPA production and biomass production rates (steepest slope) were calculated as 48 mg PPA/L/h, and 175 mg biomass/L/h, respectively (Figure 4.5).

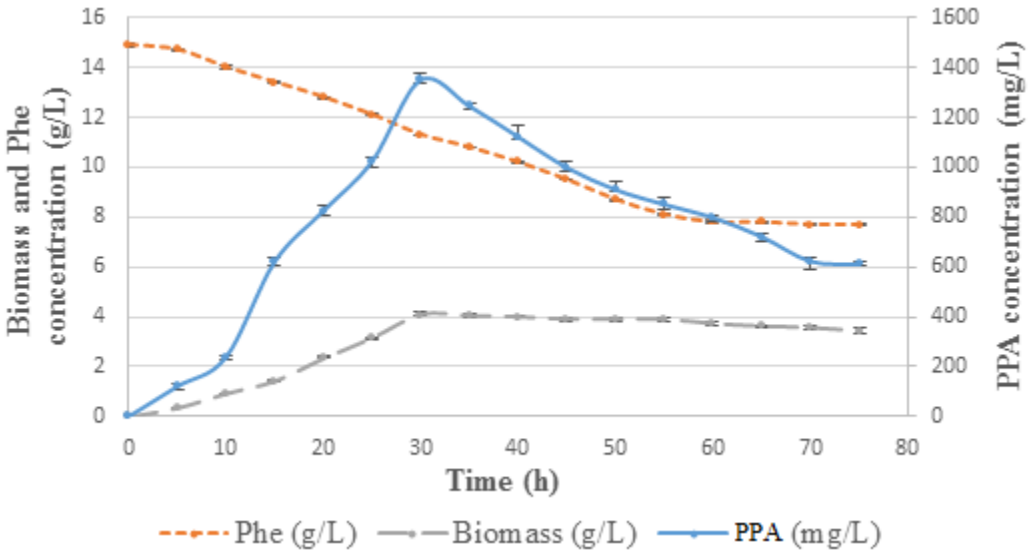

Figure 4.5. Phenylalanine consumption, PPA and biomass accumulation under optimum medium conditions for PPA production (119.39 g/L glucose, 3.68 g/L yeast extract, 14.84 g/L phenylalanine).

Whereas, under the determined optimum fermentation medium for biomass production, maximum PPA and biomass production rates were calculated as 42 mg PPA/L/h and 179 mg biomass/L/h, respectively (Figure 4.6), which shows slightly higher biomass production but remarkably less PPA production.

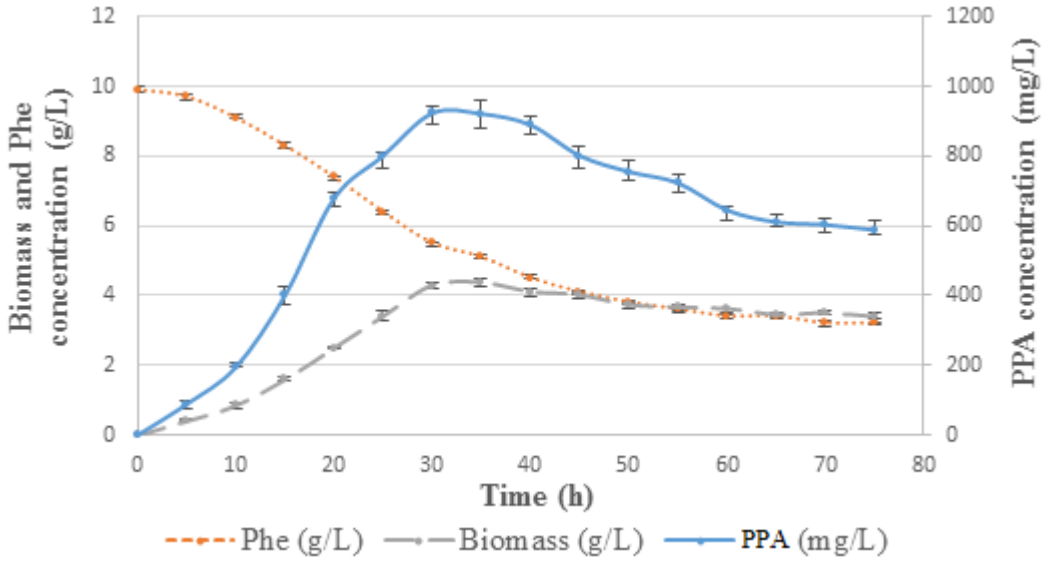

Figure 4.6. Phenylalanine consumption, PPA and biomass accumulation under optimum medium conditions for biomass production (163.83 g/L glucose, 10.75 g/L yeast extract, 9.84 g/L phenylalanine).

4.4.4. Comparison of PPA Productions in Shake-flask and Batch Bioreactors

Tukey's statistical test was used to compare maximum PPA productions on shake-flask fermentations and batch bioreactor fermentations after growth and medium parameter optimizations (Table 4.5). Tukey's method showed that maximum PPA concentrations are significantly different among three different fermentation conditions.

Table 4.5. Tukey's method comparison of maximum PPA production in shake-flask and batch bioreactor fermentations.

Source	DF	SS	MS	F	P
Factors	2	1553522	776761	46605.66	0.000
Error	6	100	17		
Total	8	1553622			

S = 4.082 R-Sq = 99.99% R-Sq(adj) = 99.99%

Grouping Information Using Tukey Method

Factors	N	Mean	Grouping
Optimization of fermentation medium	3	1349	A
Optimization of growth parameters	3	1054	B
Shake flask fermentations	3	358	C

4.5. Conclusions

In conclusion, the Box-Behnken design was used to determine optimum medium conditions for PPA and biomass productions in this study. These conditions for PPA production were determined as 119.4 g/L of glucose, 3.68 g/L of yeast extract, and 14.85 g/L of phenylalanine, whereas 163.8 g/L glucose, 10.75 g/L yeast extract, and 9.84 g/L phenylalanine for biomass production. Under the specific optimum conditions maximum PPA and biomass concentrations were measured as 1349 mg/L and 4.35 g/L, respectively. Overall, PPA production was increased2.8 fold after optimization of medium parameters compared to shake-flask fermentations and 0.3 fold compared to optimized growth parameters bioreactor productions. Additionally, it was shown that glucose, yeast extract, and phenylalanine concentrations were significantly effective on PPA production. However, phenylalanine concentration was found out to be insignificant on biomass production (p-value>0.05). Overall, this study has shown that scaling-up PPA production from shake-flask to reactor volume and optimization of medium parameters increased maximum PPA production. Also, this study clearly demonstrated that these results can be a step forward for commercial production.

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CHAPTER 5

ENHANCED PHENYLPYRUVIC ACID PRODUCTION WITH *PROTEUS VULGARIS* IN FED-BATCH AND CONTINUOUS FERMENTATIONS

5.1. Abstract

Alpha keto acids have important applications in the agriculture, food, and medical industries. Alpha keto acids are commonly produced by microbial deamination of amino acids during fermentation processes. However, reported fermentation studies in the literature have been usually performed at the shake-flask scale with low production levels. In our previous studies, phenylpyruvic acid (PPA) production was improved by optimization of growth and medium parameters in batch fermentation by using bench-top bioreactors. In this study, PPA production was further enhanced with fed-batch and continuous fermentations. As a result, maximum PPA concentrations increased from 1350 mg/L (batch fermentation) to 2958 mg/L utilizing fed-batch fermentation. Furthermore, PPA productivity was increased from 48 mg/L/h (batch fermentation) to 104 and 259 mg/L/h by applying fed-batch fermentation and continuous fermentation at the optimum dilution rate of 0.12 h^{-1}, respectively. Overall, this study demonstrated that fed-batch and continuous fermentation significantly improved PPA production in the bench-scale bioreactors.

5.2. Introduction

Alpha keto acids are important compounds especially for production of amino acids, food additives, medicines, and agricultural chemicals. Alpha keto acids can also regulate protein turnover, so attention to their biological function has gained interest (Pailla et al., 2000).

Phenylpyruvic acid (PPA) is the alpha keto acid of phenylalanine and generally used in medicine, the food industry, and agriculture. In medicine, PPA is commonly used for diagnosis of phenylketonuria, which is an inborn genetic disease about phenylalanine metabolism. Under normal conditions, phenylalanine is metabolized to tyrosine by phenylalanine hydroxylase. However, in phenylketonuria patients this reaction leads to accumulation of phenylalanine in plasma and tissues up one hundred times higher compared to normal concentration. This situation results in excessive production of phenylpyruvic acid, phenyllactate, and phanylacetate, which are

known as phenylketones in urine. As a result, high plasma concentrations of phenylalanine disrupts brain development and causes mental retardation (Folling, 1994). Another important application of PPA, since it is the deaminated form of phenylalanine, is used in kidney patients' diets instead of the amino acid to reduce urea accumulation in their body without changing nutritional intake (Krause et al., 2010). Also, consumer interest in the application of natural products as flavor enhancers in foods has increased recently. In several studies, PPA was reported as a popular flavor enhancer in food formulations (Pazo et al., 2013; Delgado et al., 2008). It was also reported that rather than phenylalanine, PPA is more important in the development of specific aroma, taste, and textures in cheese and wine manufacturing (Casey et al., 2004). PPA is also an effective phenylalanine substitute in diets to support animal growth. It was shown that phenylalanine can be removed and 1% PPA can be used in rat diets to increase animal welfare (Bubl and Butts, 1949). Another opportunity for PPA is in the supplementation of poultry feed to adjust nitrogen intake and output. Excessive nitrogen accumulation in the manure has a negative effect on the environment, which is an economic externality for farmers to manage. In order to prevent this problem, poultry growers could add alpha keto acids in poultry diets to enhance poultry growth instead of amino acids and reduce nitrogen accumulation in the manure (Summer, 1993). However, PPA is still not being used commercially due to the high cost of production.

To date, PPA production has been studied byresearchers, but mostly in shake-flask fermentation with very low concentrations.In shake-flask fermentation, working volume is always limited and growth parameters such as pH, mechanical stirring, and aeration cannot be controlled effectively (Shuler and Kargi, 1992). These disadvantages make shake-flask fermentation systems difficult to scale up to industrial levels. For these reasons, PPA is better managed in bioreactors in order to control growth parameters and enhance the production.

There are several studies that have aimed at alpha keto acid production in the literature. For example, Chernyavskaya et al. (2000) reported 1.6 g/L pyruvic acid production by *Yarrowia lipolytica* in shake-flask scale fermentation. In another study, 35 mg/L of ketoisovaleric acid, which is the alpha keto acid of valine was produced in shake-flask fermentation (Sluis et al., 2001). Itoh et al. (1994) studied production of various alpha keto acidsin shake-flasks with oral *Eubacterium* species. In that study they reported 5.3 mg/L of PPA, 10.9 mg/L of alpha ketoglutaric acid, 2.4 mg/L of pyruvic acid, and 0.62 mg/L of alpha ketobutyric acid concentrations in shake-flask fermentation. Similarly black pigmented oral species of *Bacteroides* were studied for alpha keto acid productions by Tsuchia et al. (1990). They reported 14.7 mg/L of

alpha ketoglutaric acid, 9.2 mg/L of pyruvic acid, 10 mg/L of alpha ketobutyric acid, 22.3 mg/L of alpha ketovaleric acid, 9.8 mg/L of alpha ketoisovaleric acid, 12.9 mg/L of alpha ketoisocaproic acid, and 15.8 mg/L of PPA were produced in shake-flasks. In our previous studies, the maximum PPA concentration obtained was 358 mg/L in shake-flasks but this concentration was increased to 1054 mg/L after optimization of growth parameters (temperature, pH, and aeration) in bench-top bioreactors (Chapter 3). Maximum PPA concentration was further increased to 1349 mg/L after optimization of the fermentation medium in bioreactors (Chapter 4).

Alpha keto acid production can be further enhanced using advanced fermentation systems such as fed-batch and continuous fermentations. Fed-batch fermentations are generally used to prevent substrate inhibition or catabolite repression by intermittent feeding of the substrate. Also, this allow higher microorganism and product formation in the reactors compared to batch fermentation. On the other hand continuous fermentation provides constant environmental growth and product formation and supply uniform-quality of product. (Shuler and Kargi, 1992). There are only a few studies about fed-batch and continuous alpha keto acid production in the literature. Lee et al. (2013) studied fed-batch production of alpha ketoglutaric acid with *Corynebacterium glutamicum*. They produced 12.4 g/L alpha ketoglutaric acid in batch fermentation, yet increased to 19.5 g/L using fed-batch fermenters. In another study, Sawai et al. (2011) evaluated a membrane-integrated continuous system to produce pyruvic acid with *Torulopsis glabrata*. They reported that volumetric productivity of pyruvic acid increased to 4.2 g/l/h, which was four times higher than batch fermentations. Also, Takagi et al. (2009) reported 90 g/L of 2-keto-L-gulonic acid production in a mixed culture of *Ketogulonicigenium vulgare DSM 4025* and *Bacillus megaterium* or *Xanthomonas maltophilia* in continuous fermentation. Currently, no study has comparedfed-batch and continuous fermentation for PPA. Therefore, the aim of this study was to evaluate and enhance PPA production further by using fed-batch and continuous fermentation in bench-top bioreactors.

5.3. Materials and Methods

5.3.1. Microorganisms

The *Proteus vulgaris* (B-123) was used in this study, because it was determined to be the most productive species for PPA production in our previous study (Chapter 3). It was obtained from USDA Agricultural Research Service Culture Collection (Peoria, IL) and grown in 10 ml of trypticase soy broth (TSB) at 37^{o}C for 24 h. After incubation, cultures were stored at 4^{o}C as

working cultures and regularly transferred to sterile fresh media every 2 weeks to maintain viability. Stock cultures were kept frozen at -80^{o}C in 20% glycerolfor future studies.

5.3.2. Inoculum Preparation

Flasks containing 100 ml of TSB were inoculated with 1% (v/v) *P. vulgaris* and incubated at 37^{o}C with 150 rpm agitation for 30 h until late log phase. The inoculum, which had ~10^{6} cell/ml was used to inoculate the reactors.

5.3.3. Fermentations Conditions

All fermentation runs were performed in Sartorius Biostat B Plus bioreactor (Sartorius Stedim, Allentown, PA) equipped with a 2-L vessel with a 1-L working volume. Reactors were inoculated with a 1% (v/v) prepared inoculum. Fermentation runs were performed at 34.5^{o}C, 5.12 pH, and 0.5 vvm, as determined by our previous study (Chapter 3). The fermentation medium consisted of 119.4 g of glucose, 3.7 g of yeast extract, 5 g of NaCl, 1 g of K_2HPO_4, 0.2 g of $MgSO_4$, and 14.8 g of L-phenylalanine per liter of deionized water as suggested by our previous study (Chapter 4). Agitation was maintained at 300 rpm for all fermentation runs.

5.3.3.1. Fed-batch Fermentation

Fed-batch fermentations were conducted to study the effect of addition of phenylalanine on PPA production. Based on our previous work (Chapter 4), three fed-batch addition times were selected to study PPA and biomass production and phenylalanine consumption, with the ; early log stage (10 h), middle log stage (20 h), or late log stage (30 h) feeding. At these feeding times, phenylalanine concentrations were aimed to increase to the initial concentration (~15 g/L). Therefore, 1, 2.5, and 4 g of phenylalanine were added into the reactors at early, middle, and late log stages, respectively. Samples were collectedevery 5 h to determine PPA, phenylalanine, and biomass concentrations for a total run of 75 h.

5.3.3.2. Continuous Fermentation

Continuous fermentation was set up in a chemostat system for PPA production. Based on the specific growth rate obtained in our previous study (Chapter 4) various dilution rates between

0.05 h^{-1} and 0.15 h^{-1} were evaluated. PPA fermentation was started as a batch for 30 h until late log phase, then the system was switched to continuous fermentation by turning on inlet and outlet pumps at the specified dilution rates. Samples were collected and analyzed for PPA, phenylalanine and biomass concentrations at the steady state.

5.3.4. Analysis

Collected samples were centrifuged at 5,200 x *g* for 15 min to remove the biomass. Then, supernatant was used for the analysis of PPA and phenylalanine concentrations. Samples without centrifugation were used to determine biomass concentrations. All analyses were performed with three replications as follows:

5.3.4.1. Biomass

Absorbance of samples was measured at 620 nm using a spectrophotometer (Beckman Coulter, Fullerton, CA) to determine microbial concentration in the broth. Sterile fermentation medium was used as a blank for the measurement. Absorbance was converted to g biomass/L by using the standard curve (R^2>0.99).

5.3.4.2. Phenylpyruvic Acid

Cell-free samples were derivatized with 2,4-dinitrophenylhydrazine (DNPH) as described by Elias et al. (2008). The derivatization solution was prepared as follows: 0.2 g of DNPH was dissolved in 100 ml acetonitrile and 4 ml of 70 wt % perchloric acid was added to the solution. The sample (0.1 ml) was mixed with 0.24 ml of DNPH solution and 0.04 ml of 25% (w/v) H_2SO_4 solution. The mixture was incubated at 60ºC for 1 h. After derivatization, 0.48 ml of 60:40 acetonitrile:water mixture was added to the mixture and filtered into HPLC vials using 0.2 μm polytetrafluoroethylene (PTFE) filters (VWR, Radnor, PA). PPA concentrations were measured by high pressure liquid chromatography (HPLC) with a RP-C18 column (Sigma-Aldrich, St. Louis, MO) and UV detector (Waters, Milford, MA) at 365 nm. A gradient flow rate of 1 ml/min was used with 25 mM phosphate buffer (pH 2.2) (A) and acetonitrile (B) as described in Table 5.1. PPA standard was purchased from Sigma-Aldrich (St. Louis, MO).

Table 5.1. HPLC gradient flow table for phenylpyruvic acid and phenylalanine analysis (solvent A: 25 mM phosphate buffer (pH 2.2) and solvent B: acetonitrile for phenylpyruvic acid analysis. Solvent A: 50 mM sodium acetate (pH 7.2) and solvent B: 25% 50 mM sodium acetate (pH 7.2) and 75% methanol for phenylalanine analysis).

Time (min)	Flow (ml/min)	A (%)	B (%)
0.01	1.00	90.0	10.0
1.50	1.00	90.0	10.0
20.00	1.00	10.0	90.0
25.00	1.00	90.0	10.0
30.00	1.00	90.0	10.0

5.3.4.3. Phenylalanine

Samples were derivatized with o-phthalaldehyde (OPA) as described in Knauer (2012). A cell-free sample (0.1 ml) was mixed with 1 ml OPA and filtered into HPLC vials using 0.2 μm PTFE filters. Phenylalanine concentrations were measured at 230 nm using the same HPLC system with a 1 ml/min gradient flow of A: 50 mM sodium acetate (pH 7.2) and solvent B: 25% 50 mM sodium acetate (pH 7.2) and 75% methanol as described in Table 5.1.

5.3.4.4. Statistical Analysis

Each fermentation run was duplicated. Each sample was analyzed in triplicate and averaged. Tukey's test was used to make a single step multiple comparison of fed-batch results. MINITAB statistical software (Version 15, State College, PA) package was used for statistical analyses.

5.4. Results and Discussion

5.4.1. Fed-batch Fermentation

Fed-batch fermentations were conducted to evaluate the effect of phenylalanine addition on PPA production. Figure 5.1 shows the impact of phenylalanine addition on PPA production. Figure 5.1A shows that, addition of 1 g of phenylalanine at 10 h of fermentation increased PPA concentration from 1350 mg/L to 1398 mg/L at 30 h of fermentation. In our previous batch

fermentations, the highest PPA production was obtained at 1349 g/L. Therefore, it can be concluded that the 1 g addition of phenylalanine at 10 h of the fermentation did not improve PPA production significantly ($p<0.05$) compared to the previous batch fermentations (Chapter 4). The yield of PPA production based on phenylalanine consumption ($Y_{PPA/Phe}$) was calculated as 37.8%. Figure 5.1B shows the addition 2.5 g of phenylalanine at 20 h of fermentation increased PPA concentration to 1989 mg/L at 35 h of fermentation and $Y_{PPA/Phe}$ was calculated as 40.6%. Additionally, PPA concentration was further increased to 2958 mg/L with4 g of phenylalanine addition at 30 h of fermentation (Figure 5.1C).

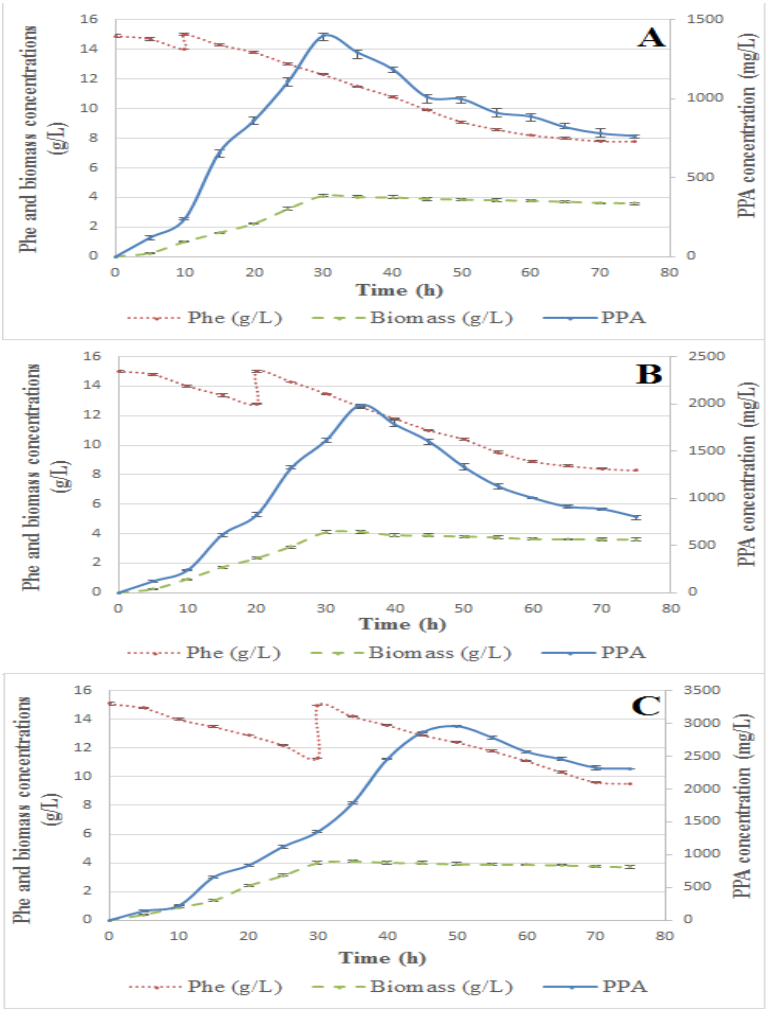

Figure 5.1. Fed-batch PPA production (A: 1 g phenylalanine addition at 10 h of fermentation. B: 2.5 g phenylalanine addition at 20 h of fermentation. C: 4 g phenylalanine addition at 30 h of fermentation). Arrows indicate the times for phenylalanine addition.

$Y_{PPA/Phe}$ was calculated as 44.8% within the late log stage phenylalanine addition fed-batch fermentation. PPA productivity of each fermentation type was shown on Table 5.2.

Table 5.2. Comparison of different fermentation methods for PPA production

Fermentation type	**Maximum PPA concentration** (mg/L)**	**PPA productivity (mg/L/h)**	**Production yield ($Y_{PPA/Phe}$) (%)**	**PPA lost in 25 h after reaching the maximum conc. (%)**
Batch fermentation (Chapter 4)	1350 ± 24	48	37.5	37
1 g of phenylalanine addition at 10 h of fed-batch fermentation	1398 ± 32	55	37.8	35
2.5 g of phenylalanine addition at 20 h of fed-batch fermentation	1989 ± 38	70	49.7	50
4 g of phenylalanine addition at 30 h of fed-batch fermentation	2958 ± 11	104	53.8	22
Continuous fermentation	2047 ± 21	259	60	N/A*

* Not applicable.

** Average of two replications.

There was an insignificant increase in productivities between batch and fed-batch PPA productions, when phenylalanine was added for 1 g at 10 h of fermentation. However, PPA productivity was remarkably increased, when 2.5 g and 4 g of phenylalanine was added at 20 and 30 h of fermentations. PPA productivities were measured as 55, 70, and 104 mg/L/h for 10 h addition, 20 h addition, and 30 h addition, respectively. In all cases, PPA concentration decreased after reaching the maximum level by 35, 50, and 22%, respectively, after 25 h of reaching the maximum PPA concentrations for each case. This may be related to the lowest biomass, since the cells were already at stationary or early death phase at 30 h of fermentation. Similarly, it was reported by Naz et al. (2013) that PPA can be further converted to phenyllactic acid and phenyl ethyl alcohol by resting cells. In all cases, PPA concentration was reduced after it reached the maximum concentration. This can be explained by consumption of PPA by microorganisms as a nutrient or further degradation of PPA to aromatic compounds or break downPPA and the

deaminase enzyme by hydrogen peroxide, which is produced during the microbial deamination reaction (Hernandez et al., 2012; Lafuente et al., 1988). In the literature, it was reported that hydrogen peroxide (H_2O_2) can negatively affect biomass, substrate, product, and enzyme during the certain fermentation, consequently effect the overall yield. Hernandez et al. (2012) reported that, hydrogen peroxide attacks keto acids and results in oxidative decarboxylation of the compound. Also, Mata et al. (2000) showed that, hydrogen peroxide was a noncompetitive inhibitor to amino acid oxidase enzyme, when D-alanine was the variable substrate and oxygen was not at saturation levels in alpha keto acid productions. Similarly, Lafuente et al. (1988) reported that, hydrogen peroxide can deactivate enzyme and/or substrate very fast and causes low yield at the end of the process during PPA productions. Also, Xu et al. (2008) reported that, hydrogen peroxide can break down pyruvic acid, which is the alpha keto acid to acetate, generating CO_2, and water and decreases the production yield in the fermentation. The effect of glucose was also evaluated, but glucose addition did not make any significant change on PPA production (data not shown). Similarly, addition of phenylalanine at higher levels in fed-batch fermentation was also studied; however no significant PPA production improvement was observed (data not shown).

Phenylalanine addition did not affect biomass production (Figure 5.1). Maximum biomass concentrations were measured as 4.10, 4.11, and 4.12 g/L/h, respectively in the fed-batch studies (Figure 5.1). Biomass production rates were calculated as 0.15 g/L/h for all fed-batch fermentation studies (Figure 5.1). Additionally, phenylalanine consumption was calculated, when the maximum PPA concentrations were obtained in the fed-batch fermentations and reported as 3.6, 4.9, and 6.7 g/L, respectively (Figure 5.1).

Application of Tukey's statistical method classified all fed-batch PPA production results separately. This means that all PPA concentrations were significantly ($p<0.05$) different than each other (Table 5.3). Therefore, it can be concluded that, phenylalanine addition and time are significant factors on PPA production ($p<0.05$).

Table 5.3. One-way Tukey's statistical method evaluation of fed-batch PPA production results.

Source	DF	SS	MS	F	P
Factors	2	3721842	1860921	29538.43	0.000
Error	6	378	63		
Total	8	3722220			

S = 7.937 R-Sq = 99.99% R-Sq(adj) = 99.99%

Factors	N	Mean
4 g of phenylalanine addition at 30th hour of fermentation	3	2958
2.5 g of phenylalanine addition at 20th hour of fermentation	3	1989
1 g of phenylalanine addition at 10th hour of fermentation	3	1398

Factors	Grouping
4 g of phenylalanine addition at 30th hour of fermentation	A
2.5 g of phenylalanine addition at 20th hour of fermentation	B
1 g of phenylalanine addition at 10th hour of fermentation	C

5.4.2. Continuous Fermentation

A continuous fermentation system was evaluated for PPA production at various dilution rates between 0.05 and 0.15 h^{-1}. Figure 5.2 shows that phenylalanine and biomass concentrations remained the same, whereas PPA concentration was increased until a dilution rate of 0.12 h^{-1}.

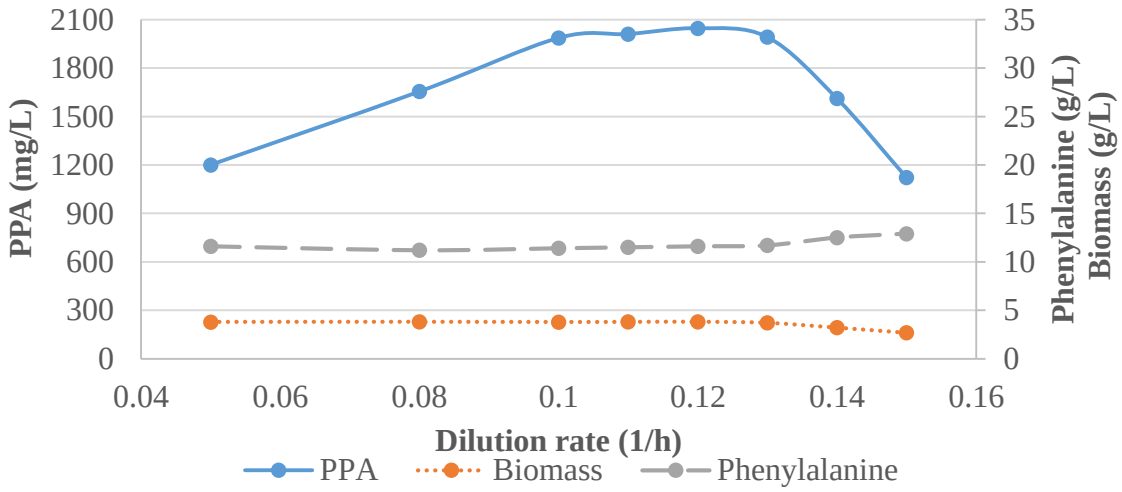

Figure 5.2. PPA, phenylalanine, and biomass production under various dilution rates in continuous fermentation.

Then, biomass and PPA concentration decreased and phenylalanine concentration increased at higher dilution rates because of wash-out effect, whereas biomass concentration was stable at ~3.8 g/L, when the fermentation wasbetween 0.05 and 0.12 h^{-1} dilution rates as expected from a continuous fermentation. Maximum biomass productivity was measured as 0.48 g/L/h, when the fermentation was running at 0.13 h^{-1} dilution rate. Similarly, phenylalanine concentration remained at 11.2-11.7 g/L until the 0.12 h^{-1} dilution rate, and phenylalanine concentration remained at constant 12.5 and 12.9 g/L, when the fermentation was at 0.14 and 0.15 h^{-1} dilution rates, respectively. The PPA concentration was measured as 1201 mg/L, at the lowest dilution rate (0.05 h^{-1}), but increased to 2047 mg/L at 0.12 h^{-1} dilution rate (Figure 5.2). Additionally, PPA productivity was measured as 259 mg/L/h, when the fermentation was at 0.13 h^{-1} dilution rate, which was 2.5 and 5 times higher than the highest PPA productivity in fed-batch and batch fermentations, respectively (Figure 5.2).

5.5. Conclusions

In conclusion, fed-batch and continuous fermentations were evaluated for PPA production by *P. vulgaris* in bench-top bioreactors in this study. It was determined that fed-batch phenylalanine additions are significantly effective on PPA production but not biomass concentration. The maximum PPA concentration was more than doubled, when 4 g of phenylalanine was added into the reactor at 30 h of fermentation (2958 mg/L) compared to batch fermentations (1350 mg/L). On the other hand, PPA productivity increased more than 5 fold in continuous fermentation (259 mg/L/h) compared to batch fermentations (48 mg/L/h). Maximum PPA concentration was almost doubled, when the fermentation was run at a maximum dilution rate, compared to 0.05 h^{-1}. This study clearly demonstrated that PPA production can be enhanced in fed-batch and continuous fermentation, which is a step forward for commercial production.

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CHAPTER 6

MODELING OF PHENYLPYRUVIC ACID FERMENTATION WITH *PROTEUS VULGARIS*

6.1. Abstract

Phenylpyruvic acid (PPA) is an important food and feed additive, which has application in agriculture, medicine and the food industry. Alpha keto acids are commonly produced by microbial deamination of amino acids during fermentation processes. In our previous study, 1350 mg/L PPA was produced in batch bioreactors after optimization of growth and medium parameters. Mathematical models of substrate consumption and product and biomass formation in PPA fermentation are useful to provide kinetic-metabolic nature of the process. In this study, the modified Gompertz and modified logistic model were used to predict PPA and biomass formation, and glucose and phenylalanine consumption. Goodness of fit was measured by calculation R^2, mean absolute error (MAE), and root mean square error (RMSE) values. It was observed that the modified Gompertz model provided better predictions in all cases with lower RMSE and MAE values compared to the modified logistic model. MAE values were obtained 0.317, 91.958, -0.624, -0.844 for biomass, PPA, phenylalanine, and glucose predictions, respectively, for the modified Gompertz model. Whereas, MAE values were 0.448, 131.709, -1.181, and -4.527 for biomass, PPA, phenylalanine, and glucose prediction, respectively, for the modified logistic model. On the other hand, R^2 values were slightly higher for the modified logistic model compared to the modified Gompertz model in PPA and biomass predictions. Overall, this study demonstrated that modeling of PPA fermentation can provide a better understanding of the fermentation kinetics and sets the stage as a tool to assist scale up to industrial production.

6.2. Introduction

Phenylpyruvic acid (PPA) is the alpha keto acid of phenylalanine, which is commonly used in many areas. In medicine, PPA is used for the diagnosis of phenylketonuria, which is a genetic disease involving phenylalanine metabolism (Folling, 1994). PPA is also used in kidney patients' diets instead of amino acids to reduce urea accumulation in their body without changing the nutritional intake (Krause et al., 2010). Additionally, natural products are gaining the interest

of consumers for flavor enhancement of foods and PPA is one of the popular flavor enhancer in food preparations. It was reported that PPA is an important compound in the development of aroma, taste, and texture in cheese and wine manufacturing (Casey et al., 2004). Moreover, PPA can be used in poultry as a feed supplement to reduce nitrogen accumulation in the manure to prevent negative effects on the environment (Summer, 1993).

PPA can be produced by microbial deamination of phenylalanine. It was reported that *Proteus*, *Morganella*, and *Provedincia* spp. can produce the deamination enzyme with high activity (Chapter 3; and Smit, 1966). There are only a few studies in the literature about PPA production. Itoh et al. (1994) reported that 5.3 mg/L of PPA, 10.9 mg/L of alpha ketoglutaric acid, 2.4 mg/L of pyruvic acid, and 0.62 mg/L of alpha ketobutyric acid were produced by using *Eubacterium nodatum.* In another study, Tsuchiya et al. (1990) produced 14.7 mg/L of alpha ketoglutaric acid, 9.2 mg/L of pyruvic acid, 10 mg/L of alpha ketobutyric acid, 22.3 mg/L of alpha ketovaleric acid, 9.8 mg/L of alpha ketoisovaleric acid, 12.9 mg/L of alpha ketoisocaproic acid, and 15.8 mg/L of PPA in the shake-flasks with black pigmented oral *Bacteroides* species. In our previous studies, 358 mg/L of PPA was produced with *Proteus vulgaris* in shake-flask fermentation and production increased to 1054 and 1350 mg/L after optimization of growth and medium parameters, respectively, in the bioreactors (Chapters 3 and 4).

Mathematical models can provide information about product and biomass formation as well as substrate consumption, which can be used to assess the fermentation processes. Dufosse et al. (2001) used the Gompertz model to study the effect of nitrogenous substrates on microbial growth. They reported the Gompertz model successfully represented microbial data in their studies. Additionally, Zhao et al. (2014) modeled the growth of *Proteus mirabilis* under various temperature conditions. They reported that the Gompertz and logistic models predicted the experimental data successfully with low mean error square values (MSE). Similarly, the Gompertz model and logistic models were used by Pongtharankul et al. (2008) and Lv et al. (2005) for nisin production. Currently, there is no study available in the literature for modeling the microbial production of PPA. Therefore, this study undertaken to model PPA and *Proteus vulgaris* biomass production and also phenylalanine and glucose consumption in the bioreactors.

6.3. Materials and Methods

6.3.1. Microorganisms and Inoculum Preparation

Proteus vulgaris (B-123) was obtained from the USDA Agricultural Research Service Culture Collection (Peoria, IL) and grown in 10 ml of trypticase soy broth (TSB) at 37^{o}C for 24 h. After incubation, working cultures were stored at 4^{o}C and regularly transferred to sterile fresh media every 2 weeks to maintain viability. Stock cultures were kept frozen in 20% glycerol at -80^{o}C for future studies. Flasks containing 100 ml of TSB were inoculated with 1% (v/v) *P. vulgaris* and incubated at 37^{o}C with 150 rpm agitation for 30 h until late log phase. A concentration of 10^{6} cell/ml was used to inoculate the bioreactors.

6.3.2. Batch Fermentation in Bioreactors

Sartorious Biostat B Plus bioreactors (Sartorious, Allentown, PA) equipped with a 2-L vessel and 1-L working volume were used in the experiments. The fermentation medium consisted of 120 g of glucose, 3.6 g of yeast extract, 5 g of NaCl, 1 g of K_2HPO_4, 0.2 g of $MgSO_4$, and 15 g of L-phenylalanine per liter of deionized water. Temperature, pH, and aeration were maintained at 34.5^{o}C, 5.12, and 0.5 vvm, as indicated by our previous optimizing studies (Chapter 3 and 4). Additionally, agitation was maintained at 300 rpm for all fermentation runs. Fermentation was initiated by adding 1% (v/v) inoculum and samples were taken every 5 h until 75 h then analyzed for biomass, PPA, glucose, and phenylalanine concentrations.

6.3.3. Analysis

Samples collected during the fermentation was first subsampled to determine biomass content and then centrifuged at 5,200 x *g* for 15 min to remove the biomass. The supernatant was used for analysis of PPA, glucose, and phenylalanine concentrations.

6.3.3.1. Biomass

Absorbance of sample was measured at 620 nm by using a spectrophotometer (Beckman Coulter, Fullerton, CA) to determine microbial concentration in the broth. Sterile fermentation

medium was used as the blank for the measurement. Absorbencies were converted to g/L using a standard curve.

6.3.3.2. Phenylpyruvic Acid

Cell-free samples were derivatized with 2,4-Dinitrophenylhydrazine (DNPH) as described in the literature (Elias et al., 2008). The derivatization solution was prepared as follows: 0.2 g of DNPH was dissolved in 100 ml acetonitrile and 4 ml of 70 wt % perchloric acid was added to the solution. Samples (0.1 ml) were mixed with 0.24 ml of DNPH solution and 0.04 ml of 25% (w/v) H_2SO_4 solution. The mixtures were incubated at 60ºC for 1 h. After derivatization, 0.48 ml a of 60:40 acetonitrile:water mixture was added to the mixtures and filtered into high pressure liquid chromatography (HPLC) vials using 0.2 μm polytetrafluoroethylene (PTFE) filters (VWR, Radnor, PA). Phenylpyruvic acid concentrations were measured using Waters (HPLC with a RP-C18 column (Sigma-Aldrich, St. Louis, MO) and UV detector (Waters, Milford, MA) at 365 nm. Flow was adjusted to 1 ml/min gradient flow of A: 25 mM phosphate buffer (pH 2.2) and solvent B: acetonitrile. The PPA standard was purchased from Sigma-Aldrich (St. Louis, MO).

6.3.3.3. Phenylalanine

Samples were derivatized with o-phthalaldehyde (OPA) as described by Elias et al., (2008). The cell-free sample (0.1 ml) was mixed with 1 ml OPA and filtered into HPLC vials using 0.2 μm PTFE filters. Phenylalanine concentrations were measured at 230 nm using the same HPLC system with a 1 ml/min gradient flow of A: 50 mM sodium acetate (pH 7.2) and solvent B: 25% 50 mM sodium acetate (pH 7.2) and 75% methanol.

6.3.3.4. Glucose

Glucose concentrations were measured using high pressure liquid chromatography (HPLC) with a refractive index detector (Waters, Milford, MA). Glucose determinations were performed using Aminex HPX-87H column (Bio-Rad, Richmond, CA) with 0.8 ml/min isocratic flow of 0.012 N sulfuric acid. The detector and column temperature were maintained at as 35 and

65ºC, respectively. The cell-free samples were filtered 13 mm diameter, 0.2 μm pore sized filters (PALL Life Sciences, Port Washington, NY).

6.3.3.5. Mathematical Models

The growth curve of the bacteria and PPA formation usually exhibits a sigmoid pattern with lag, exponential, and stationary phases. Therefore, the modified Gompertz (Equation 6.1) and modified logistic model (Equation 6.2) were proposed to predict PPA and biomass production as well as phenylalanine and glucose consumption in thefermentation. Zwietering et al. (1990) reported the modified Gompertz and modified logistic equations an equation 6.1 and equation 6.2, respectively, as below.

$$A_t = A_m \exp\left\{-\exp\left[\frac{B_m e}{A_m}(\lambda - t) + 1\right]\right\} \quad \text{.....Equation 6.1}$$

$$A_t = \frac{A_m}{\left\{1+\exp\left[\frac{4B_m}{A_m}(\lambda - t)+2\right]\right\}} \quad \text{.....Equation 6.2}$$

where A_t= The concentration of the studied factor at "t" time

A_m= The maximum concentration of the studied factor

B_m= The production or consumption rate of the studied factor

λ= Duration of lag phase (h)

t= Sampling time (h)

All these parameters were calculated from the batch fermentation, which was run under optimum conditions as described in Chapter 4. Maximum PPA and biomass concentrations at the end of the log phase and maximum glucose and phenylalanine concentrations at the beginning of lag phase were assigned as A_m. B_m values were obtained by the measurement of the maximum production or consumption rate of the factors. λ was calculated by the determining of the time to reach log phase.

6.3.3.6. Validation of the Mathematical Models

Predicted data, obtained from the modified Gompertz model and the modified logistic models were compared with the experimental data by calculation of root mean square error (RMSE), and mean absolute error (MAE). Also, R^2 and slope were evaluated by plotting the predicted versus experimental data.

6.4. Results and Discussion

6.4.1. Modeling of Biomass Production

Comparisons of the experimental and predicted *Proteus vulgaris* growth curves with modified the Gompertzand the modified logistic models are shown on Figure 6.1.

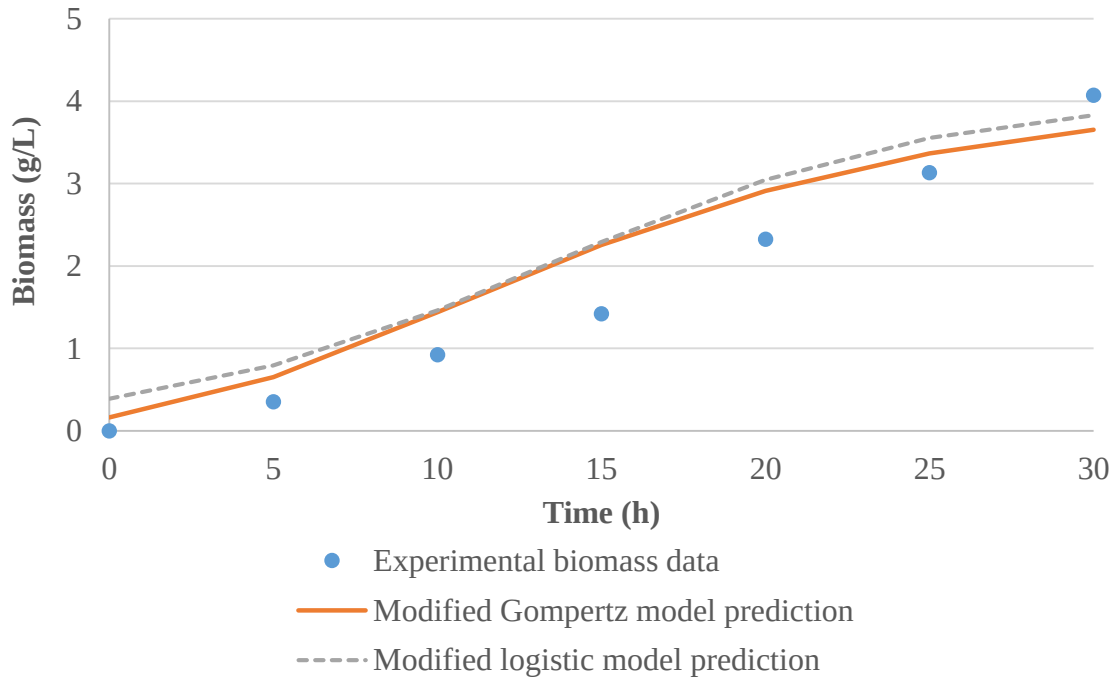

Figure 6.1. Growth curve of *Proteus vulgaris* fitted with the modified Gompertzand modified logistic model.

The modified Gompertz model fitted the experimental data slightly better during lag and exponential phases compared to modified logistic model. Contrarily, the modified logistic model predict the maximum biomass concentration better than the modified Gompertz model in the stationary phase. The maximum biomass concentration at 30 h of fermentation was predicted as 3.65 and 3.83 g/L from the modified Gompertzand modified logistic model, respectively. Additionally, better lower asymptote prediction was obtained utilizing modified Gompertz model. A lower asymptote (initial biomass concentration) was predicted as 0.1625 and 0.3880 g/L with the modified Gompertz and modified logistic model, respectively.

Calculated RMSE, MAE, R^2, and slope values for the two models are shown in Table 6.1. Since the same input data was used, the RMSE and MAE values can be used to compare the performances of the two models. The modified Gompertz model provided lower RMSE and MAE values (0.486 and 0.317) compared to modified logistic model (0.555 and 0.448) for the biomass prediction, respectively. On the other hand, higher R^2 and slope values were determined for the modified logistic model (0.949 and 0.8819) compared to modified Gompertz model (0.933 and 0.8727) (Table 6.1).

Table 6.1. RMSE, MAE, R^2, and slope values about biomass prediction for the modified logistic and modified Gompertz models.

Model	**RMSE (g/L)**	**MAE (g/L)**	**R^2**	**Slope**
Modified logistic	0.555	0.448	0.949	0.8819
Modified Gompertz	0.486	0.317	0.933	0.8727

6.4.2. Modeling of PPA Production

PPA production with *P. vulgaris* was also modeled using the modified Gompertzand modified logistic model (Figure 6.2).

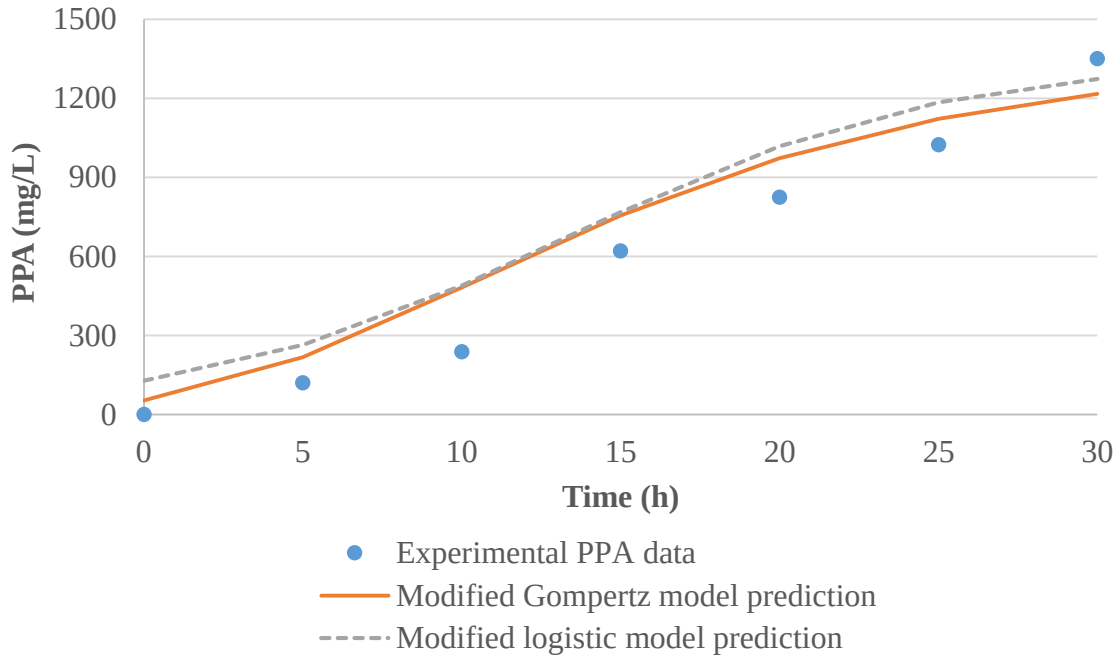

Figure 6.2. PPA production fitted with the modified Gompertz model and modified logistic model.

PPA prediction by both models showed a very similar trend with the biomass prediction. Both models over-predicted the experimental PPA data except for 30 h. Also, the modified logistic model always predicted higher values compared to modified Gompertz model. Predicted values by both models were very close to each other at t=10 and t=15 h. The maximum PPA concentration (1350 mg/L) at 30 h of fermentation was predicted as 1216 and 1274 mg/L by the modified Gompertz and modified logistic model, respectively. Lower asymptote was predicted as 54 and 128 mg/L by the modified Gompertz and modified logistic model, respectively. RMSE, MAE, R^2, and slope values obtained for PPA prediction from both models were shown on Table 6.2.

Since both models over-predicted PPA production, positive MAE values were obtained for the modified Gompertz model, and modified logistic model as 91.958 and 135.709, respectively. Lower RMSE value was obtained for PPA production from modified Gompertz model compared to modified logistic model. However, slightly higher R^2 and slope values were determined for the modified logistic model (0.966 and 0.8833) compared to the modified Gompertz model (0.953 and 0.8758), respectively (Table 6.2).

Table 6.2. RMSE, MAE, R^2, and slope values about PPA prediction for modified logistic and modified Gompertz model.

Model	RMSE (mg/L)	MAE (mg/L)	R^2	Slope
Modified logistic	165.441	135.709	0.966	0.8833
Modified Gompertz	141.452	91.958	0.953	0.8758

6.4.3. Modeling of Phenylalanine Consumption

Consumption of the phenylalanine was also predicted by using the modified Gompertz and modified logistic models (Figure 6.3). Phenylalanine consumption was lower-predicted by both models for all data points. However modified logistic model always showed higher off- values compared to modified Gompertz model predictions. Differences between the predictions of the both models decreased by the time increased. Initial phenylalanine concentration (14.9 g/L) was predicted as 14.01 and 13.21 g/L by modified Gompertz model and modified logistic model, respectively. On the other hand, phenylalanine concentration at 30 h of the fermentation (11.3 g/L) was predicted as 11.1 and 10.8 g/L by modified Gompertz model and modified logistic model, respectively.

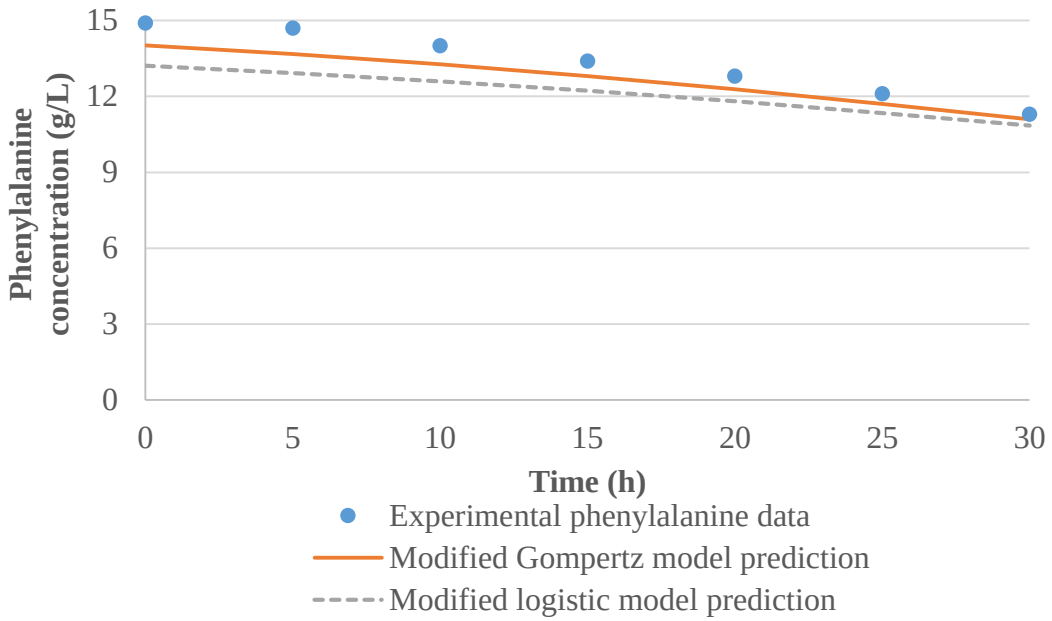

Figure 6.3. Phenylalanine consumption fitted with the modified Gompertz model and modified logistic model.

Because of lower-predictions, negative MAE values were obtained for both models. Similar to previous predictions, modified Gompertz model resulted in lower RMSE and MAE values compared to modified logistic model. RMSE value was obtained as 0.677 for modified Gompertz model, whereas this value almost doubled for the modified logistic model (1.264). Also, MAE value was -0.624 for modified Gompertz model but -1.181 for the modified logistic model. R^2 values calculated as the same for the both models. Also, slope of the modified Gompertz model was significantly higher than slope of the modified logistic model (Table 6.3).

Table 6.3. RMSE, MAE, R^2, and slope values about phenylalanine prediction for modified logistic and modified Gompertz model.

Model	RMSE (g/L)	MAE (g/L)	R^2	Slope
Modified logistic	1.264	-1.181	0.997	0.6385
Modified Gompertz	0.677	-0.624	0.997	0.7931

6.4.4. Modeling of Glucose Consumption

Additionally, consumption of the glucose was predicted by using modified Gompertz model and modified logistic model (Figure 6.4). Both models successfully represented glucose consumption. Initial glucose concentration (120 g/L) was predicted as 118.46 and 111.64 g/L for modified Gompertz model and modified logistic model, respectively. Both models predicted the glucose concentration as 78 g/L at the 30 h of fermentation, whereas the experimental data was 76 g/L. It can be seen from Figure 6.4 that modified Gompertz model predicted experimental glucose data more successfully compared modified logistic method.

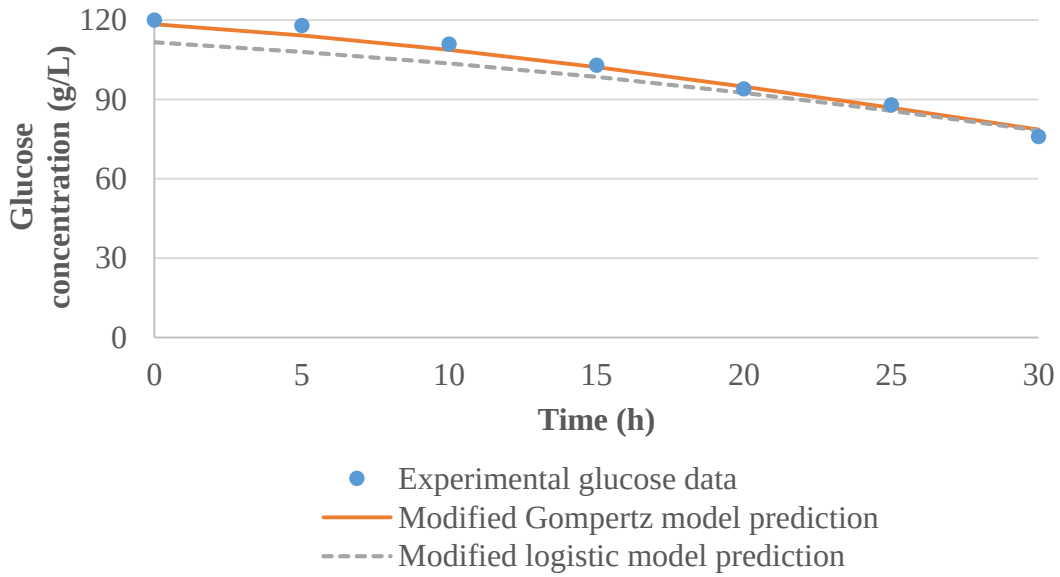

Figure 6.4. Glucose consumption fitted with the modified Gompertz model and modified logistic model.

RMSE, MAE, R^2, and slope values about glucose prediction for modified logistic and modified Gompertz model were shown on Table 6.4. Modified Gompertz model showed demonstrated RMSE and MAE values (2.130 and -0.844) for glucose prediction compared modified logistic method (6.046 and -4.527), respectively. Even though both models provided the same R^2 value as 0.994, higher slope value was obtained by modified Gompertz model compared to modified logistic method.

Table 6.4. RMSE, MAE, R^2, and slope values about glucose prediction for modified logistic and modified Gompertz model.

Model	RMSE (g/L)	MAE (g/L)	R^2	Slope
Modified logistic	6.046	-4.527	0.994	0.7410
Modified Gompertz	2.130	-0.844	0.994	0.8903

6.5. Conclusions

In this study, PPA and biomass production, and glucose and phenylalanine consumption by *P. vulgaris* were predicted with modified Gompertz model and modified logistic model. Both models over-predicted biomass and PPA production, whereas negative MAE values were obtained for phenylalanine and glucose consumption predictions. For all predictions modified Gompertz model predicted the experimental data with lower RMSE and MAE values compared to modified logistic model. However, modified logistic model provided higher R^2 values in biomass and PPA predictions compared to modified Gompertz model. These models can be used for further development of PPA production and scale-up of fermentations.

Nomenclature

Parameters used in the models	Symbols	Value	Unit
Maximum specific growth rate for biomass production	B_m	0.17	h^{-1}
Maximum specific PPA production rate	B_m	57	g/L/h
Maximum specific phenylalanine consumption rate	B_m	0.133	g/L/h
Maximum specific glucose consumption rate	B_m	1.65	g/L/h
Maximum biomass concentration	A_m	4.071	g/L
Maximum PPA concentration	A_m	1350	mg/L
Maximum glucose concentration	A_m	120	g/L
Maximum phenylalanine concentration	A_m	14.9	g/L
Duration of lag phase	λ	1.5	h^{-1}
e		2.71	

6.6. References

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CHAPTER 7

SCREENING OF PHYTASE PRODUCERS AND OPTIMIZATION OF CULTURE CONDITIONS FOR SUBMERGED FERMENTATION

7.1. Abstract

Phytase (myo-inositol-hexakisphosphate phosphohydrolase) is an enzyme, which breaks down phytate to inositol and orthophosphoric acid. Phytase has been used as feed additive, and in some medical applications for years. To date, phytase production has been usually performed as a solid-state fermentation with small production volumes. Therefore, the aim of this study was to increase the phytase activity in submerged fermentations by screening several microorganism strains based on the literature to select the most productive phytase producer and optimizing growth parameters such as temperature, pH, and aeration level using response surface methodology (RSM). As a result, among the four different microorganisms evaluated, *Aspergillus ficuum* (NRRL 3135) was selected as the most productive strain. Optimum temperature, pH, and aeration values were determined as 33ºC, 4.5, and 0.9 vvm, respectively, for *A. ficuum* in batch submerged phytase fermentations. Under these conditions, phytase activity was measured as 2.27 U/ml. Therefore, this is a unique study showing the production of phytase with *A. ficuum* successfully in submerged fermentation as opposed to the traditional solid-state fermentation.

7.2. Introduction

Phytate is the primary storage of phosphate in plants, which is especially abundant in legumes, cereals, pollens, and nuts. It plays an important role during germination by liberating high amounts of phosphorus, which are used for ATP synthesis (Vohra and Satyanarayana, 2003). However, six reactive groups of phytate make it a polyanionic chelating agent which reacts with proteins, amino acids, and important divalent cations such as Ca^{+2}, Mg^{+2}, Zn^{+2}, Cu^{+2}, Fe^{+2}, and Mn^{+2} in humans and animals (Haefner et al., 2005). Therefore, this may result in malnutrition and several health problems such as iron deficiency, bone weakness, tooth decay, and digestion problems (Hurrel et al., 2003; Sanson et al., 1981). Additionally, phytate also causes some environmental problems. While ruminant animals sustain the microflora, which releases inorganic phosphorus from phytate, monogastric animals such as chickens and pigs can produce little or no phytase in their intestine. Since, monogastric animals are generally fed with soybean and other

meals, which has high concentration of phytate, excessive phosphorus accumulation occurs in their manure. This causes problems such as water pollution, algal blooms, fish kills, and changing of fauna and flora in the environment (Mullaney et al., 2000).

Phytase, catalyzes hydrolysis of phytate to inositol and orthophosphoric acid (Liu et al., 1998). It has been used as food and feed additive to prevent the adverse effects of phytate as described above. Nelson et al. pretreated a corn–soya diet with phytase of *A. niger* (Nelson et al., 1968). When microbial phytase was added to low phosphorus diets for broilers, the availability of phosphorus increased by 60% and the amount of phosphorus in the droppings decreased by 50%. Moreover, after 21 days of phytase supplementation body weight of male and female chicks increased by 13.2 and 5.8%, respectively. Supplementation of the low phytate diet with phytase also increased the relative retention of total P^{-3}, Ca^{+2}, Cu^{+2}, and Zn^{+2} by 12.5, 12.2, 19.3, and 62.3%, respectively (Sebastian et al., 1996). Phytase has been also used to produce inositol phosphates and phospholipids, which play a role in transmembrane signaling and transfer of Ca^{+2} from intracellular reserves (Billington, 1993). Inositol phosphate derivatives can be used as enzyme stabilizers and enzyme substrates for metabolic investigation, as enzyme inhibitors and therefore potential drugs, and as chiral building blocks (Laumen and Ghisalba, 1994).

Phytase can be secreted by several microorganisms including bacteria, yeasts, and molds. Bacterial phytase are generally produced from *Lactobacillus*, *Escherichia, Pseudomonas,* and *Klebsiella* spp. Bacterial phytase is the most active at pH 6.0-6.5 and at 60°C. Other isolates have been tested for extracellular phytase productions such as *Mucor, Penicillium, Aspergillus,* and *Rhizopus* spp. Liu et al. (1998) evaluated 58 different fungi for the production of extracellular phytase and found that, *A. ficuum* as the most efficient producer. Only a few yeast species such as *Saccharomyces cerevisiae*, *Schwanniomyces castellii,* and *Schizophyllum commune* have been reported as phytase producers (Nelson et al., 1968).

To date, phytase production has been mostly performed as a solid state fermentation. Salmon et al. (2012) used different agroindustrial residuals and studied solid state phytase production by using *S. commune*. Similarly, phytase production was studied by Gibson (1987) on starch media and by Han et al. (1988) on cereal grains and legume seeds and by Ebune et al. (1995) on canola meal by using *A. ficuum*. However, solid state phytase productions are not very flexible to scale-up for commercial productions. Moreover, costly and complex extraction steps are used to separate and purify the enzyme, even it is produced extracellularly. Additionally, lack of stirring during fermentation causes heterogeneity in phytase properties in the solid state

productions. Therefore, there is a need to produce phytase in submerged liquid fermentation. For example, Haritha and Sambasivarao produced phytase under submerged fermentation conditions by using *Rhizopus oligsporus* (Haritha and Sambasivarao, 2009). Soni and Khire (2007) produced and partially characterized phytase from *A. niger* NCIM 563. Also, Mittal et al. (2012) produced phytase under submerged conditions by using orange peel flour as substrate and *Klebsiella* as microorganism. However, all these studies were performed in shake-flask scale and fermentation parameter optimization was not studied to maximize phytase production. As it is well known, working volume is always limited in the shake-flask fermentations and growth parameters cannot be controlled easily such as pH control, mechanical stirring and effective aeration, which makes shake-flask fermentations cannot be scaled up at industrial productions. Therefore, microbial phytase production and optimization of fermentation parameters should be studied for submerged fermentation in bioreactors.

The aim of this study was to select the most productive phytase producer microbial strain from among several microorganisms, which have been reported as phytase producer in the previous studies and to increase the phytase activity by optimization of the growth parameters such as temperature, pH, and aeration in submerged fermentations by using a Box-Behnken design.

7.3. Materials and Methods

7.3.1. Microorganisms and Media

Lactobacillus plantarum (B-4496), *Lactobacillus acidophilus* (B-4495), *Lactobacillus amylovorus* (B-4540), and *Aspergillus ficuum* (NRRL 3135) were obtained from Agricultural Research Service Culture Collection (Peoria, IL). *Lactobacillus* strains were grown in 10 ml of MRS medium (Neogen, Lansing, MI) at 37^{o}C for 24 h and stored at 4^{o}C as working cultures. *A. ficuum* was grown on potato dextrose agar (PDA) (Difco, Sparks, MD) slants at 30^{o}C for 6 days and stored at 4^{o}C as working cultures. All cultures were regularly transferred to sterile fresh media every 2 weeks to maintain viability. Stock cultures for future studies were kept frozen in 20% glycerol at -85^{o}C.

Phytase selective agar medium was used to perform microorganism screening. Medium includes 20 g of glucose, 4 g of Na-phytate (A&Z Food Additives Co. Ltd., Zhejiang, China), 2 g of $CaCl_2$, 5 g of NH_4NO_3, 0.5 g of KCl, 0.5 g of $MgSO_4(7H_2O)$, 0.01 g of $FeSO_4(7H_2O)$, 0.01 g of

$MnSO_4(7H_2O)$, and 15 g of agar per liter of deionized water. After pH was adjusted to 7, medium was autoclaved and plated (Sumengen, 2011).

7.3.2. Screening of Strains for Phytase Production

Based on the previous studies in the literature, *L. plantarum*, *L. acidophilus*, *L. amylovorus*, and *A. ficuum* were selected for screening. All *Lactobacillus* species were grown at 37°C for 24 h in 10 ml of MRS medium. *A. ficuum* were grown on PDA plates for 6 days at 30°C. At the end of the incubation, *A. ficuum* spores were suspended by 2 ml of 0.1% peptone water per plates. Then, 20 µl of each inoculum, which had ~10^6 CFU/ml was used to inoculate on phytase selective agar plates by using point inoculation and plates were incubated at their specific temperatures for 2 days. Microorganisms, which produced phytase, created clear zones on the phytase selective agar plates. To be sure if the zones were generated by phytase activity or microbial acid production, phytase selective agar plates were subjected to washing with several solutions. First, all plates were washed with distilled water to remove the microorganisms. Next, plates were covered with 2% cobalt chloride solution and incubated for 5 min at room temperature. Then, cobalt chloride solution was discarded and the plates were covered with equal volume of freshly prepared 6.25% ammonium molybdate and 0.42% ammonium vanadate mixture. After 5 min incubation at room temperature, solution was discarded and still existing clear zones represented phytase activity (Sumengen, 2011). Diameters of clear zones were measured to compare phytase activity of each microorganism.

In order to validate phytase selective agar results, shake-flask phytase fermentation was also performed for each strain. Shake-flask phytase fermentation medium consisted of 80 g of glucose, 0.5 g of KCl, 0.5 g of $MgSO_4$, 0.1 g of $Fe_2(SO_4).3H_20$, 0.02 g of $MnSO_4.H_2O$, 8.6 g of $NaNO_3$, 3 g of $(NH_4)_2SO_4$ and 10 g of Na-phytate per liter of deionized water. Flasks include 100 ml of medium were inoculated with 1% (v/v) inoculum, which had ~10^6 CFU/ml and incubated at 200 rpm for 6 days at 37°C for *L. plantarum*, *L. acidophilus*, and *L. amylovorus* and 30°C for *A. ficuum*. Aliquot samples were taken every 12 h during 144 h fermentation and analyzed to determine phytase activity.

7.3.3. Batch Fermentation in Submerged Bioreactors and Optimization of Growth Parameters

Selected microorganism strain (*A. ficuum*) was used for submerged fermentation and growth conditions were optimized to obtain maximum phytase production in Sartorious Biostat B Plus bioreactor (Allentown, PA) equipped with a 2-L vessel with 1-L working volume. The same medium composition, which was used for shake-flask fermentations was also used for reactor fermentations. Batch fermentation was conducted according to three factors Box-Benkhen design of RSM by using MINITAB statistical software (Version 15, State College, PA) (Table 7.1). The growth factors evaluated were temperature (25-40°C), pH (4-7), and aeration rate (0-1.5 volume of air/volume of broth/minute (vvm)). Temperature and pH intervals were set based on literature review and aeration interval was set based on preliminary experiments. Agitation speed was maintained to 300 rpm for all fermentation runs. *A. ficuum* spores present on each PDA petri dishes were grown for 6 days at 30°C, were suspended by adding 7 ml of 0.1% peptone water solution and used as inoculum. Fermentations were started by adding 30 ml of *A. ficuum* inoculum, which had 10^6 spores/ml. Samples were collected (2 ml) from the reactors every 24 h to determine phytase activity and glucose concentration during 6 days.

7.3.4. Validation of the Model

Fermentations were run under the conditions identified as optimum with three replications to validate suggested optimum condition by RSM. Results, which were obtained from fermentations, were compared with predicted value from the model. If the values are close each other with a minor standard deviation, the model will be accepted as representative.

7.3.5. Analysis

Samples were collected and centrifuged at 5,200 x *g* for 15 min to remove the biomass. Then, supernatant was used for phytase activity and glucose concentration analyses. Biomass concentration could not be measured in the collected samples due to bulk microbial growth in the reactors.

7.3.5.1. Phytase Activity

Phytase activity was determined as described by Kim et al. (1998) with minor modifications. Cell-free broth (0.125 ml) was mixed with 0.125 ml of 1.5 mM Na-phytate in 0.1 M sodium acetate solution and mixtures were incubated in the water bath at 55ºC for 30 min. After incubation, reaction was stopped by adding 0.25 ml of 15% tricholoroacetic acid (TCA) solution into the tubes. Then, 2 ml of color regent was added, which was prepared freshly with; 2:1:1:1 ratio of water: 2.5 % ammonium molybdate: 6 N H_2SO_4: 10 % ascorbic acid and tubes were incubated at 55ºC for 30 min. After cooling down to room temperature, absorbances were measured at 700 nm by using a spectrophotometer (Beckman Coulter, Fullerton, CA). Uninoculated fermentation medium was used as the blank for the measurement. The obtained data was used to calculate the activity unit of phytase (U/ml), which was defined as the amount of phosphorus liberated in μmole from 1.5 mM phytate per min under the assay conditions.

7.3.5.2. Glucose Concentration

Glucose concentrations were measured using high pressure liquid chromatography (HPLC) with a refractive index detector (Waters, Milford, MA). Glucose determination was performed by using Aminex HPX-87H column (Bio-Rad, Richmond, CA) with 0.8 ml/min isocratic flow of 0.012 N sulfuric acid. The detector and column temperature were maintained at as 35 and 65ºC, respectively. The cell-free samples were filtered by using 13 mm diameter, 0.2 μm pore sized filters (PALL Life Sciences, Port Washington, NY).

7.3.5.3. Statistical Analysis

MINITAB Statistical Software package was used for statistical analyses. Analysis of variance (ANOVA) was performed for investigating statistically significant differences between phytase activities at different growth parameters and terms, which have *p*-value lower than 0.05 were considered as significant.

7.4. Results and Discussion

7.4.1. Microorganism Screening

There were several microorganisms, which can produce phytase have been mentioned in the literature, however it is important to identify the best strain for submerged fermentation. Four

of phytase producer strains were selected for screening several microorganisms based on the literature review. Microorganisms, which produced phytase, created clear zones on phytase selection agar media and the most productive strain was selected by comparing average diameter of clear zones (Figure 7.1).

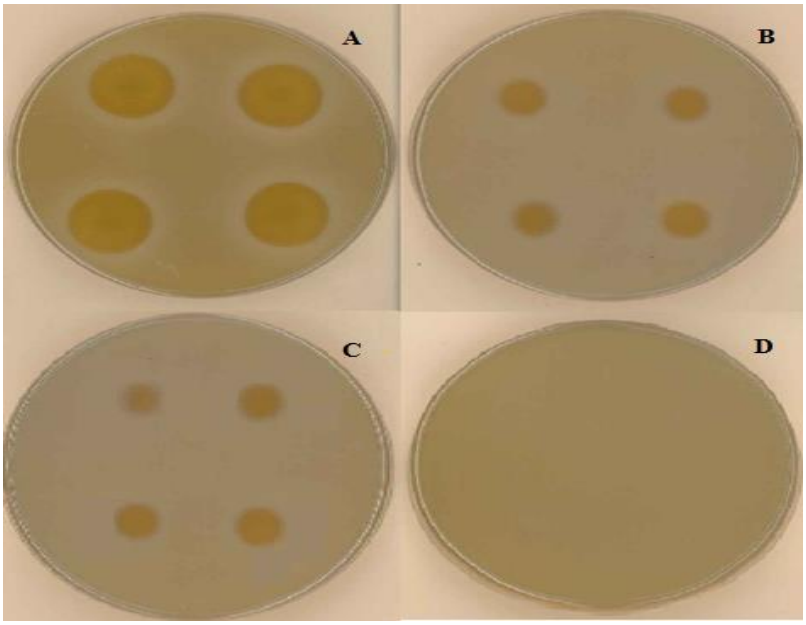

Figure 7.1. Phytase selective agar results. *A. ficuum* (A), *L. plantarum* (B), *L. acidophilus* (C), and *L. amylovorus* (D).

Among four microorganisms, *A. ficuum* showed the largest clear zones by 2 cm. *L. plantarum* and *L. acidophilus* had 1.1 and 0.9 cm diameter as the clear zones respectively, whereas there was no clear zone was observed for *L. amylovorus* on the phytase selective agar plate. Phytase selective agar results were validated by performing shake-flask fermentations for screened microorganisms. *A. ficuum* showed the highest the phytase activity by 1.02 U/ml, whereas *L. plantarum* and *L. acidophilus* produced 0.41 and 0.28 U/ml activity within 120 h, respectively. Similar to phytase selective agar results, there was no phytase activity determined for *L. amylovorus* (Figure 7.2).

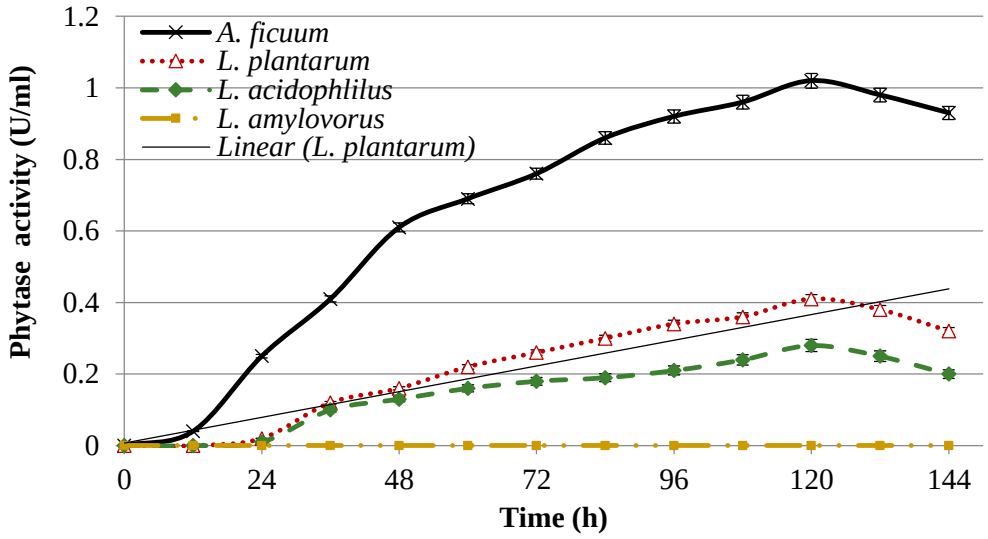

Figure 7.2. Phytase production in shake-flask fermentation.

Microorganism screening result was also supported by the literature. Vohra and Satyanarayana (2003) showed that phytase, which was secreted by *A. ficuum* gave the lowest K_m value among 29 microorganisms. Similarly, Shieh and Ware (1968) studied 24 different *Aspergillus* species and concluded that *A. ficuum* (NRRL 3135) was the most yielded microorganism. In the literature, *Aspergillus* species have been generally used for solid state phytase productions however, this study demonstrated that, *A. ficuum* is the most productive strain for even in submerged shake-flask fermentations among the screened microorganisms.

7.4.2. Optimization of Growth Parameters

Growth parameters for *A. ficuum* such as temperature, pH, and aeration were optimized in 2-L batch bioreactor under submerged fermentation conditions by using a Box-Behnken design. Table 7.1 shows the overall Box-Benhken design, experimental, predicted activity values, and glucose residuals for different fermentation conditions.

Table 7.1. Box-Behnken response surface design and phytase activity and residual glucose results.

Run Order	Temperature (ºC)	pH	Aeration (vvm)	Measured Phytase Activity (U/ml)	Predicted Phytase Activity (U/ml)	Glucose residual (g/L)
1	32.5	5.5	0.75	2.14	2.13	28
2	25.0	5.5	0	0.72	0.52	55
3	40.0	4.0	0.75	1.76	1.62	38
4	32.5	5.5	0.75	2.12	2.13	26
5	25.0	7.0	0.75	0.67	0.80	45
6	25.0	5.5	1.50	0.88	0.96	40
7	32.5	4.0	0	1.10	1.30	38
8	40.0	5.5	0	0.77	0.70	51
9	32.5	5.5	0.75	2.13	2.13	28
10	32.5	7.0	1.50	1.10	0.89	40
11	25.0	4.0	0.75	1.34	1.33	34
12	32.5	7.0	0	0.73	0.79	38
13	40.0	5.5	1.50	0.74	0.94	45
14	40.0	7.0	0.75	0.65	0.66	63
15	32.5	4.0	1.50	1.94	1.87	32

A. ficuum produced high phytase activity (2.13 U/ml) at 32.5ºC and phytase activity was decreased below and above this temperature. It can be seen on the Table 7.1, phytase activity was less than 1 U/ml, when fermentation was run at 25 or 40ºC, except for run # 3 (40ºC, 4 pH, and 0.75 vvm aeration) and # 11 (25ºC, 4 pH, and 0.75 vvm aeration). Also, phytase was produced with high activity under low pH conditions. When fermentations were run at 40ºC and 0.75 vvm aeration, but different pH conditions, phytase activity was obtained as 1.76 U/ml at pH 4 (run # 3) and 0.65 U/ml at 7 pH (run # 14). Similarly, results obtained from the fermentations conducted at 25ºC at different pH values; phytase activity was 1.34 U/ml, when fermentation was run at 4 pH (run # 11) and it was decreased to 0.67 U/ml, when pH was maintained to 7 at the same temperature and aeration level (run # 5). Additionally, phytase activity was increased by aeration, up to a certain level. For example, when the fermentation was conducted at 25ºC, 5.5 pH, and 1.5 vvm aeration conditions (run # 6), phytase activity was 0.88 U/ml, however it was obtained as 0.72 U/ml, when there was no aeration (run # 2).

Vohra and Satyanarayana (2003) suggested the growth conditions as optimum for phytase production with *A. ficuum* are 27°C and pH 5 in shake-flask submerged fermentation, which are similar to the optimum values of this study. However, there is no information about aeration level for submerged phytase production in their study, which was determined as 0.9 vvm in this current study. Aeration was not only necessary for oxygen supplementation for the microorganisms but also it provided better agitation in the fermentation broth.

7.4.3. Response Surface Model

A second order polynomial equation (Equation 7.1) and ANOVA table (Table 7.2) were created by MINITAB software to show the predicted values, effects of temperature, pH, and aeration for batch phytase production.

Phytase (Unit/ml) = $-17.5115 + 0.9083 \times (T) + 1.5891 \times (pH) + 2.7411 \times (A) - 0.013 \times (T \times T) - 0.1313 \times (pH \times pH) - 1.1037 \times (A \times A) - 0.0097 \times (T \times pH) - 0.0087 \times (T \times A) - 0.1058 \times (pH \times A)$ where "T" is temperature and "A" is aeration. ……**Equation 7.1**

R^2 predicted coefficient (0.8780) indicates how well the model prediction responses for the new observations and R^2 (0.9564) indicates how well the model fits the experimental data. To show the good fit, experimental and predicted values were plotted (not shown) and the slope of the best fitted line was determined as 0.96, which is very close to "1".

Also it can be seen on the Table 7.1 that, model predicts better, 72when the fermentation was run under mid conditions (run # 1, # 4, and # 9) (32.5°C, 5.5 pH, and 0.75 vvm aeration) than low temperature conditions (run # 2 (25, 5.5 pH, and 0 vvm aeration), # 5 (25°C, 7 pH, and 0.75 vvm aeration) and # 6 (25°C, 5.5 pH, and 1.5 vvm aeration)).

ANOVA showed that all main effects and two way interactions except for Temperature×pH, Temperature×aeration, and pH×aeration were significant (Table 7.2).

Table 7.2. ANOVA table of growth parameters for phytase production by *A. ficuum* in a submerged fermentation bioreactor.

Terms	Coefficients	Standard Error Coefficient	*p*-values
Constant	-17.5115	3.09780	0.002
Temperature	0.9083	0.13629	0.001
pH	1.5891	0.61533	0.049
Aeration	2.7411	0.84534	0.023
Temperature*Temperature	-0.013	0.00193	0.001
pH*pH	-0.1313	0.04818	0.041
Aeration*Aeration	-1.1037	0.19272	0.002
Temperature*pH	-0.0097	0.00926	0.345
Temperature*Aeration	-0.0087	0.01852	0.660
pH*Aeration	-0.1058	0.09258	0.305

Among the main effects, temperature was the most effective growth parameter on phytase production since it has the lowest *p*-value. Also, application of optimization in MINITAB suggested that the maximum phytase activity can be obtained as 2.27 U/ml at 33^{o}C, 4.5 pH and 0.9 vvm aeration conditions. In the literature, Soni and Khire (2007), and Papagianni et al. (2001) reported 40 U/ml and 15 U/ml phytase activities with *A. niger*. However their results seem to be questionable. Although the basic principle of the spectrophotometric phytase analysis was depended on colorimetric measurement of the amount free phosphate released, KH_2PO_4 was added to the fermentation media. Due to KH_2PO_4, which was present in the medium, extra color occurrence due to presence of KH_2PO_4 might have affected the results. Moreover, Soni and Khire (2007) mentioned that they used buffer and enzyme solution as blank in their studies. Many nutrients in the fermentation medium may impact color occurrence in phytase activity assay. Therefore, uninoculated fermentation medium should be used as blank, which was the case in this study. On the other hand, Mittal et al. (2012) also performed phytase production in a similar medium, which did not include any phosphate source and they reported 0.9 U/ml phytase activity, which is about 2.5 times lower than the activity was obtained in this study (2.27 U/ml).

Phytase activity trends with changing growth parameters were shown on Figure 7.3. It can be seen from Figure 7.3A that, phytase was produced with high activity between 30-35^{o}C, when pH was around 4. Phytase activity decreased sharply higher than pH 5 values Figure 7.3B).

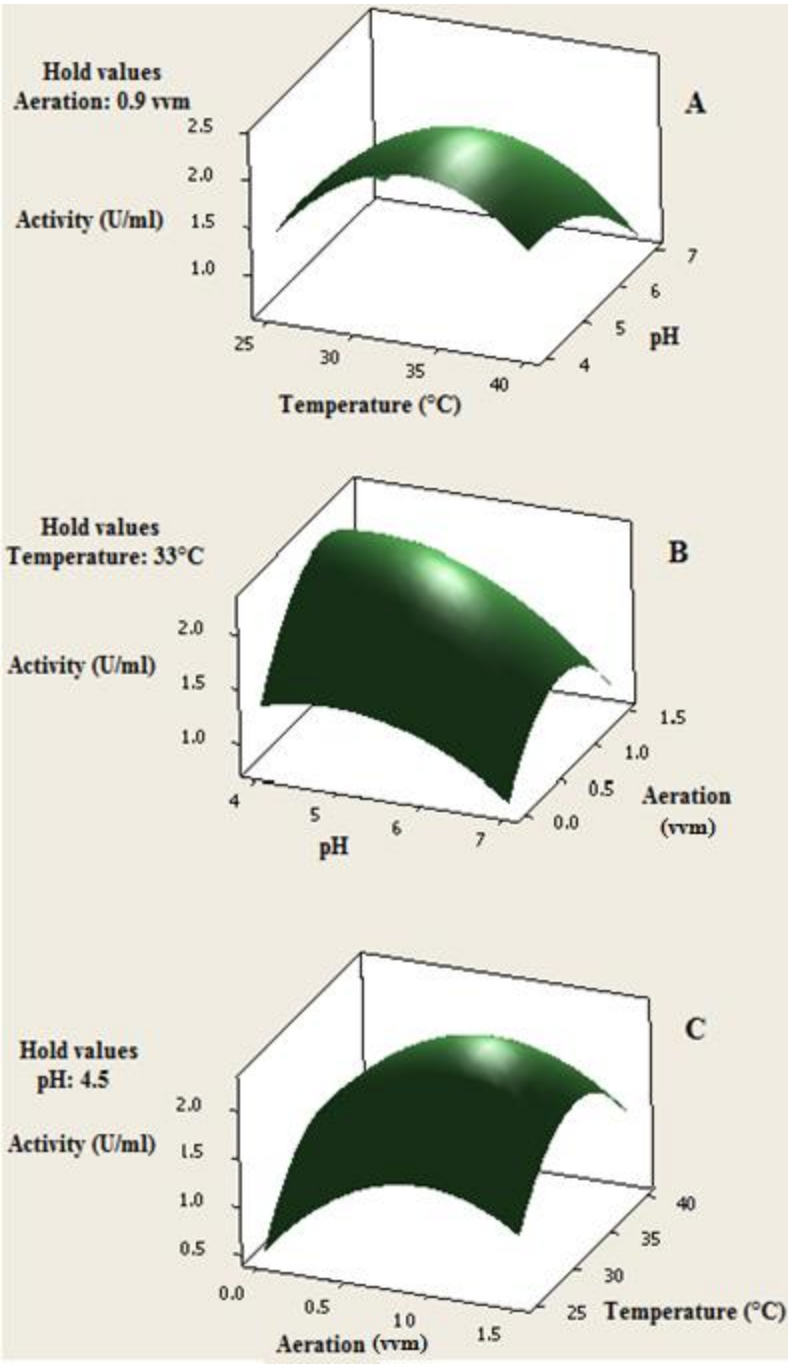

Figure 7.3. Phytase activity trends with changing growth parameters.

Additionally, it can be seen from Figure 7.3C, that low aeration rate affects phytase activity more than high levels. Therefore, submerged phytase fermentation systems with *A. ficuum*, must be definitely aerated. Therefore, this study suggests that, phytase activity can be obtained in high levels with 0.8-0.95 vvm aeration interval.

Phytase activity and glucose consumption under the conditions identified as optimum was shown in Figure 7.4. Phytase activity rapidly increased 24 h after inoculation and it started to decrease after 120 h. Similarly, glucose consumption rate was increased 24 h after inoculation and 28 g/L glucose leftover in 120 h of fermentation, when the highest phytase activity obtained. Maximum substrate consumption rate and maximum phytase activity production rate was calculated as 0.5 g glucose/h and 0.022 U/ml/h, respectively.

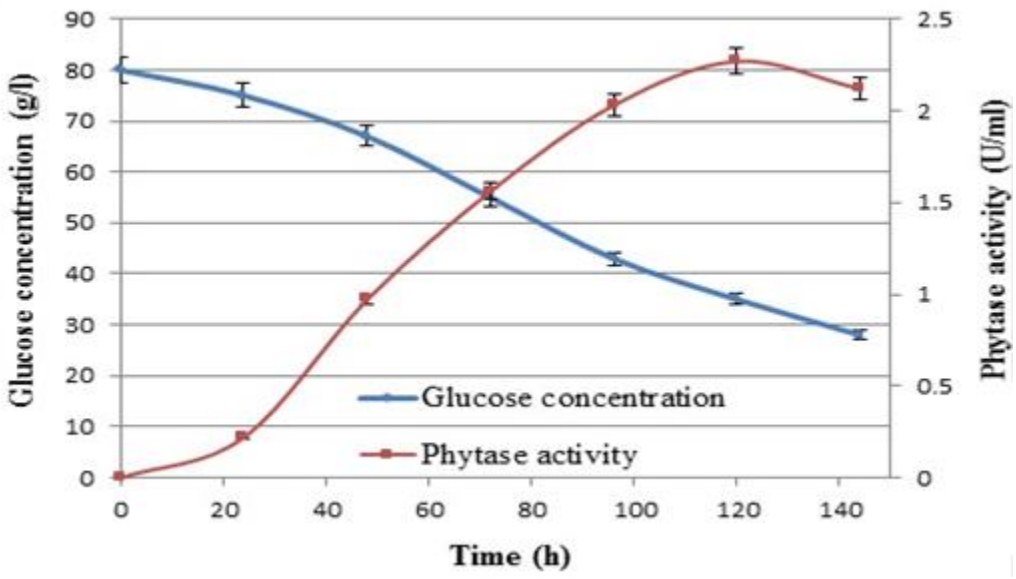

Figure 7.4. Phytase activity and glucose consumption trend under conditions identified as optimum.

7.4.4. Validation of the Optimum Conditions

Batch fermentation was conducted in triplicate at the conditions identified as optimum, which were 33^{o}C, pH 4.5, and 0.9 vvm for validation purpose. The phytase activity obtained from the experimental run was found as 2.22±0.12 U/ml, which was very similar to the predicted activity value from the model (2.27 U/ml).

7.5. Conclusions

In conclusion, the most productive phytase producer microorganism selection and optimization of growth parameters for phytase production in 2-L batch reactors were performed. *A. ficuum* was identified as the most productive strain for phytase production among *A. ficuum*, *L. plantarum*, *L. acidophilus*, and *L. amylovorus*. Moreover, Box-Behnken design was used to determine optimum growth conditions, which were determined as 33°C, 4.5 pH, and 0.9 vvm aeration. Maximum phytase activity was found as 2.27 U/ml at these conditions identified as the optimum.

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CHAPTER 8

OPTIMIZATION OF FERMENTATION MEDIUM AND ASSAY CONDITIONS TO IMPROVE PHYTASE PRODUCTION WITH *ASPERGILLUS FICUUM* IN SUBMERGED FERMENTATION

8.1. Abstract

Phytase, is an important feed additive, which is used in diets to increase the absorption of divalent ions, amino acids, and proteins and to reduce phosphorus release in the manure to prevent negative effects on the environment. To date, phytase has been mostly produced in solid state fermentations with low production volumes. Thus, there is a need to produce phytase in submerged fermentations, which can be scaled-up for commercial productions. Additionally, optimization of the fermentation medium has not been well documented in the literature. Therefore, this study has been undertaken to improve *Aspergillus ficuum* phytase production in submerged fermentations by optimizing important nutrients in the fermentation medium (glucose, Na-phytate, and $CaSO_4$) using the Box-Behnken design of Response Surface Methodology. Effects of temperature and pH on phytase activity were also studied. The optimum glucose, Na-phytate, and $CaSO_4$ concentrations were determined to be 126, 14, and 1.1 g/L, respectively. Additionally, pH 5.5 and 55^{o}C were determined as optimum for the produced *A. ficuum* phytase activity. Under these conditions, phytase activity was increased to 3.45 U/ml, which is about 50% higher than the previous results. Furthermore, the lowest activity loss was observed at 4^{o}C storage conditions during 1 week of storage.

8.2. Introduction

Phytate is the major phosphate source in plants. Especially, legumes, cereals, pollen, and nuts contain high levels of phytate in their structure. Phytate is generally used during germination for ATP synthesis in the plants (Vohra and Satyanarayana, 2003). Nevertheless, phytate has several negative effects on animal and human health. A major problem with phytate consumption in the diets is that, phytate is a strong chelating agent, which binds proteins, amino acids, and divalent ions such as Ca^{+2}, Mg^{+2}, Zn^{+2}, Cu^{+2}, Fe^{+2}, and Mn^{+2} in vivo and creates insoluble salts. Consequently, absorption and utilization of these nutrients are significantly reduced (Haefner et

al., 2005). For these reasons, excessive phytate in the diet may cause some several health problems such as iron deficiency, bone weakness, tooth decay, and digestion problems (Hurrell et al., 2003; Sanson et al., 1981). Additionally, there are several environmental issues associated with phytate consumption. Monogastric animals such as chickens and pigs are not able to break down and utilize phytate, since they do not have the necessary enzymes or microflora in their digestion systems (Mullaney et al., 2000). Since, these monogastric animals are generally fed diets with phytate rich ingredients such as wheat, rice, and corn, excessive phosphorus can accumulate in their manure, which causes environmental problems such as water pollution, algal blooms, fish kills, and changing of fauna and flora in the environment (Mullaney et al., 2000). However, farmers and the feed industry can overcome this problem with diets supplemented with phytase enzyme, which decrease phytate content in the feed. Phytase can be obtained from plants, but more commonly from microorganisms. Phytase application in the feed formulation can save feed industry around $2 billion per year by improving nutrient utilization in the diet (Cowieson et al., 2012). Bacteria and yeasts, but most commonly molds have been used for phytase productions in several studies. The first generation of commercial phytase was produced in 1991using *Aspergillus niger*, which reduced phytate content in the diets by 35-40% (Cowieson et al., 2012). Nelson et al. used phytase, produced by *A. niger* to pretreat a corn–soybean diets for broilers. They showed that the phosphorus availability increased by 60% and phosphorus content decreased by 50% in the manure. Moreover, phytase improved body weight of male and female broilers by 13.2 and 5.8%, respectively after 21 days on a diets supplemented with phytase (Nelson et al., 1968). Furthermore, the positive effect of phytase application on absorption of other essential nutrients was shown by Sebastian et al. (1996). They demonstrated that phytase supplementation increased the relative retention of total P^{-3}, Ca^{+2}, Cu^{+2}, and Zn^{+2} by 12.5, 12.2., 19.3, and 62.3%, respectively.

To date, phytase production has been mostly performed in solid state fermentation. However, solid state phytase production is not very flexible to scale-up for commercial production and requires costly and complex extraction steps. Additionally, solid-state fermentations have very low homogeneity comparing to submerged systems, which causes heterogeneity in phytase properties. Overall, there is a need to enhance the uniformity of the enzyme and to optimize the fermentation medium to improve phytase activity. In our previous study, *Aspergillus ficuum* phytase activity was measured as 1.02 U/ml in shake-flask production. As an improvement, after scaled up 1-L (working volume) bioreactor, phytase activity increased to 2.27 U/ml by optimizing of the growth parameters (Chapter 7). Other studies optimized the

fermentation medium composition for phytase production with different microorganism. Lan et al. (2002) reported that rice bran, which has high phytate content added to the fermentation medium, increased phytase production by *Mitsuokella jalaludinii* significantly. They measured phytase activity as 5.08 U/ml, when 5% rice bran was used in the medium and activity rose to 12.93 U/ml, at20% rice bran addition. They also showed that glucose can be utilized more rapidly, comparing to other carbon sources in their fermentations. However, it results in accumulation of organic acids and consequently reduction in pH of fermentation medium, which suppresses enzyme synthesis (Lan et al., 2002). In another study, Sunitha et al. (1999) reported that phytase production with *E. coli* increased by 1.58 times, when Luria Bertani (LB) broth was supplemented with 2 g/l glucose comparing no glucose added LB medium. Additionally, Sasirekha et al. (2012) studied effect of several carbon sources on phytase production by *Pseudomonas sp*. They reported that phytase productions were very similar among each other, when glucose (0.727 U/ml), sucrose (0.739 U/ml) or lactose (0.724 U/ml) was used as carbon source. On the other hand, slightly lower phytase production was obtained (0.704 U/ml), when maltose was used as carbon source in the fermentation medium. Moreover, effect of phosphorus concentration on phytase production was studied by Dahiya et al. (2009). They reported that, when inorganic phosphate was reduced in the fermentation medium, extracellular phytase production was increased significantly with *Aspergillus sp*. 5990. In their study, phytase activity was measured as around 18 U/ml, when 0.05% inorganic phosphate was used in the fermentation medium. However, phytase activity decreased to almost 0.35 U/ml, when inorganic phosphate concentration was increased to 0.20%. Also various agricultural wastes such as orange peel, rice bran, wheat bran, and corn cobs were used as phytate source in fermentation medium in several studies. For example, orange peel (0.062-0.082% phytate) was used as phytate source in phytase production by *Klebsiella sp.* DB3 (Mittal et al., 2011). In this study, it was reported that phytase activity increased from 0.6 to 3.15 U/ml, when 2% orange peel bran was used in the fermentation medium. Liu et al. (2011) studied the effect of K^+, Ca^{2+} and Mg^{2+} concentrations in the fermentation medium for phytase production by *Pichia pastoris*. They reported that 13.25 g/l of K_2SO_4, 1.03 g/l of $CaSO_4 \cdot 2H_2O$, and 17.94 g/l of $MgSO_4 \cdot 7H_2O$ concentrations are the optimum concentrations to maximize phytase production. Under these conditions phytase activity was measured as 161.64 U/ml, which was significantly higher comparing to the activity was measured in standard minimal salt medium (73.31 U/ml).

Even though there have been improvements in phytase production in each case, fluctuations in phytase activity and medium conditions, are important issues. Determination of

phytase activities vary depending on several issues such as reaction pH, temperature, incubation time, mineral content, substrate concentration, total reaction volume, the blank, etc. (Selle and Ravindran, 2007). These differences result in different activity calculation formulas in each case. Because of these reasons, assay conditions for each phytase, which was isolated from different species, should be optimized specifically.

Therefore, this study is undertaken in order to shed some light on phytase fermentation medium optimization, enzyme assay and storage conditions. The main goal is to improve phytase production by *A. ficuum* in submerged fermentation by optimization of medium as well as determination of optimal pH and temperature for enzyme activity and optimum temperature for storage.

8.3. Materials and Methods

8.3.1. Microorganism and Medium

There are several phytase producer microorganisms including molds, yeasts, and bacteria have been reported. *Aspergillus ficuum* (NRRL 3135) was used in this study as suggested by our previous study (Chapter 7). *A. ficuum* was obtained from Agricultural Research Service Culture Collection (Peoria, IL) and grown on potato dextrose agar (PDA) (Difco, Sparks, MD) slants for 6 days at 30^{o}C and stored at 4^{o}C as the working culture. In order to maintain viability, *A. ficuum* was regularly transferred to sterile fresh agar slant biweekly.

Base phytase fermentation medium included 100 g of glucose, 0.5 g of KCl, 0.1 g of $FeSO_4(7H_2O)$, 0.5 g of $MgSO_4(7H_2O)$, 0.01 g of $MnSO_4(7H_2O)$, 8.6 g of $NaNO_3$, 3 g $(NH_4)_2SO_4$ and 10 g of Na-phytate (A&Z Food Additives Co. Ltd., Zhejiang, China) per litter of deionized water.

8.3.2. Inoculum Preparation

A. ficuum spores were grown on 25 PDA plates for 6 days at 30°C. After incubation, spores were suspended by adding 7 ml of sterile 0.1% peptone water to each plate and the resulting solution (~10^6 spores/ml) used as the inoculum.

8.3.3. Batch Fermentation

Based on a Box-Benkhen design, phytase production was performed in 15 different media compositions. Samples were collected from the reactors every 24 h to determine phytase activity and glucose residuals during 6 days. All runs were performed in Sartorius Biostat B Plus bioreactor (Allentown, PA) equipped with a 2-L vessel and 1-L working volume. Reactors were inoculated with 3% prepared inoculum. Fermentation runs were performed at 33ºC, 4.5 pH and 0.9 vvm aeration as suggested by our previous studies (Chapter 7). Agitation was maintained at 300 rpm for all fermentation runs.

8.3.4. Optimization of Fermentation Medium

Glucose, Na-phytate, and $CaSO_4$ concentrations seemed to be important in the fermentation medium based on the literature review. In order to find optimum concentrations for these nutrients, Box-Benkhen design of Response Surface Methodology (RSM) was used between the concentration ranges of 100-200, 5-20, and 0-1.36 g/L for glucose, Na-phytate and $CaSO_4$, respectively, which were selected based the literature and our preliminary studies (Table 8.1). Observed phytase activities were statistically analyzed by using RSM optimizer module of MINITAB statistical software (Version 15, State College, PA).

8.3.5. Validation of the Model

After determining the optimum concentrations, three fermentation runs were performed at the determined optimum conditions to validate results. Observed and predicted values were compared to each other and root mean square error (RMSE) and mean absolute error (MAE) values were determined to validate the RSM model.

8.3.6. Effect of Storage Temperature on Phytase Activity

Samples were collected at the highest activity under optimum conditions and centrifuged at 5,200 x *g* for 15 min to remove the biomass. Then, 1 ml of supernatant was transferred into glass vials and stored at -20, 4, 25, and 33ºC. One sample from each storage condition was taken to measure phytase activity daily until 10^{th} day of storage, then every other day. Samples were kept at room temperature for an hour before the analysis performed. Activity losses from each

sample were compared to each other to determine the best temperature condition for *A. ficuum* phytase storage.

8.3.7. Analysis

8.3.7.1. Phytase Activity

Phytase, which was produced under optimum conditions was collected from the reactor and used to determine optimum pH and temperature values in the enzyme assay. In order to determine the optimum temperature for the enzyme assay, samples were incubated with Na-phytate solution in water bath, which was set up to 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, or 80ºC. Similarly, to determine the optimum pH for the enzyme assay, pH values of all assay solutions and samples were adjusted to 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, or 9. Enzyme assay was performed under the determined optimum temperature and pH as described by Kim et al. with minor modifications (Kim et al., 1998). Briefly, cell-free broth (0.125 ml) was mixed with 0.125 ml of 1.5 mM Na-phytate in 0.1 M sodium acetate solution and mixtures were incubated in the water bath at 55ºC for 30 min. After incubation, reaction was stopped by adding 0.25 ml of 15% tricholoroacetic acid (TCA) solution into the tubes. Then, 2 ml of color regent was added, which was prepared freshly with; 2:1:1:1 ratio of water: 2.5 % ammonium molybdate: 6 N H_2SO_4: 10% ascorbic acid and tubes were incubated at 55ºC for 30 min. After cooling down to room temperature, absorbances were measured at 700 nm by using a spectrophotometer (Beckman Coulter, Fullerton, CA). Uninoculated fermentation medium was used as the blank for the measurement. The obtained data was used to calculate the activity unit of phytase (U/ml), which was defined as the μmole of phosphorus liberated from 1.5 mM phytate per min under the set assay conditions.

8.3.7.2. Glucose Concentration

Glucose concentrations were measured using high pressure liquid chromatography (HPLC) with a refractive index detector (Waters, Milford, MA). Glucose determination was performed by using Aminex HPX-87H column (Bio-Rad, Richmond, CA) with 0.8 ml/min isocratic flow of 0.012 N sulfuric acid. The detector and column temperature were maintained at as 35 and 65ºC, respectively. The cell-free samples were filtered by using 13 mm diameter, 0.2 μm pore sized filters (PALL Life Sciences, Port Washington, NY).

8.3.7.3. Statistical Analysis

MINITAB Statistical Software package was used for statistical analyses. Analysis of variance (ANOVA) performed to investigate significant differences between phytase activities at different medium compositions. The terms, which have *p*-value lower than 0.05 were considered as significant. Also, R^2, RMSE, and MAE values were calculated to show if the model represents the process successfully.

8.4. Results and Discussion

In this study, *A. ficuum* phytase activity assay was improved by determining the optimum pH and temperature values and phytase fermentation was enhanced in submerged bioreactors by optimization of the fermentation medium. Moreover, effect of storage temperature on phytase activity was studied.

8.4.1. Optimum Temperature and pH of Enzyme Assay

Enzymes show different catalytic activities under different temperature and pH conditions. Therefore, it was very important to determine the optimum pH and temperature values for *A. ficuum* phytase to maximize the catalytic activity. In order to determine the optimum reaction temperature of *A. ficuum* phytase, activities were measured at a temperature range between 25 and 80^{o}C. Figure 8.1 shows that *A. ficuum* phytase activity increased as temperature increased until 55^{o}C and remained almost the same at 60^{o}C. However, a sharp decrease in the activity was observed, when the assay was performed at 65^{o}C or above. Therefore, optimum temperature was determined as 55^{o}C for the produced phytase.

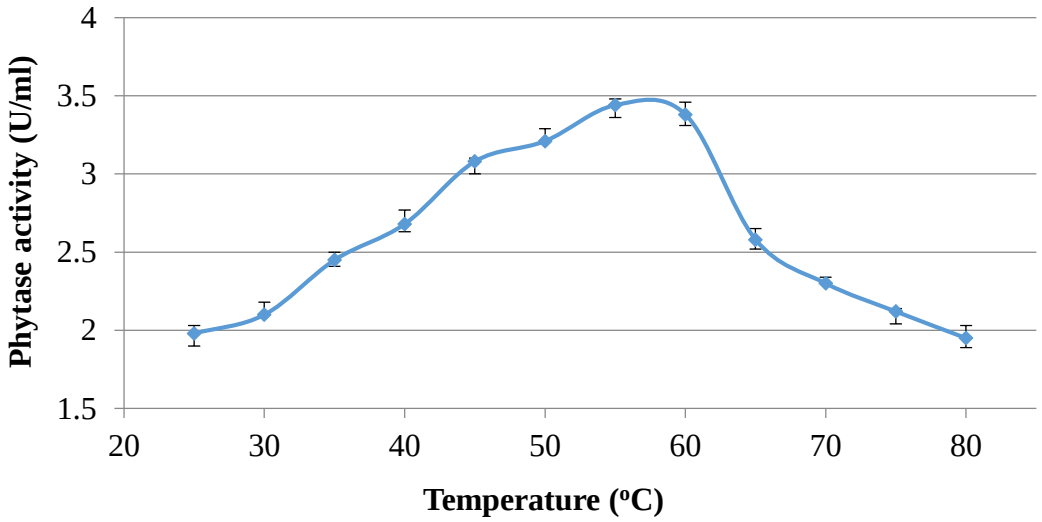

Figure 8.1. Optimum temperature for the phytase activity assay.

Similarly, effect of pH was evaluated for the enzyme activity at a pH range between 2.0 and 9.0. *A. ficuum* phytase was active in a very wide pH spectrum. It was observed that *A. ficuum* phytase had a high activity, when pH was adjusted to 2.5 and slightly higher at 5.5 (Figure 8.2). Therefore, optimum pH value was selected as 5.5 for the enzyme assay.

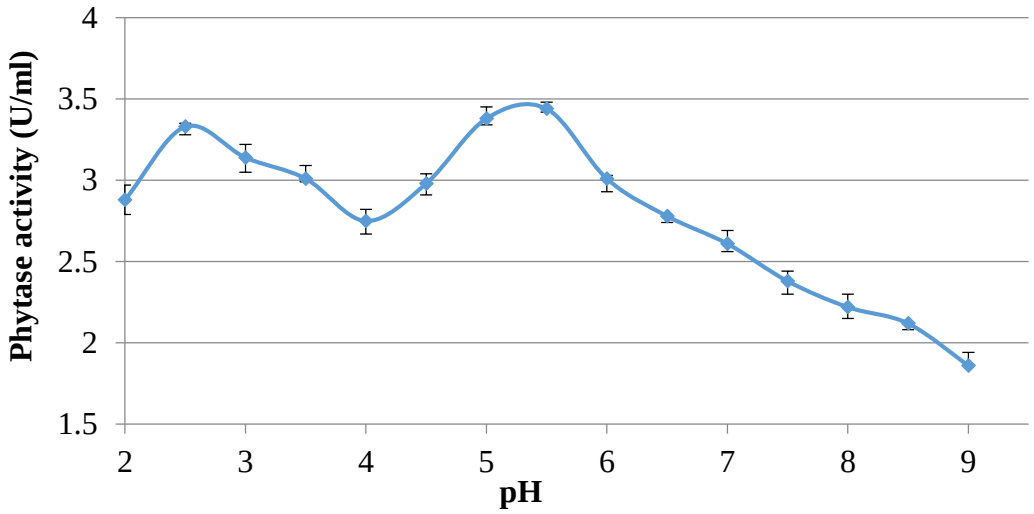

Figure 8.2. Optimum pH for the phytase activity assay.

These results were very similar the ones reported Zhang et al. (2010). They also reported that optimum catalytic pH of phytase, which is produced by A. *ficuum* NRLL 3135 was 2.5 and 5-5.5 and optimum temperature was 58^{o}C. These characteristics of *A. ficuum* phytase are promising for the food industry that phytase can maintain its activity in a wide spectrum so it can benefits animal and human during all gastrointestinal metabolism.

8.4.2. Optimization of Fermentation Medium

In this study, glucose, Na-phytate, and $CaSO_4$ concentrations in the fermentation medium were chosen as study of interest for the medium optimization. Table 8.1 shows the overall Box-Behnken design, the experimental, and predicted phytase activity values, glucose residuals and glucose consumption percentages for different medium preparations. Phytase activity was always less than 3 U/ml, when glucose concentration in the fermentation medium was 200 g/L (run # 2, 3, 5, and 6). Under these conditions, it was also observed that the medium became very viscous and thick. This may cause inefficient agitation and aeration conditions and also decrease in nutrient mass transfer rates and consequently resulted in lower phytase activities. Among all runs, phytase activity was measured the lowest as 1.88 U/ml, when 200 g/L glucose was used in the medium (Run #5). On the other hand, the lowest phytase activities were measured as 2.12 and 2.01 U/ml, when 100 g/L and 150 g/L of glucose was used in the medium, respectively. When Na- phytate was used in low concentrations in the reactors (5 g/L), phytase activity was always below than 2.85 U/ml (run # 2, 9, 10, and 12). However, when the fermentation medium had 20 g/L of Na-phytate, a dark color occurrence was observed in the reactors comparing to other runs under these conditions, phytase activities were less than 3.02 U/ml (run # 3, 7, 13, and 14). Additionally, it was observed that $CaSO_4$ has an important effect on phytase activity. Phytase activity was only up to 2.12 U/ml, when there was no $CaSO_4$ added in the medium (run # 5, 10, 11, and 14). However, phytase activity increased to 3.40 U/ml, when 1.36 g/L of $CaSO_4$ was added in the fermentation medium (run # 1).

Table 8.1. Effect of different growth parameter combinations on batch phytase production with *A. ficuum*.

Run Order	Glucose (g/L)	Na-phytate (g/L)	$CaSO_4$ (g/L)	Measured Phytase Activity (U/ml)	Predicted Phytase Activity (U/ml)	Glucose Residual (g/L)	Glucose Consumption Percentages (%)
1	100	12.50	1.36	3.40	3.30	56	44
2	200	5.00	0.68	2.30	2.36	130	35
3	200	20.00	0.68	2.47	2.31	136	32
4	150	12.50	0.68	3.28	3.26	91	39
5	200	12.50	0.00	1.88	1.98	120	40
6	200	12.50	1.36	2.62	2.61	142	29
7	100	20.00	0.68	3.02	2.96	50	50
8	150	12.50	0.68	3.25	3.26	86	43
9	150	5.00	1.36	2.85	2.79	95	37
10	150	5.00	0.00	2.09	1.93	86	43
11	100	12.50	0.00	2.12	2.12	62	38
12	100	5.00	0.68	2.38	2.54	52	48
13	150	20.00	1.36	2.85	3.01	76	49
14	150	20.00	0.00	2.01	2.06	104	31
15	150	12.50	0.68	3.25	3.26	88	41

A. ficuum resulted in the highest glucose consumption by 50% at 100 g/L of glucose, 20 g/L of Na-phytate, and 0.68 g/L of $CaSO_4$ concentrations, but phytase activity was not the highest measured activity (3.02 U/ml) (Figure 8.1). The reason for this might be that glucose might be used to produce more biomass than phytase. Unfortunately, biomass was not measured during the experiments due to bulk of *A. ficuum* in the reactor medium. Overall, the highest phytase activity was measured as 3.40 U/ml, when 100 g/L of glucose, 12.5 g/L of Na-phytate, and 1.36 g/L of $CaSO_4$ concentrations were used in the fermentation medium (run #1).

There are several studies were mentioned in the literature effect of these compounds on the phytase production, however optimization of the concentrations in the fermentation medium has not been studied well. In literature, there are several studies showed that glucose is one of the most commonly used carbon source for phytase productions (Sunitha et al., 1999; Sasirekha et al., 2012; Hosseinkhani et al., 2009). Similarly, agroindustrial wastes, which includes high phytate concentration was used in the fermentation media as phytate sources for phytase productions in

several studies (Lan et al., 2002; Mittal et al., 2011). Only study that available about optimization of $CaSO_4.2H_2O$ concentration for phytase production was studied by Liu et al. (2011) and they reported the optimum concentration as 1.03 g/L in the phytase fermentation medium for *Pichia pastoris*, which is very close to our results.

8.4.3. Response Surface Model

ANOVA table (Figure 8.2) and a second order polynomial equation (Equation 8.1) were created by MINITAB software to show the effects of glucose, Na-phytate, and $CaSO_4$ on batch phytase production with *A. ficuum*.

Phytase (Unit/ml) = $-2.16292 + 0.04224 \times (G) + 0.22817 \times (P) + 2.45581 \times (C) - 0.00013 \times (G \times G) - 0.00687 \times (P \times P) - 0.91453 \times (C \times C) - 0.00031 \times (G \times P) - 0.00397 \times (G \times C) + 0.00392 \times (P \times C)$,where "G" is glucose, ''P'' is Na-phytate and ''C'' is $CaSO_4$ concentrations. ……**Equation 8.1**

Regression coefficient (R^2) was determined as 0.9622 for the model whereas R^2 adj was given as 0.8942. To show the good fit, experimental and predicted values were plotted (not shown) and the slope of the best fitted line was determined as 0.9645, which is very close to "1". Additionally, RMSE and MAE values were calculated as 0.095 and 0.075, respectively, which are very low and show the model represents the process successfully. Additionally, ANOVA showed that glucose, Na-phytate, and $CaSO_4$ concentrations are all significantly effective on phytase activity (Table 8.2). Among the main effects, $CaSO_4$ was the most effective medium ingredient on phytase activity with the lowest *p*-value by 0.005.

Table 8.2. ANOVA table of medium parameters for phytase production by *A. ficuum* in a submerged fermentation bioreactor.

Terms	Coefficients	Standard Error Coefficient	*p*-values
Constant	-2.16292	0.959119	0.0074
Glucose	0.04224	0.011035	0.012
Na-phytate	0.22817	0.053086	0.008
$CaSO_4$	2.45581	0.502154	0.005
Glucose x Glucose	-0.00013	0.000035	0.013
Na-phytate x Na-phytate	-0.00687	0.001552	0.007
$CaSO_4$ x $CaSO_4$	-0.91453	0.188392	0.005
Glucose x Na-phytate	-0.00031	0.000224	0.220
Glucose x $CaSO_4$	-0.00397	0.002464	0.168
Na-phytate x $CaSO_4$	0.00392	0.016428	0.821

Application of optimization in MINITAB suggested that the maximum phytase activity can be obtained as 3.45 U/ml, if the fermentation is performed with 126 g/L of glucose, 14 g/L of Na-phytate, and 1.1 g/L of $CaSO_4$ concentration under the evaluated conditions (Figure 8.3).

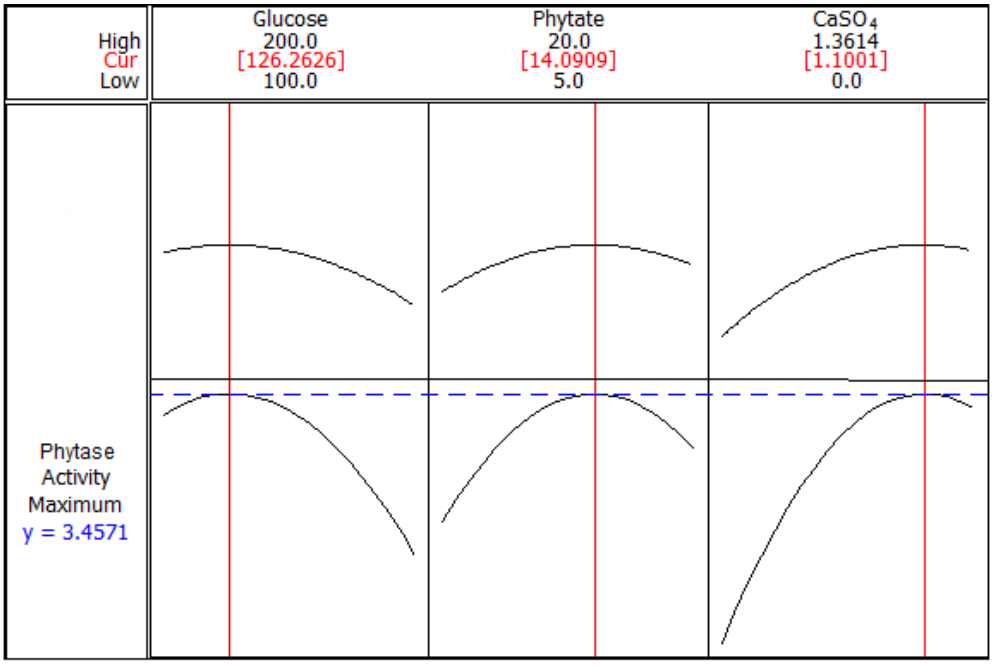

Figure 8.3. Optimum glucose, Na-phytate, and $CaSO_4$ concentrations in base medium for phytase production with *A. ficuum* NRRL 3135.

Figure 8.4 shows phytase activity trends by varying medium composition. Phytase was produced with high activity between 100-150 g/L of glucose concentrations. However, activity decreased remarkably after 150 g/L of glucose concentration (Figure 8.4A). Fig. 4b shows that phytase activity was increased by adding Na-phytate into the fermentation medium until around 15 g/L. After this level, the phytase activity continuously decreased. Therefore, 14 g/L of phytate was determined as optimum phytate concentration. However, it can be seen in Figure 8.4B that, phytase can be still produced around 3 U/ml even 5 g/L phytate and more than 1 g/L $CaSO_4$ were used in to the fermentation medium. Figure 8.4C shows the effect of $CaSO_4$ on phytase activity and after 1.1 g/l of $CaSO_4$, phytase activity stays the same. Therefore, it is concluded there is no need to use $CaSO_4$ more than 1.1 g/L in the fermentation medium. These graphs especially help to design economic fermentation media, which is an important concern especially in industrial scales. In this study, since Na-phytate is more expensive in unit weight comparing to glucose and $CaSO_4$, phytase activity still can be obtained in desired values after concentration of phytate was decreased and concentration of other compounds were adjusted. For example it can be seen in the Figure 8.4A that, phytase activity still can be obtained around 2.5 U/ml even 5 g/L phytate was added in the fermentation medium until glucose concentration was increased to around 200 g/L and $CaSO_4$ concentration was maintained at 1.1 g/L. Similarly, phytase activity can be measured almost 3 U/ml, when phytate concentration was decreased to 5 g/L and $CaSO_4$ was increased to 1.5 g/L while glucose concentration was maintained at 126 g/L (Figure 8.4B). Since $CaSO_4$ is significantly effective on phytase activity, the highest phytase activity can be measured around 2.25 U/ml, if $CaSO_4$ will not be used in the fermentation medium (Figure 8.4C).

8.4.4. Validation of the Optimum Conditions

The model was validated under the optimized conditions. Batch fermentations were conducted in triplicate at the determined optimum conditions, which were 126 g/L of glucose, 14 g/L of Na-phytate, and 1.1 g/L of $CaSO_4$ in the fermentation medium. Under these conditions, the highest phytase activity was measured as 3.39±0.10 U/ml (Figure 8.5), which was very similar compared to the predicted value from the model. This clearly demonstrated the model represents the fermentation successfully.

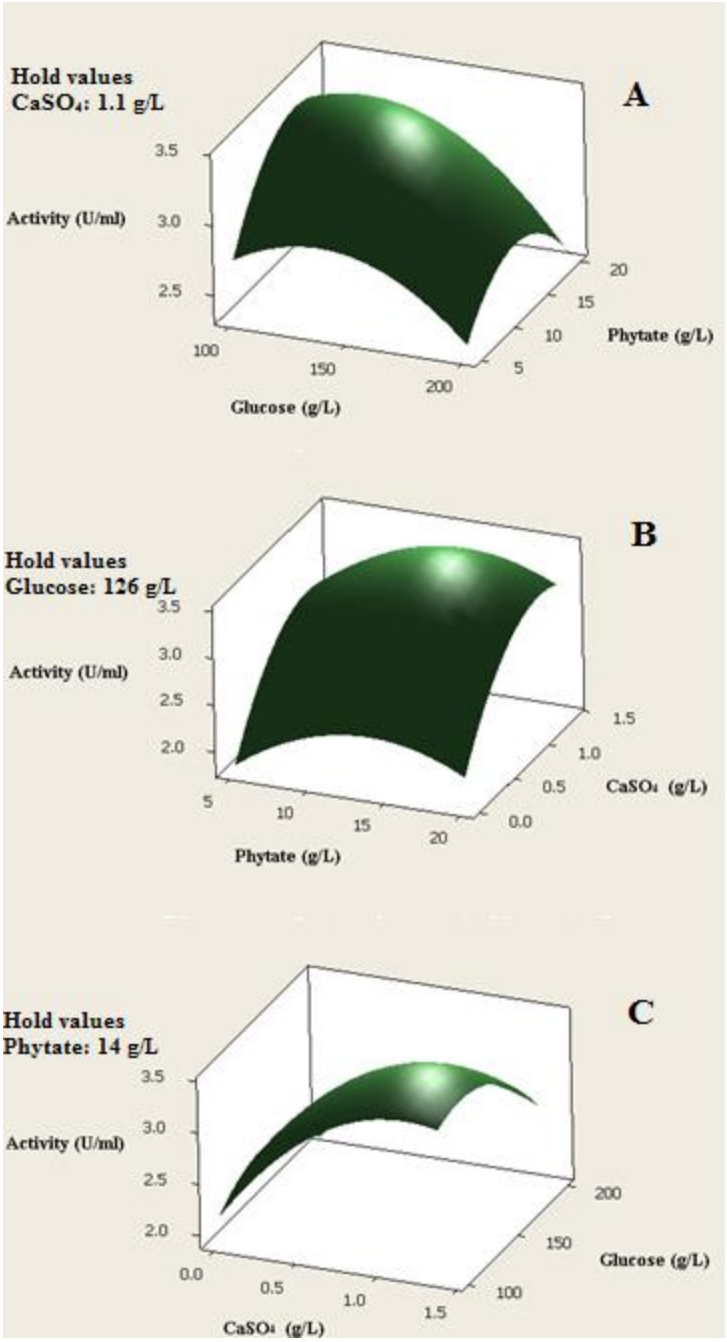

Figure 8.4. Phytase activity trends with changing medium composition.

Additionally, maximum glucose consumption rate and maximum phytase activity production rate were calculated as 0.70 g glucose/L/h and 0.0426 U/ml/h, respectively under optimized conditions (Figure 8.5).

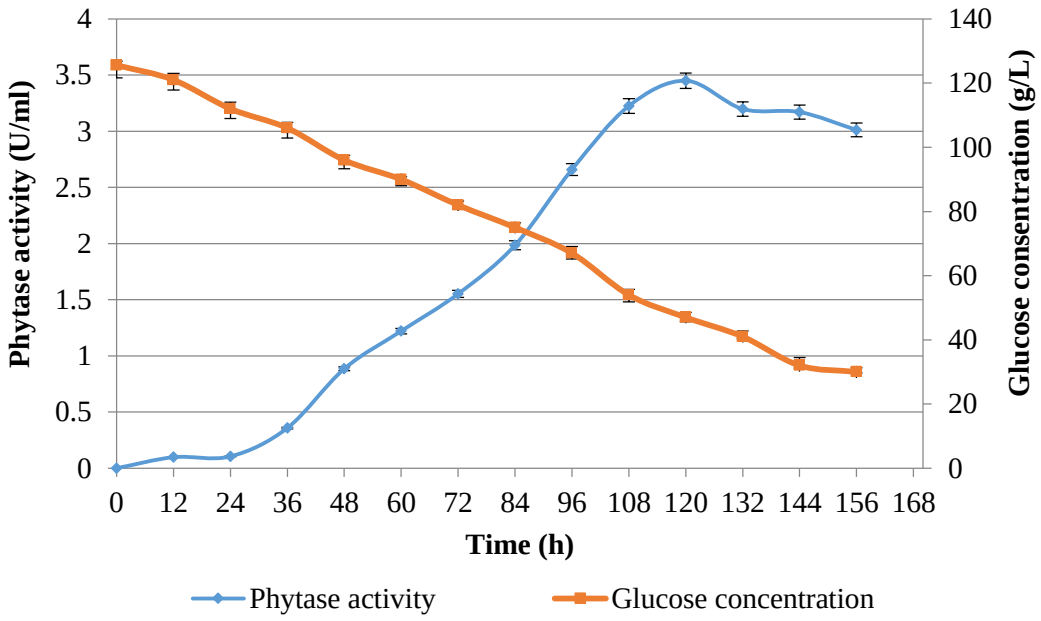

Figure 8.5. Glucose consumption and phytase activity under optimum conditions.

8.4.5. Effect of Storage Temperature on Phytase Activity

Effect of various storage temperatures on phytase activity was studied and results were shown on Figure 8.6. The lowest phytase activity loss was observed under 4°C during first week of the storage. The reason that more activity losses were measured in the samples, which were stored at -20°C during the first week of storage, may be the negative effect of occurrence of ice crystals in the vial. These ice crystals may damage enzyme structure partially and decreased the activity. However, after 7 days, it was observed that samples retained their activity more, when stored at -20 compared to storing at 4°C. Therefore, it can be concluded that samples must be stored at 4°C for one week storage and -20°C should be preferred for longer storage periods. On the other hand, samples, which were kept at room temperature and optimum fermentation temperature lost their activity faster comparing to samples stored at -20 and 4°C.

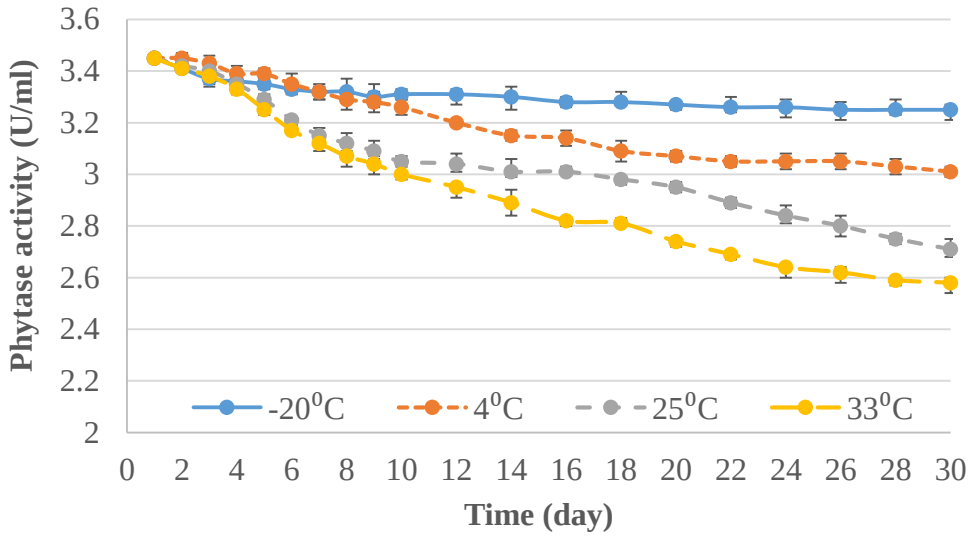

Figure 8.6. Effect of storage temperature on *A. ficuum* phytase activity.

8.5. Conclusions

In conclusion, this study showed that optimum pH and temperature were 5.5 and 55ºC for *A. ficuum* phytase. Also, 126 g/L of glucose, 14 g/L of Na-phytate, and 1.1 g/L of $CaSO_4$ were determined as optimum in the fermentation medium composition. Under this condition, phytase activity was increased to 3.45 U/ml from 2.27 U/ml, which is about 50% increase compared to the base fermentation medium results. A mathematical model, which created by MINITAB successfully represented the process, can be used for future studies and scale-up purposes. Also, it was determined that pH 5.5 and 55ºC as optimum for the produced *A. ficuum* phytase activity. Additionally, it was shown that 4ºC was the best condition for less than 1 week storage period for *A. ficuum* phytase. Overall, this study shows that, fungal phytase can be produced in submerged fermentation successfully rather than solid state and enzyme activity can be further increased by more improvements on media and process conditions.

8.6. References

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CHAPTER 9

ENHANCED SUBMERGED *ASPERGILLUS FICUUM* PHYTASE PRODUCTION BY IMPLEMENTATION OF FED-BATCH FERMENTATIONS

9.1. Abstract

Phytase, is an important feed and food additive, which is both used in animal and human diets. Phytase has been used to increase the absorption of several divalent ions, amino acids, and proteins in the bodies and to decrease the excessive phosphorus release in the manure to prevent negative effects on the environment. To date, microbial phytase has been mostly produced in solid state fermentations with insignificant production volumes. There are only a few studies in the literature that phytase productions were performed in submerged bench-top reactor scale. In our previous studies, growth parameters (temperature, pH, and aeration) and important fermentation medium ingredients (glucose, Na-phytate, and $CaSO_4$) were optimized. This study was undertaken for further enhancement of phytase production with *Aspergillus ficuum* in bench-top bioreactors by conducting fed-batch fermentations. The results showed that 60 g of glucose addition and 10 g of Na-phytate addition at 96 h of fermentation increased phytase activity to 3.84 and 4.82 U/ml, respectively. Therefore, the maximum phytase activity was further enhanced with glucose addition by 11% and Na-phytate addition by 40% compared to batch phytase fermentations. Additionally, it was observed that fed-batch glucose and Na-phytate additions lowered the decrease of phytase activity after reaching the maximum levels compared to the batch fermentations.

9.2. Introduction

Phytate is a major phosphorus source in plants, which especially serves for ATP synthesis during germination (Vohra et al., 2003). However, phytate has a high negatively charged molecular structure, which chelates amino acids, proteins and divalent ions such as Ca^{+2}, Mg^{+2}, Zn^{+2}, Cu^{+2}, Fe^{+2}, and Mn^{+2} in vivo (Haefner et al., 2005). Consequently, high phytate consumption causes health problems such as iron deficiency, bone weakness, tooth decay, and

digestion problems (Hurrell et al., 2003; Sanson et al., 1981). Another problem about phytate consumption is that, monogastric animals such as chickens and pigs are not able to break and utilize phytate due to lack of necessary microflora in their digestion systems. Therefore, excessive accumulation of phosphorus in monogastric animals' manure causes environmental problems such as water pollution, algal blooms, fish kills, and changing of fauna and flora (Mullaney et al., 2000). Cowieson et al. (2012) reported the supplementation of phytase reduced phytate content in the diets by 35-40%. In another study, Nelson et al. (1968) used phytase, pretreat a corn–soybean diet for broilers and reported that phosphorus availability increased by 60% and phosphorus content decreased by 50% in the manure. It was also reported that phytase helped to improve body weight of male and female broilers by 13.2 and 5.8%, respectively after 21 days on a diet supplemented with phytase (Nelson et al., 1968). Effect of phytase on absorption of minor nutrients was studied by Sebastian et al. (1996). They demonstrated that phytase supplementation increased the relative retention of total P^{-3}, Ca^{+2}, Cu^{+2}, and Zn^{+2} by 12.5, 12.2., 19.3, and 62.3%, respectively, in broilers (Sebastian et al., 1996).

To date, phytase productions have been generally performed in small scale solid state fermentations with lack of optimizations and novel techniques. There are a few studies are available about submerged phytase fermentation. For example, Sasirekha et al. (2012) studied optimization of phytase fermentation medium for *Pesudomonas sp.* and produced 0.739 U/ml phytase activity, when sucrose was used as carbon source in the medium. Also, Kumar et al. (2012) studied optimization of phytase production by using *Achromobacter sp*. PB-01 in batch fermentations and increased phytase activity by 11 fold compared to initial conditions. Moreover, Shah et al. (2009) reported that optimization of the fermentation medium for phytase production with *Aspergillus niger* NCIM 563 in submerged batch fermentations doubled phytase activity. In another study, submerged phytase production with *Aspergillus niger* NCIM 56 was performed by Bhavsar et al. (2013) and they reported almost 6-fold increase in phytase activity after performing mutations on the microorganism and modifications on the fermentation medium. Additionally, Liu et al. (2011) doubled *Pichia pastoris* phytase activity by optimization of the submerged fermentation medium. In our previous studies, phytase activity with *A. ficuum* was increased from 1.02 U/ml to 2.27 U/ml and 3.45 U/ml in batch fermentations after optimization of growth parameters and fermentation medium, respectively (Chapters 7 and 8). There are important fluctuations in the phytase activity calculations in the literature. The reason is that all researchers are using different chemicals and analytical conditions for enzyme activity assays, which directly affect activity calculations. Additionally, several questionable phytase productions were

performed in the literature. For example, Soni and Khire (2007) and Papagianni et al. (2001) reported 40 and 15 U/ml phytase activity in their studies. However, they used KH_2PO_4 in their fermentation medium, which also cause color occurrence in spectrophotometric phytase activity assay. Moreover, Soni and Kahire (2007) reported that instead of using uninoculated fermentation medium as blank, they used buffer and enzyme solutions as blank in their analysis. All these result in significant fluctuations in reporting phytase activity values. On the other hand, Mittal et al. (2012) performed phytase production in a similar fermentation medium with our studies and reported 0.9 U/ml phytase activity, which was 2.5 times lower than our results (2.27 U/ml).

In order to improve phytase activity, more complex fermentations than batch fermentations such as fed-batch and continuous fermentations can be used. Fed-batch fermentations are generally used to prevent substrate inhibition or catabolite repression by intermittent feeding of the substrate. Also, fed-batch fermentations allow higher microorganism and product formation in the reactors compared to batch fermentations. On the other hand continuous fermentations provide a chemically-balanced system with constant environmental growth and product formation also supply uniform-quality of product (Shuler and Kargi, 1992). A few fed-batch and continuous studies were performed about microbial phytase production. For example, Jin et al. (2007) set an artificial neural network pattern recognition (ANNPR) model for fed-batch production with recombinant *Pichia pastoris* phytase. In this study, they maintained the methanol concentration in the reactor between 0-20 g/L to induce cell growth and phytase production. They reported that, phytase activity increased about four times compared to previous studies. Another fed-batch phytase production was studied by Kleist et al. (2003). In this study, phytase production with *Escherichia coli* was enhanced by keeping the glucose concentration constant at low oxygen levels (5-10%). They reported that phytase activity increased 1.5 fold compared to initial studies. There is still a need that more detailed studies must be conducted about fed-batch phytase productions to increase phytase production to make it available for industrial productions. Overall, the goal of this study is to improve phytase production by *A. ficuum* in submerged fermentation by conducting fed-batch fermentations.

9.3. Materials and Methods

9.3.1. Microorganisms

Aspergillus ficuum (NRRL 3135) was used in this study as suggested by our previous study (Chapter 7). *A. ficuum* was obtained from (USDA) Agricultural Research Service Culture Collection (Peoria, IL) and grown on potato dextrose agar (PDA) (Difco, Sparks, MD) slants for 6 days at 30ºC and stored at 4ºC as the working culture. In order to maintain viability, *A. ficuum* was regularly transferred to sterile fresh agar slant biweekly.

9.3.2. Inoculum Preparation

A. ficuum spores were grown on 25 PDA plates for 6 days at 30°C. After incubation, spores were suspended by adding 7 ml of sterile 0.1% peptone water and the resulting solution (~10^6 spores/ml) has been collected and used as the inoculum.

9.3.3. Fed-batch Fermentation Conditions

All runs were performed in Sartorius Biostat B Plus bioreactor (Allentown, PA) equipped with a 2-L vessel with 1-L working volume. Reactors were inoculated with 3% prepared inoculum. Fermentation runs were performed at 33ºC, 4.5 pH and 0.9 vvm aeration conditions (Chapter 7). As suggested by our previous study (Chapter 8), the medium consists of 126 g of glucose, 0.5 g of KCl, 0.1 g of $FeSO_4(7H_2O)$, 0.5 g of $MgSO_4(7H_2O)$, 0.01 g of $MnSO_4(7H_2O)$, 8.6 g of $NaNO_3$, 3 g $(NH_4)_2SO_4$, 1.1 g/L $CaSO_4$, and 14 g of Na-phytate (A&Z Food Additives Co. Ltd., Zhejiang, China) per litter of deionized water. Agitation was maintained at 300 rpm for all fermentation runs.

Fed-batch processes were conducted to study effect of addition of glucose and Na-phytate on phytase productions. Based on previous studies (Chapter 8), three fed-batch addition points were selected to evaluate; are early increase of phytase activity stage (72 h), middle stage (96 h), and late stage (120 h). Glucose concentrations were aimed to increase to the initial concentration in these points. Therefore, based on glucose consumption measurement, 45, 60, and 80 g of glucose were dissolved in 50 ml of DI water, pH was adjusted to 4.5 autoclaved and then added into the reactors to bring the glucose concentrations to the initial levels. Similarly, effect of Na-phytate addition on phytase production was studied by adding of 5 and 10 g/L of Na-phytate into

the reactors at these three stages. Samples were collected from the reactors every 24 h to determine phytase activity and glucose residuals during 9 days of fermentation.

9.3.4. Analysis

9.3.4.1. Phytase Activity

Enzyme assay was performed under the determined optimum temperature and pH as described by Kim et al. (1998) with minor modifications. Briefly, cell-free broth (0.125 ml) was mixed with 0.125 ml of 1.5 mM Na-phytate in 0.1 M sodium acetate solution and mixtures were incubated in the water bath at 55ºC for 30 min. After incubation, reaction was stopped by adding 0.25 ml of 15% tricholoroacetic acid solution into the tubes. Then, 2 ml of color regent was added, which was prepared freshly with; 2:1:1:1 ratio of water: 2.5 % ammonium molybdate: 6 N H_2SO_4: 10 % ascorbic acid and tubes were incubated at 55ºC for 30 min. After cooling to room temperature, absorbancies were measured at 700 nm by using a spectrophotometer (Beckman Coulter, Fullerton, CA). Uninoculated fermentation medium was used as the blank for the measurement fed-batch glucose addition fermentations. However, since phytate addition is effective on phytase activity measurement, samples, which were collected from the fermentations were boiled at 100ºC for 30 min to deactivate the phytase enzyme and used as blank after cooling down for fed-batch fermentations. The obtained data was used to calculate the activity unit of phytase (U/ml), which was defined as the μmole of phosphorus liberated from 1.5 mM phytate per min under the set assay conditions.

9.3.4.2. Glucose Concentration

Glucose concentrations were measured using high pressure liquid chromatography (HPLC) with a refractive index detector (Waters, Milford, MA). Glucose determination was performed by using Aminex HPX-87H column (Bio-Rad, Richmond, CA) with 0.8 ml/min isocratic flow of 0.012 N sulfuric acid. The detector and column temperature were maintained at as 35 and 65ºC, respectively. The cell-free samples were filtered by using 13 mm diameter, 0.2 μm pore sized filters (PALL Life Sciences, Port Washington, NY).

9.3.4.3. Statistical Analysis

MINITAB statistical software (Version 15, State College, PA) package was used for statistical analyses. Tukey's test was used to make a single step multiple comparison of fed-batch results. Also, 2-sample t test was used to compare the results between 5 and 10 g Na-phytate addition fermentation results.

9.4. Results and Discussion

In this study, effect of glucose and phytate addition on phytase production with *A. ficuum* was studied in bench-top bioreactors. Various amounts of glucose and phytate were added into the bioreactors in various time intervals to see the effect on phytase production. It was shown that both glucose and phytate additions are significantly effective on phytase activity.

9.4.1. Fed-batch Glucose Addition

Glucose was added into the reactors at 45, 60, and 80 g at 72, 96, and 120 h of fermentation, respectively, to bring the glucose concentrations to the initial levels. Effect of fed-batch glucose addition at various times of the fermentation was shown on Figure 9.1.

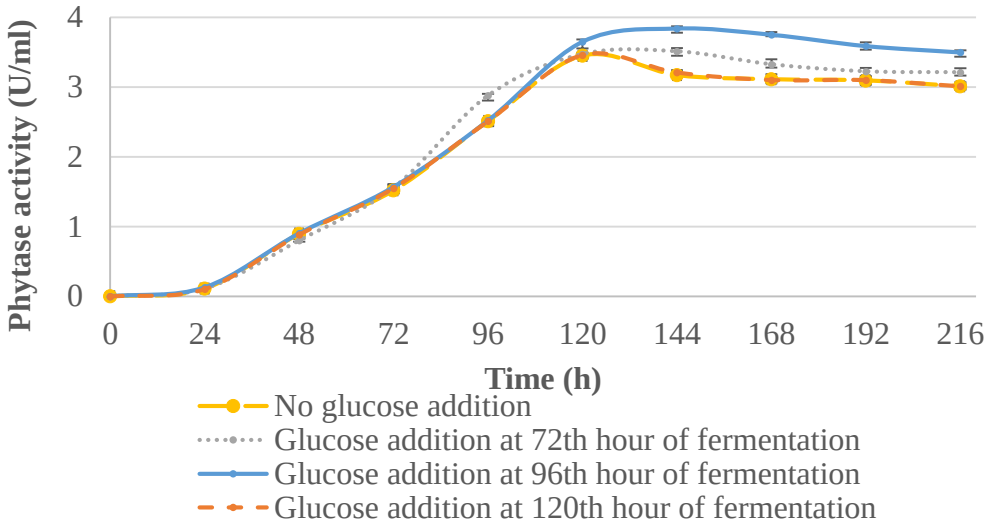

Figure 9.1. Effect of addition 45 g of glucose at 72 h of fermentation, addition 60 g of glucose at 96 h of fermentation, and addition 80 g/L of glucose at 120 h of fermentation on phytase activity.

Glucose addition at 72 h of fermentation resulted in 3.51 U/ml phytase activity at 144 h of fermentation. On the other hand, phytase activity was further increased to 3.84 U/ml by glucose addition at 96 h of fermentation. However, addition of glucose at 120 h of fermentation did not affect phytase activity. In this fed-batch study, the maximum phytase activity remained the same as 3.46 U/ml, which was similar to the values obtained in previous batch fermentations (Chapter 8). This can be explained by that since phytase is a growth related product, *A. ficuum* can use these nutrients more efficiently in early stages than late stages of growth. Also, it was determined that phytate addition was more effective on phytase activity than glucose addition. Maximum phytase productivity increased to 0.05 U/ml/h, when glucose addition was done at 72 and 96 h of fermentation. However, productivity remained the same (0.04 U/ml/h), when glucose was added at 120 h of the fermentation. Also, it was observed that, phytase activity did not decrease significantly through following couple of days in all cases compared to the control fermentation. Phytase activities decreased by 5.3, 2.3, and 7.2%, respectively, in 24 h after reaching the maximum levels. Glucose consumptions were measured as 130, 128, and 134 g/L, respectively at 216 h of the fed-batch fermentations (data not shown).

Application of Tukey's statistical method showed that, addition of 60 g of glucose at 96 h of fermentation was significantly ($p<0.05$) different then addition 45 g of glucose at 72 h of fermentation and addition 80 g of glucose at 120 h of fermentation fed-batch studies (Table 9.1).

Table 9.1. One-way ANOVA and Tukey's statistical method evaluation of fed-batch glucose addition on phytase production results.

Source	DF	SS	MS	F	P
Factor	2	0.255800	0.127900	213.17	0.000
Error	6	0.003600	0.000600		
Total	8	0.259400			

S = 0.02449 R-Sq = 98.61% R-Sq(adj) = 98.15%

Factor	N	Mean	Grouping
60 g of glucose addition at 96th hour of fermentation	3	3.84	A
45 g of glucose addition at 72th hour of fermentation	3	3.51	B
80 g of glucose addition at 120th hour of fermentation	3	3.46	B

Addition of 60 g of glucose at 96 h of fermentation was grouped as “A” on the other hand, addition 45 g of glucose at 72 h of fermentation and addition 80 g of glucose at 120 h of fermentation were grouped as “B”. Therefore, it can be concluded that, glucose addition amount and time are significantly effective on phytase production.

9.4.2. Fed-batch Na-phytate Addition

Na-phytate was added into the reactors at 5 and 10 g amounts at the same studied time intervals for glucose addition. As shown on Figure 9.2, 5 g of Na-phytate addition increased phytase activity in all cases.

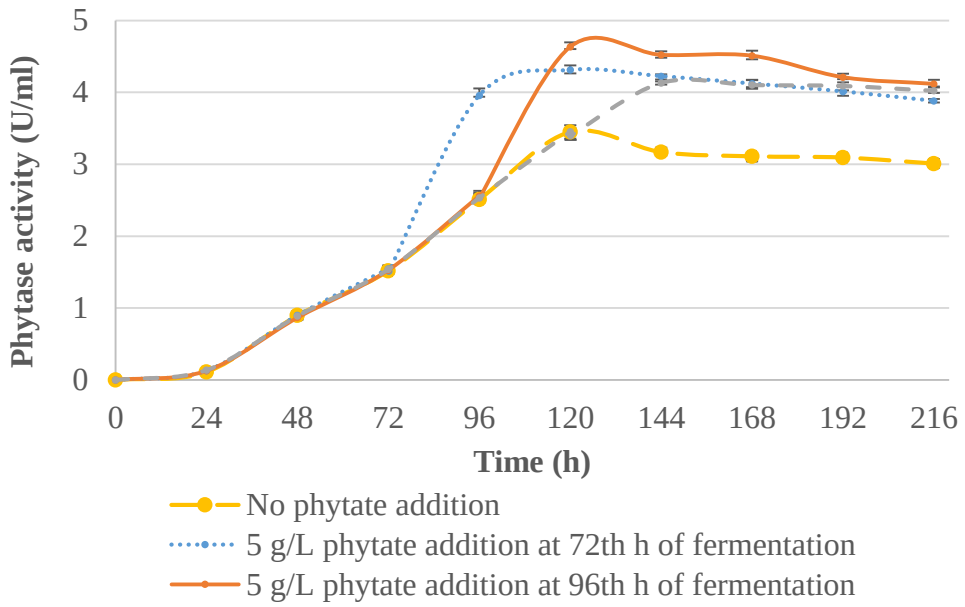

Figure 9.2. Effect of 5 g of Na-phytate addition at 72, 96, and 120 h of fermentation on phytase activity.

The highest phytase activity was measured as 4.63 U/ml by addition of 5 g Na-phytate at 96 h of fermentation. Similar to glucose addition also 5 g of Na-phytase addition in early stages enhanced phytase activity more compared to late stage additions. Addition 5 g of Na-phytate at 72 h of fermentation increased phytase activity by 156% (from 1.54 U/ml to 3.96 U/ml) in 24 h.

Also, addition 5 g of Na-phytate at 96 h of fermentation increased phytase activity by 81% in 24 h. However, addition 5 g of Na-phytate at 120 h of fermentation increased phytase activity only by 20% in 24 h. Additionally the maximum phytase activity increased by 25, 34, and 20% compared to batch fermentations, when 5 g Na-phytate addition was performed at 72, 96, and 120 h of fermentation, respectively. Also, it was reported that phytase productivities were also increased by Na-phytate addition in the fermentations except for 120 h addition. Phytase productivities were measured as 0.1, 0.086, and 0.03 U/ml/h, when 5 g of Na-phytate was added into the fermentations at 72, 96, and 120 h of fermentation, respectively. Furthermore, 5 g Na-phytate addition also lowered phytase activity lost after reaching the maximum levels. It was reported that phytase activities reduced by 2.0, 2.4, and 0.7%, respectively within 24 h after reaching the maximum levels.

Tukey's statistical method showed addition of 5 g of Na-phytate at 72, 96, or 120 h of fermentation were significantly (p-value <0.05) different than each other (Table 9.2). This proves that, there is a significant effect of Na-phytate addition time on phytase activity.

Table 9.2. One-way ANOVA and Tukey's statistical method evaluation of fed-batch addition of 5 g of Na-phytate addition on phytase activity results.

Source	DF	SS	MS	F	P
Factor	2	0.384800	0.192400	412.29	0.000
Error	6	0.002800	0.000467		
Total	8	0.387600			

S = 0.02160 R-Sq = 99.28% R-Sq(adj) = 99.04%

Factor	N	Mean	Grouping
5 g of phytate addition at 96th hour of fermentation	3	4.63	A
5 g of phytate addition at 72th hour of fermentation	3	4.31	B
5 g of phytate addition at 120th hour of fermentation	3	4.13	C

Additionally, effect of 10 g fed-batch phytate addition on phytase activity was also studied and results were shown on Figure 9.3. Maximum phytase activity was obtained as 4.83 by addition 10 g of Na-phytate at 96 h of fermentation. The maximum phytase activities were measured as 4.7 and 4.23 U/ml, when 10 g of Na-phytate was added at 72 and 120 h of the fermentations, respectively. The reason that phytase addition increased phytase activity more compared to glucose addition may be related to that phytate is the substrate of phytase enzyme so

it encourages fungi to produce phytase, whereas glucose serves as a carbon source and primarily supports microbial growth. This shows that phytate amount is a significant parameter in phytase fermentation. Therefore, high phytate included agrochemical wastes such as corn cob, when bran etc. can be used as phytate sources in the fermentations to enhance phytase activity. Lan et al. (2002) studied rice bran added phytase production with *Mitsuokella jalaludinii* and reported that phytase activity increased from 5.08 U/ml, to 12.93 U/ml, when 20% rice bran was added in the fermentation medium. Also, Mittal et al. (2011) modified phytase fermentation medium with 2% orange peels and increased *Klebsella* sp. DB3 phytase activity from 0.6 to 3.15 U/ml.

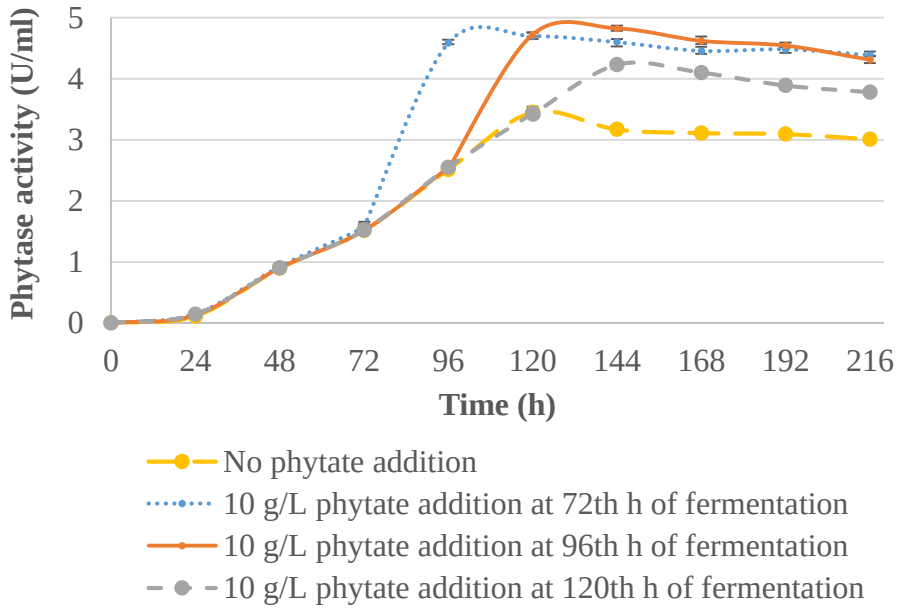

Figure 9.3. Effect of 10 g of Na-phytate addition at 72, 96, and 120 h of fermentation on phytase activity.

Phytase productivities were measured as 0.126, 0.091, and 0.033 U/ml/h, when 10 g of Na-phytate were added into the reactors at 72, 96, or 120 h of fermentation, respectively. Similar to 5 g of phytate addition, also 10 g of phytate addition provided more rapid increases in phytase activity in early state additions than late stage addition. Phytase activities were increased by 194, 86, and 24% after 24 h by addition of 10 g of Na-phytate at 72, 96, and 120 h of fermentation, respectively. After reaching the maximum levels, phytase activities decreased by 2.1, 4.2, and 3.0%, respectively, within 24 h, when 10 g Na-phytate was added into the reactors. Furthermore,

Tukey's test showed that, addition of 10 g of Na-phytate at 72, 96, and 120 h of fermentation were significantly (p-value <0.05) different then each other (Table 9.3).

Table 9.3. One-way ANOVA and Tukey's statistical method evaluation of fed-batch addition of 10 g of Na-phytate addition on phytase activity results.

Source	DF	SS	MS	F	P
Factor	2	0.588422	0.294211	980.70	0.000
Error	6	0.001800	0.000300		
Total	8	0.590222			

S = 0.01732 R-Sq = 99.70% R-Sq(adj) = 99.59%

Factor	N	Mean	Grouping
10 g of phytate addition at 96th hour of fermentation	3	4.825	A
10 g of phytate addition at 72th hour of fermentation	3	4.700	B
10 g of phytate addition at 120th hour of fermentation	3	4.231	C

Comparison of effects of 5 g and 10 g addition of phytate at 96 h of fermentation on phytase activity was evaluated by conducting 2 sample t-test. End of the test, p-value was given as 0.005 in MINITAB (data not shown). This concludes that there is a significant difference between effects of 5 g and 10 g addition of phytate at 96 h of fermentation on phytase activity. Therefore, not only addition time, but also phytate amount is a significant parameter on enhancing phytase activity.

9.5. Conclusion

In conclusion, this study showed that fed-batch glucose and Na-phytate addition enhanced submerged phytase productions with *A. ficuum* in bench-top bioreactors. Maximum phytase activities were measured as 3.84, 4.63, and 4.83 U/ml, when 45 g of glucose, 5 g of Na-phytate, and 10 g of Na-phytate was added into the bioreactors, respectively at 96 h of the fermentations. These results showed that maximum phytase activities were increased by 11, 34, and 40%, respectively, compared to the batch fermentation. Additionally, Tukey's method results showed that Na-phytate addition was significantly more effective on phytase activity than glucose addition. Overall, this study shows that, fungal phytase can be produced in submerged fermentation successfully rather than solid state.

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CHAPTER 10

MICROPARTICLE ENHANCED *ASPERGILLUS FICUUM* PHYTASE PRODUCTION IN SUBMERGED FERMENTATION

10.1. Abstract

Phytase, is an important feed additive, which can be both used in animal and human diets. It has been used to increase the absorption of several divalent ions, amino acids and proteins and to reduce unabsorbed phosphorus release in the manure to prevent negative effects on the environment. To date, microbial phytase has been mostly produced in solid state fermentations with corresponding low volumes. Therefore, this study aimed to enhance *Aspergillus ficuum* phytase production in submerged fermentations by evaluation of the effect of various microparticle type (aluminum oxide and talcum) and levels (0-25 g/L) in the fermentation medium. It was observed that microparticles prevented bulk fungal pellet growth in the fermentation medium and decreased average fungal pellet size and significantly ($p<0.05$) increased phytase activity during the submerged fermentations. The significantly higher ($p<0.05$) phytase activities were measured as 2.01 and 2.93 U/ml with addition of 15 g/L of talcum and aluminum oxide, respectively, in shake-flasks fermentations compared to control (1.02 U/ml). Phytase activity was further enhanced to 6.49 U/ml within 96 h of fermentation in the bioreactors with the addition of 15 g/L of talcum, whereas the maximum phytase activity was only 3.45 U/ml at 120 h of fermentation for the control (without microparticle) in bioreactor. In conclusion, talcum addition as the microparticle enhanced phytase fermentation by 88% within a shorter fermentation time.

10.2. Introduction

Phytate is the major phosphate source in plants, which is especially abundant in legumes, cereals, pollen, and nuts. In the plants, phytate is generally used during germination for ATP synthesis (Vohra et al., 2003). However, phytate is a very strong chelating agent, which has several negative effects on animal and human health. Phytate can bind proteins, amino acids, and divalent ions such as Ca^{+2}, Mg^{+2}, Zn^{+2}, Cu^{+2}, Fe^{+2}, and Mn^{+2} in vivo and creates insoluble salt forms. As a result of this, absorption and utilization of these nutrients decreases (Haefner et al., 2005). For these reasons, phytate consumption in the diets may have some several health problems such as iron deficiency, bone weakness, tooth decay, and digestion problems (Hurrell et

al., 2003; Sanson et al., 1981). Additionally, there are several environmental issues have been also reported about phytate consumption. Monogastric animals such as chickens and pigs are not able to hydrolyze phytate, because they do not have the necessary microflora in their digestion systems (Mullaney et al., 2000). Since these monogastric animals are generally fed with phytate rich ingredients such as wheat, rice, and corn, excessive amount of phosphorus accumulates in their manure. This manure phosphorus may cause environmental problems such as water pollution, algal blooms, fish kills, and changing of fauna and flora (Mullaney et al., 2000). However, these problems can be overcome, if the diets are supplemented with phytase to help to hydrolyze feed phytate. In several studies, the positive effect of phytase application on animal growth and the environment were shown. Nelson et al. (1968) used phytase, produced from *Aspergillus niger* to pretreat a corn–soybean diet for broilers. They showed that the phosphorus availability increased by 60% and phosphorus content decreased by 50% in the manure. Additionally, phytase helped to improve body weight gain of male and female broilers by 13.2 and 5.8%, respectively, after 21 days on a diet supplemented with phytase. Another positive effect of phytase application was shown by Sebastian et al. (1996) to increase in absorption of minor nutrients. They demonstrated that phytase supplementation increased the relative retention of total P^{-3}, Ca^{+2}, Cu^{+2}, and Zn^{+2} by 12.5, 12.2., 19.3, and 62.3%, respectively, in broilers.

Phytase can be obtained from plants, but more commonly from microorganisms for industrial purposes. Molds were most commonly used for phytase production in several studies. Cowieson et al. (2012) used *Aspergillus niger* phytase in diets of the animal and reduced phytate content by 35-40%. In another study, Shah et al. (2009) reported that optimization of the fermentation medium for phytase production with *Aspergillus niger* NCIM 563 in submerged batch fermentation doubled phytase activity compared to initial values. Similarly, submerged phytase production with *Aspergillus niger* NCIM 56 was performed by Bhavsar et al. (2013) and they reported almost 6-fold increase in phytase activity after performing mutations on the microorganism and modifications on the fermentation medium. In our previous studies, phytase activity with *A. ficuum* was increased from 1.02 U/ml to 2.27 U/ml and 3.45 U/ml in batch fermentations after optimization of growth parameters and the fermentation medium, respectively (Chapters 7 and 8). However, the cultivation of filamentous microorganisms is commonly accompanied by several problems, such as clumpy growth and insufficient mass transfer, which may result in reduced productivity. In order to overcome these problems, microparticles can provide a solution. Microparticle addition is a novel approach for cultivation of filamentous microorganisms and product formation to increase the overall yield of the process (Kaup et al.,

2008; Driouch et al., 2012; Walisko et al., 2012). Additionally, the morphology of filamentous microorganisms during fermentation is an important issue for desired product formation, supplying substrate, and creating effective agitation in the bioreactors. Microparticles can provide precise control of filamentous microorganism morphology during fermentation preventing bulk fungal growth (Walisko et al., 2012). To date, microparticles such as talcum (magnesium silicate), aluminum oxide, and titanium oxide were used in several studies to increase the yield of filamentous microorganisms. For example, Driouch et al. (2012) studied *Aspergillus niger* fermentation in the presence of 0-50 g/L $TiSiO_4$. They reported that, 25 g/L of $TiSiO_4$ addition increased fructofuranosidase and glucoamylase enzyme production by 3.7 fold and 9.5 fold, respectively compared to control in shake-flask systems. Additionally, they studied effect of microparticles on microbial morphology and reported that pellet diameter was reduced from 1.7 mm to 0.3 mm, when a 25 g/L concentration of $TiSiO_4$ was added into fermentation medium (Driouch et al., 2012). In another study, Kaup et al. (2008) studied effect of aluminum oxide and talcum as microparticles on chloroperoxidase production by *Caldariomyces fumago*. They observed that particles around 500 µm diameter did not make any difference in growth morphology or production, however particles smaller than 42 µm diameters dispersed *C. fumago* to single hypae, which enhanced enzyme production by 5-fold. Also, Driouch et al. (2011) reported that, fructofuranosidase production by *A. niger* was enhanced by 3.5-fold in the presence of either 10 g/L of talcum or 20 g/L aluminum oxide in the fermentation medium compared to the control. Therefore, this study is undertaken to further enhance *A. ficuum* phytase production by evaluating talcum and aluminum oxide as microparticles.

10.3. Materials and Methods

10.3.1. Microorganism

Aspergillus ficuum (NRRL 3135) was used as the phytase producer (Chapter 7), which was obtained from USDA Agricultural Research Service Culture Collection (Peoria, IL) and grown on potato dextrose agar (PDA, Difco, Sparks, MD) slants for 6 days at 30^{o}C and stored at 4^{o}C as the working culture. In order to maintain viability, *A. ficuum* was regularly transferred to sterile fresh agar slants bi-weekly.

10.3.2. Inoculum Preparation

A. ficuum spores were grown on 25 PDA plates for 6 days at 30°C. After incubation, spores were suspended by adding 7 ml of sterile 0.1% peptone water and the resulting solution (~10^6 spores/ml) has been collected and used as the inoculum for the fermentations.

10.3.3. Shake-flask Fermentation

Shake-flasks, containing 100 ml of fermentation medium were supplemented with 0, 5, 10, 15, 20, and 25 g/L of aluminum oxide or talcum. The base fermentation medium included 126 g of glucose, 0.5 g of KCl, 0.1 g of $FeSO_4(7H_2O)$, 0.5 g of $MgSO_4(7H_2O)$, 0.01 g of $MnSO_4(7H_2O)$, 8.6 g of $NaNO_3$, 3 g $(NH_4)_2SO_4$, 1.1 g $CaSO_4$, and 14 g of Na-phytate (A&Z Food Additives Co. Ltd., Zhejiang, China) per litter of deionized water, as suggested by our previous study (Chapter 8). The pH was adjusted to 6.8 and flasks were autoclaved for 15 min. Thereafter, flasks were inoculated with a 3% prepared spore suspension and incubated at 33ºC and 200 rpm for total of 144 h. Samples were collected from each flask in every 12 h and analyzed for phytase activity.

10.3.4. Microbial Imaging of Pellets in Shake-flask Fermentation

Biomass samples collected from the shake-flasks with various microparticle concentrations at 72 h of fermentation were washed with a 0.9% NaCl solution to remove excessive microparticles and medium from the pellets. Thereafter, selected average sizes of pellets from the each sample were visually analyzed using a light microscope (1242MM, Van Guard, Kirkland, WA).

10.3.5. Batch Fermentation in Bioreactors

Batch fermentations with talcum (average particle size is 10 µm) as microparticles were performed in Sartorius Biostat B Plus bioreactor (Sartorius, Allentown, PA) equipped with a 2-L vessel with 1-L working volume. The same base fermentation medium at the shake-flask fermentations were used, but supplemented with 5, 10, 15, 20, and 25 g/L talcum microparticles. Then, the reactors were autoclaved for 30 min and inoculated with 3% prepared inoculum after cooling. The fermentations were run at 33ºC, pH 4.5, 0.9 vvm aeration, with 300 rpm agitation as

suggested by our previous study (Chapter 7). Samples were collected (2 ml) from the reactors every 12 h for 6 days.

10.3.6. Phytase Activity Analysis

Samples were centrifuged at 5,200 x *g* for 15 min (Galaxy 5D, VWR, Radnor, PA) to remove the biomass. Then, supernatant was used for phytase activity analyses. Enzyme assay was performed under the optimum temperature and pH as described by Kim et al. (1998) with minor modifications. Briefly, cell-free broth (0.125 ml) was mixed with 0.125 ml of 1.5 mM Na-phytate in a 0.1 M sodium acetate solution and mixtures were incubated in the water bath at 55^{o}C for 30 min. After incubation, the reaction was stopped by adding 0.25 ml of 15% tricholoroacetic acid solution into the tubes. Then, 2 ml of color regent was added, which was prepared freshly with; a 2:1:1:1 ratio of water: 2.5 % ammonium molybdate: 6 N H_2SO_4: 10% ascorbic acid and tubes were incubated at 55^{o}C for 30 min. After cooling down to room temperature, absorbance were measured at 700 nmusing a spectrophotometer (Beckman Coulter, Fullerton, CA). Uninoculated fermentation medium was used as the blank for the measurement. The obtained data was used to calculate the activity of phytase (U/ml), which was defined as the μmoles of phosphorus liberated from 1.5 mM phytate per min under the set assay conditions.

10.3.7. Statistical Analysis

MINITAB Statistical Software package was used for statistical analyses. A 2 sample t-test was used to show if there is a significant difference between fermentation results. Additionally, Tukey's method was used to compare shake-flask and reactor fermentations results.

10.4. Results and Discussion

In this study, the effect of two different microparticles on *A. ficuum* phytase production was studied in shake-flask fermentation and bench-top bioreactors.

10.4.1. Effect of Aluminum Oxide and Talcum Microparticles on Phytase Activity in Shake-flask Fermentation

Shake-flask fermentations demonstrated that phytase activity increased by addition of aluminum oxide or talcum to the fermentation medium (Figure 10.1). Both aluminum oxide and talcum addition increased phytase activity until 15 g/L of microparticles. It was calculated that addition of 15 g/L of aluminum oxide and 15 g/L of talcum increased the phytase activity to 2.01 U/ml (97% increase) and 2.93 U/ml (185% increase), respectively, compared to control, which yielded only 1.02 U/ml phytase activity. Therefore, these results suggested that talcum is a better microparticle for phytase production. However, higher than 15 g/L microparticle concentrations resulted in a decrease of the enzyme activity. For example, addition of 20 g/L of aluminum oxide and talcum in shake-flask fermentations decreased the maximum phytase activity to 1.58 and 1.69 U/ml, respectively. Similarly, when 25 g/L talcum or aluminum oxide was used, phytase activity decreased to almost the levels of control (Figure 10.1). Kaup et al. (2008) also reported that presence of 0.5 to 10 g/L talcum enhanced chloroperoxidase production in their studies. Although, higher concentrations decreased enzyme activity, still produced in higher levels than the control.

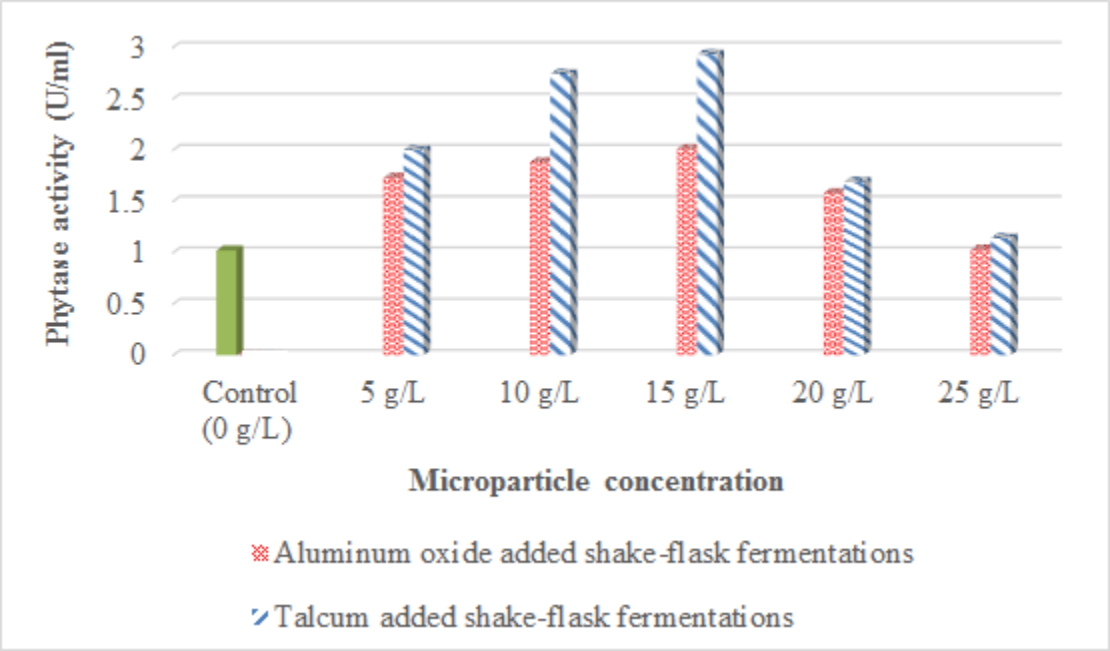

Figure 10.1. Maximum phytase activities in shake-flasks with microparticle addition and control fermentation.

Moreover, it is important to note that it was also observed that the highest phytase activities were obtained at 96 h of the fermentation with addition of aluminum oxide and talcum at all levels except for 5 g/L, whereas the control phytase fermentation provided the maximum phytase activity at 120 h of fermentation in shake-flask fermentations (data not shown). Additionally, the highest aluminum oxide and talcum added shake-flask fermentation results were compared by conducting 2 way t-test and result showed that there is a significance difference between phytase activity means of addition of 15 g/L of aluminum oxide and talcum in shake-flask fermentations (p-value<0.05).

10.4.2. Effect of Aluminum Oxide and Talcum Microparticles on Fungal Morphology in Shake-flask Fermentation

Fungal pellet size decreased with greater microparticle concentration in the shake-flask fermentations (Figure 10.2). It was observed that addition of 5 g/L of both aluminum oxide and talcum did not change the pellet size significantly compared to the control. However, higher levels of microparticles provided smaller fungal pellet sizes.

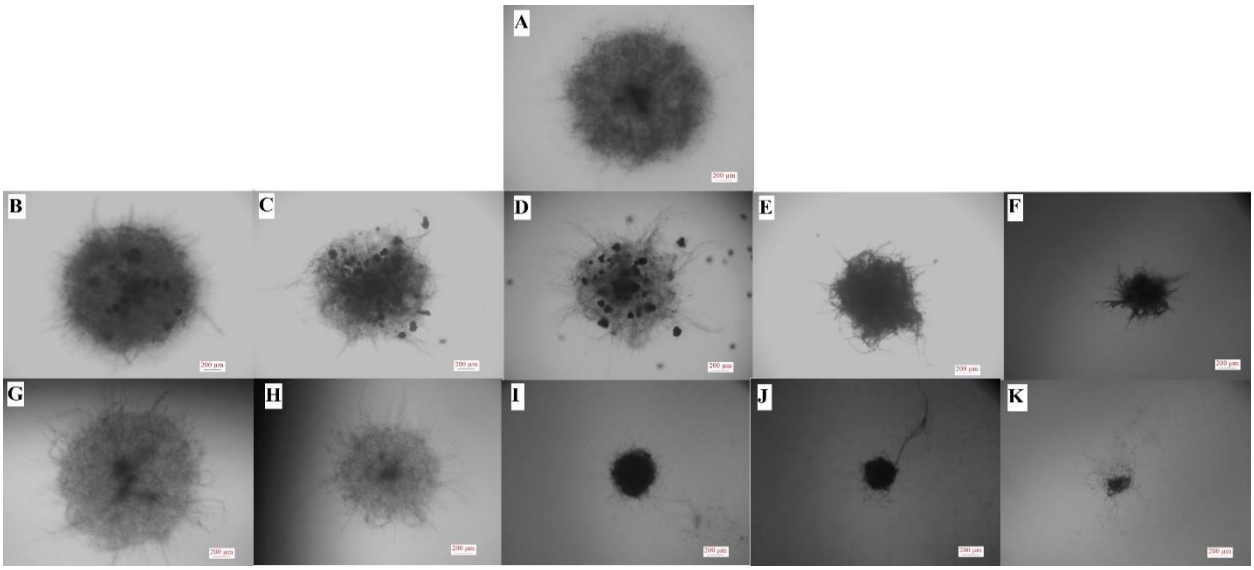

Figure 10.2. Effect of microparticles on *A. ficuum* morphology. (Control (A). Addition of aluminum oxide: 5 g/L (B), 10 g/L (C), 15 g/L (D), 20 g/L I, 25 g/L (F). Addition of talcum: 5 g/L (G), 10 g/L (H), 15 g/L (I), 20 g/L (J), 25 g/L (K)).

Effect of microparticles on average fungal pellet size was shown on Figure 10.3. Average fungal pellet radius was measured 800 µm for control fermentations. Also, it was observed that addition of 5 g of talcum or aluminum oxide did not change the pellet size remarkably. On the other hand, 15 g/L of aluminum oxide and talcum additions decreased average fungal pellet radius to 500 and 200 µm, respectively. It was also determined that addition of the same amount of talcum provided smaller fungal pellets compared to aluminum oxide added shake-flask fermentations.

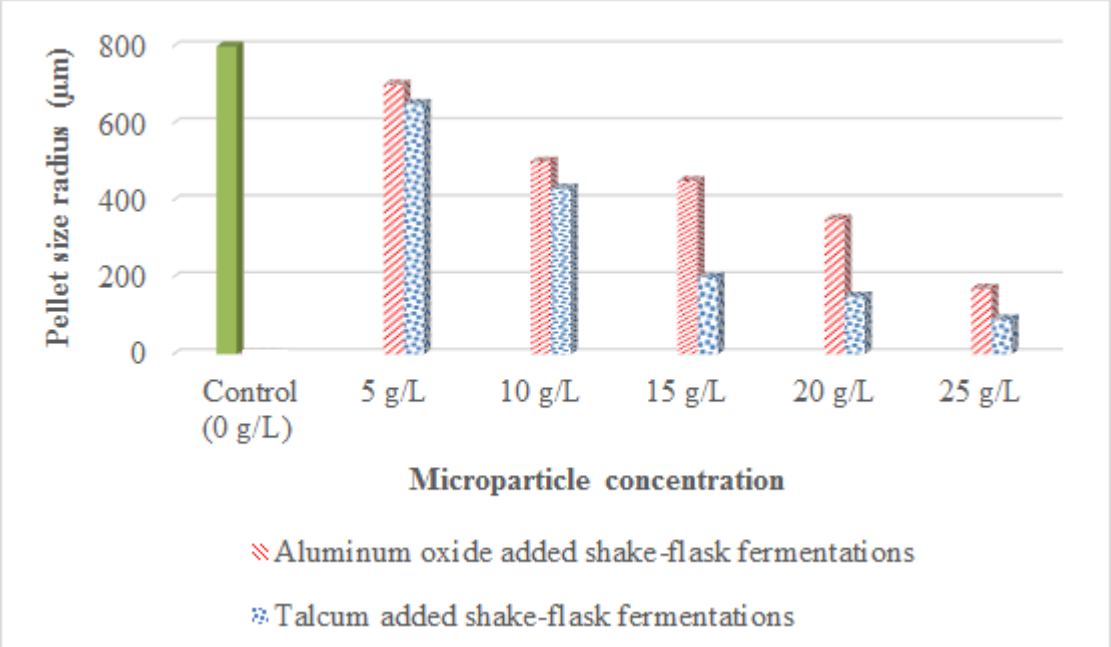

Figure 10.3. Effect of microparticles on *A. ficuum* pellet size.

This can be explained by the differences of mean particle sizes of these two microparticles. Since talcum has a smaller diameter compared to aluminum oxide particles, it provides smaller pellet sizes in the fermentation medium compared to aluminum oxide added fermentations (Figure 10.4).

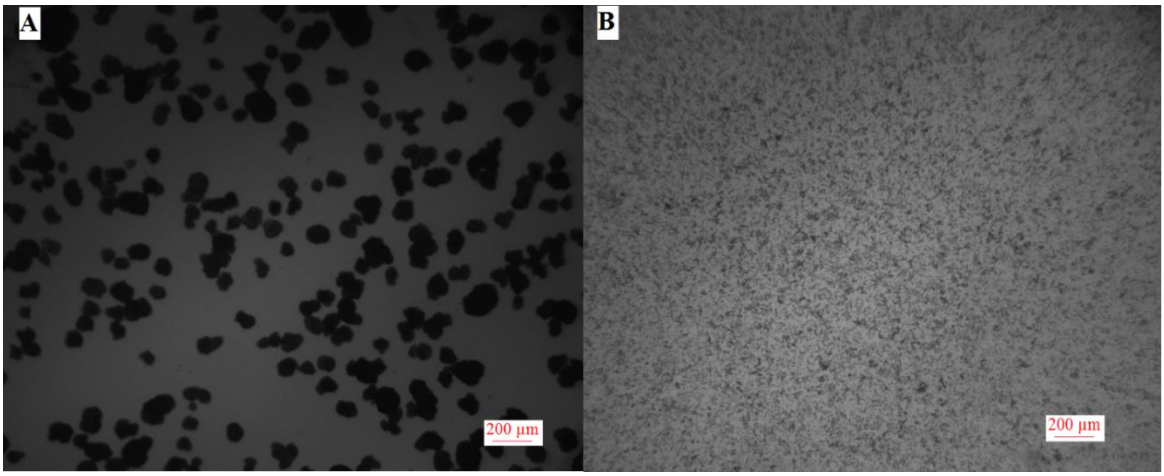

Figure 10.4. Size differences between two microparticles (A: Aluminum oxide, B: Talcum).

The average diameter of talcum and aluminum oxide particles were reported as 10 and 63-200 µm, respectively by the manufacturers. Driouch et al. (2011) also mentioned that the physical properties of the microparticles play an important role in fermentation yield and fungal morphology. They also mentioned that average talcum particles has 6µm diameter size, whereas alumina 14µm.

It was also determined that addition of higher than 15 g/L of microparticle negatively affected microbial growth. There were visually remarkable differences for the fungal growths with microparticle added and without in the shake-flask fermentations. This was also supported by pH changes based on the different levels of fungal growth in the shake-flasks. Initial pH was adjusted to 6.8 in shake-flasks and pH decreased to 3.29 in control flask at 72 h of fermentation. On the other hand, pH values were decreased to 3.49, 3.76, 4.08, 4.39, and 4.60 for 5, 10, 15, 20, and 25 g/L of talcum added shake-flask fermentations, respectively. Similarly, pH values were decreased to 4.07, 4.64, 4.82, 4.93, and 5.02 for 5, 10, 15, 20, and 25 g/L of aluminum oxide added shake-flask fermentations, respectively. Therefore, it can be concluded that while microparticles provide smaller pellet size and higher productivity, on the other hand in higher concentrations they can prevent microbial growth and decrease the yield of fermentations.

10.4.3. Fermentation in Bench-top Bioreactor with Talcum Addition

Since higher phytase production was obtained with addition of talcum compared to aluminum oxide in shake-flask fermentations, only talcum was studied for batch fermentation in bench-top bioreactor (Figure 10.5). The highest phytase activity was measured as 6.49 U/ml in batch fermentations by 15 g/L of talcum addition, whereas phytase activity in the control was only 3.45 U/ml. The maximum phytase activities were obtained as 4.32 and 5.00 U/ml in 5 and 10 g/L talcum additions, respectively. Also, similar to the shake-flask fermentation, greater than 15 g/L talcum addition reduced phytase activity; the maximum phytase activities were 4.00 and 3.56 U/ml at 20 and 25 g/L of talcum, respectively. Therefore, addition of 15 g/L of talcum increased phytase activity in bioreactor by 88% compared to the control in bioreactor. Also, it was observed that the maximum production rate of phytase increased to 0.08 U/ml/h for 15 g/L of talcum compared to 0.036 U/ml/h for the control. Similar to the shake-flask fermentation, the highest phytase activities were obtained at 96 h of all levels except for 5 g/L of talcum additions, while it was observed at 120 h of fermentation for the control (Figure 10.5).

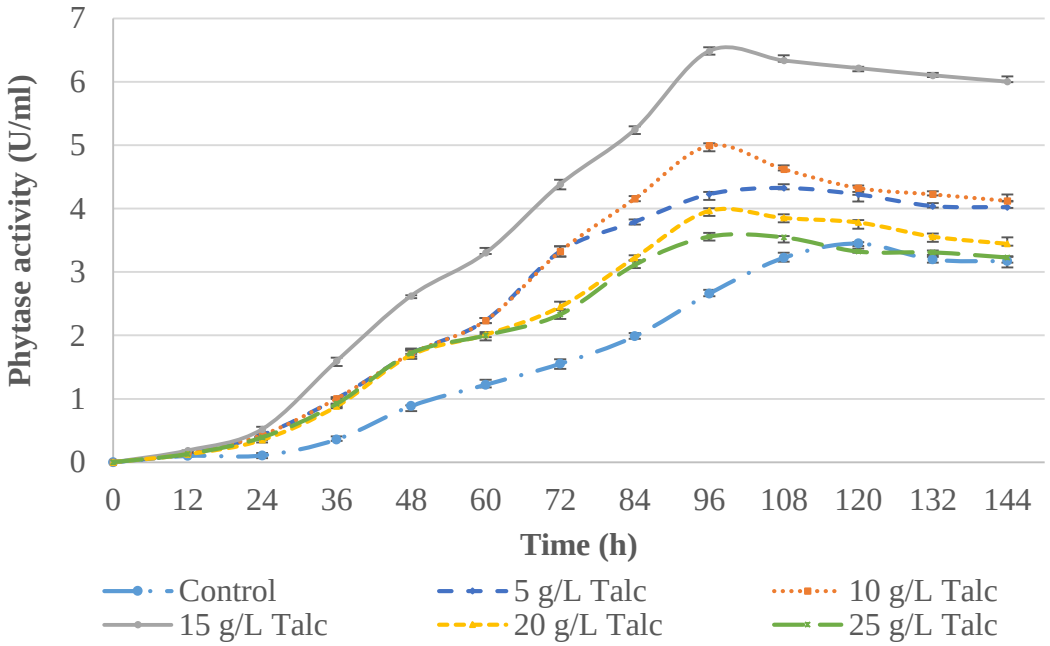

Figure 10.5. Effect of addition of talcum on phytase activity in batch fermentation.

Tukey test result showed that phytase activity significantly increased (p-value <0.05) with microparticle addition in shake-flask and bioreactor scale fermentations (Table 10.1). Mean values of phytase activities for batch bioreactor and shake-flask fermentations are grouped differently, which shows that mean values are significantly different among each other.

Table 10.1 One-way ANOVA and Tukey's statistical method evaluation of microparticle addition on phytase fermentation.

Source	DF	SS	MS	F	P
Factors	2	33.59040	16.79520	35989.71	0.000
Error	6	0.00280	0.00047		
Total	8	33.59320			

S = 0.02160 R-Sq = 99.99% R-Sq(adj) = 99.99%

Factors	N	Mean	Grouping
15 g/L talc added batch reactor fermentations	3	6.49	A
15 g/L talc added shake-flask fermentations	3	2.93	B
15 g/L aluminum oxide added shake-flask fermentations	3	2.01	C

10.5. Conclusions

In this study, the effects of aluminum oxide and talcum were studied on phytase production with *A. ficuum*. Phytase activity increased by addition of 15 g/L of aluminum oxide and talcum to 2.01 and 2.93 U/ml, respectively, compared to the control (1.02 U/ml) in shake-flask fermentations. Also, microparticle addition prevented bulk fungal growth in the fermentations. It was also determined that addition of talcum provided smaller fungal pellets compared to aluminum oxide because of smaller particle size. The highest phytase activity was measured as 6.49 U/ml by addition of 15 g/L of talcum in bioreactors. Additionally, it was found that microparticle addition decreased the time needed to reach the highest phytase activity from 120 h to 96 h. In conclusion, this study clearly demonstrated that fermentation of fungal productions with microparticles can enhance the phytase fermentation in fermentations.

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CHAPTER 11

PHYTASE PRODUCTION BY *ASPERGILLUS FICUUM* IN FED-BATCH AND CONTINUOUS FERMENTATION WITH MICROPARTICLE ADDITION

11.1. Abstract

Phytase is an important feed additive, which can be used in diets for several reasons. Phytase can increase the absorption of several divalent ions, amino acids, and proteins in bodies and reduce the excessive phosphorus release in the manure to prevent the negative effects on the environment. To date, microbial phytase production has been mostly produced by solid state fermentation with low production volumes. In our previous studies, phytase fermentation was studied in batch fermentation and enhanced with microparticle addition. The goal of this study was to evaluate fed-batch and continuous fermentation for *Aspergillus ficuum* phytase production with the addition of talcum as a microparticle. The results showed that phytase activity almost doubled in fed-batch and continuous fermentations by addition of 15 g/L of talcum compared to the control. It was also shown that talcum provided smaller fungal pellets and a more homogenous fermentation broth compared to the control. Additionally, average pellet radius decreased from 500 to 100 µm with the addition of 15 g/L of talcum in to the bioreactor. Furthermore, talcum addition increased phytase productivity and the optimum dilution rate in the continuous fermentation from 0.293 to 0.621 U/ml/h and from 0.09 to 0.1 h^{-1}, respectively, compared to the control. This study demonstrated that talcum addition significantly enhanced fungal phytase production in the fed-batch and continuous fermentation, which has merit for industrial scale production.

11.2. Introduction

Phytate is the major phosphate source in plants, which is generally used during germination for ATP synthesis (Vohra et al., 2003). However, phytate has very strong chelating properties, which can bind proteins, amino acids, and divalent ions such as Ca^{+2}, Mg^{+2}, Zn^{+2}, Cu^{+2}, Fe^{+2}, and Mn^{+2} in vivo and generating insoluble salts, that have negative effects on animal

and human health such as iron deficiency, bone weakness, tooth decay, and digestion problems (Haefner et al., 2005; Sanson et al., 1981). Additionally, monogastric animals such as chickens and pigs cannot utilize phytate, because they do not have the necessary microflora in their digestion systems (Mullaney et al., 2000). Since these monogastric animals are generally fed with phytate rich ingredients such as wheat, rice, and corn, excessive amount of phosphorus accumulates in their manure causes serious environmental problems such as water pollution, algal blooms, fish kills, and changing of fauna and flora (Mullaney et al, 2000). These problems can be overcome if the diets are supplemented with phytase to decrease phytate content of the feed. Positive effect of phytase application on animal growth and environment were shown in the literature. Sebastian et al. (1996) reported the increase in absorption of minor nutrients in the body by application of phytase in diets. They demonstrated that phytase supplementation increased the relative retention of total P^{-3}, Ca^{+2}, Cu^{+2}, and Zn^{+2} by 12.5, 12.2., 19.3, and 62.3%, respectively, in broilers. In another study, Nelson et al. (1968) used *Aspergillus niger* phytase to pretreat a corn–soybean diet for broilers and showed that the phosphorus availability increased by 60% and the phosphorus content decreased by 50% in the manure. Furthermore, phytase helped to improve body weight of male and female broilers by 13.2 and 5.8%, respectively, after 21 days on a diet supplemented with phytase. Also, Cowieson et al. (2012) reported the application of *A. niger* phytase in the animal diets and reduced phytate content by 35-40%.

Among the various microorganisms, molds are most commonly used for phytase production in the literature. Especially, *Aspergillus* species have been commonly used for phytase production. Shah et al. (2009) studied the optimization of the fermentation medium for phytase production with *A. niger* NCIM 563 in submerged batch fermentations and doubled phytase activity compared to initial values. In another study, Bhavsar et al. (2013) produced phytase with *A. niger* NCIM 56 and reported a 6-fold increase in phytase activity after performing mutations on the microorganism and modifications on the fermentation medium. In our previous studies, phytase activity generating from *A. ficuum* was measured as 1.02 U/ml in shake-flask fermentation. Phytase activity was increased to 2.27 U/ml and 3.45 U/ml in batch fermentations after optimization of growth parameters and fermentation medium, respectively (Chapter 7 and 8). Phytase production was also studied in fed-batch and continuous fermentations in the literature. For example, Jin et al. (2007) set an artificial neural network pattern recognition (ANNPR) model for fed-batch production with recombinant *Pichia pastoris* phytase. In their study, they maintained the methanol concentration in the reactor between 0-20 g/L to induce cell growth and phytase production. They reported that phytase activity increased more than four-fold

compared to their previous studies. In another fed-batch phytase production was studied by Kleist et al. (2003) with *Escherichia coli*. They enhanced the production by keeping the glucose concentration constant at low oxygen levels. They reported 120 U/ml phytase production in a short fermentation time (14 h) under %5-10 oxygen concentrations. Additionally, Hidayat et al. (2006), studied continuous acid phosphatase production by *A. niger* N402A. They showed that *A. niger* produced the highest specific acid phosphatase activity at 0.04 h^{-1} dilution rate as 150 U/g. However, the highest specific acid phosphatase productivity was measured as 7.5 U/g/h at 0.13 h^{-1} dilution rate.

Although *Aspergillus* species are commonly used for phytase production, the cultivation of filamentous microorganisms is accompanied by several problems, such as clumpy growth and insufficient mass transfer, which may result in reduced productivity. Recently, microparticles have gained attention to overcome these problems. Cultivation of filamentous microorganisms with microparticles has been implemented to ensure a more precise control of filamentous microorganism morphology and increase the overall yield of the process (Kaup et al., 2008; Walisko et al., 2012). There are several studies in the literature reporting the yield improvement in fungal fermentations with microparticles. Driouch et al. (2012) studied the effect of addition of $TiSiO_4$ on fructofuranosidase and glucoamylase production with *A. niger*. They reported that 25 g/L of $TiSiO_4$increased fructofuranosidase and glucoamylase productions by 3.7 fold and 9.5 fold, respectively, compared to control suspended cell fermentations in shake-flasks. Furthermore, the fungal pellet diameter was reduced from 1.7 mm to 0.3 mm, when 25 g/L concentration of $TiSiO_4$ was added to the medium (Driouch et al., 2012). Additionally, Driouch et al. (2011) enhanced *A. niger* fructofuranosidase activity by 3.5 fold in the presence of 10 g/L talcum or 20 g/L aluminum oxide in the fermentation medium compared to the control. Similarly, the merits of aluminum oxide and talcum microparticles were shown by Kaup et al. (2008) for chloroperoxidase production by *Caldariomyces fumago*, whichincreasedfive folds compared to the control group. Also, in our previous study *A. ficuum* phytase production increased significantly with theaddition of 15 g/L talcum from 1.02 to 2.93 U/ml in shake-flask and from 3.45 to 6.49 U/ml in bioreactor fermentation (Chapter 10). Therefore, the aim of this study was to further enhance *A. ficuum* phytase production by the addition of talcum microparticles in fed-batch and continuous fermentations and compare with the control fermentation.

11.3. Matherials and Methods

11.3.1. Microorganism

Aspergillus ficuum NRRL 3135 was recommended as the best microorganism for phytase production in our previous study (Chapter 7), which was obtained from USDA Agricultural Research Service Culture Collection (Peoria, IL). It was grown on potato dextrose agar (PDA), (Difco, Sparks, MD) slants for 6 days at 30^{o}C and stored at 4^{o}C as the working culture. In order to maintain viability, *A. ficuum* wastransferred to sterile fresh agar slant bi-weekly.

11.3.2. Inoculum Preparation

A. ficuum spores were grown on 25 PDA plates for 6 days at 30°C. After incubation, spores were suspended by adding 7 ml of sterile 0.1% peptone water and the resulting solution (~10^6 spores/ml) was collected and used as the inoculum.

11.3.3. Phytase Fermentations

Fed-batch and continuous fermentations were performed in Sartorius Biostat B Plus bioreactors (Sartorius, Allentown, PA), equipped with a 2-L vessel with 1-L working volume. The base fermentation medium consisted of 126 g of glucose, 0.5 g of KCl, 0.1 g of $FeSO_4(7H_2O)$, 0.5 g of $MgSO_4(7H_2O)$, 0.01 g of $MnSO_4(7H_2O)$, 8.6 g of $NaNO_3$, 3 g $(NH_4)_2SO_4$, 1.1 g $CaSO_4$, and 14 g of Na-phytate (A&Z Food Additives Co. Ltd., Zhejiang, China) per litter of deionized water, as suggested by our previous study (Chapter 8). For the experimental treatment group, 15 g/L talcum (Sigma Aldhrich, St. Louise, MO) was added, which was also determined as the optimum concentration from our previous study (Chapter 10) and autoclaved for 30 min. Reactors were inoculated with 3% prepared inoculum and fermentations were run at 33^{o}C, pH 4.5, 0.9 vvm aeration, and 300 rpm agitation (Chapter 7).

11.3.4. Fed-batch Fermentations

Fed-batch phytase fermentations were carried out to study the effect ofglucose and phytate additions on phytase production. Based on previous studies, four fed-batch addition points were selected to evaluate, which are, 48, 72, 96, and 120 h of fermentation (Chapter 8).

Glucose concentrations were targeted to increase to the initial concentration level while 10 g of phytate was added into the fermentation medium at these selected times. Therefore, based on glucose consumption data; 30, 45, 60, and 80 g of glucose and 10 g of phytate were dissolved in 100 ml of DI water. Then, pH was adjusted to 4.5, autoclaved and then transferred into the reactors. Samples were collected (2 ml) from the reactors every 24 h for 9 days and analyzed for phytase activity

11.3.5. Continuous Fermentation

Continuous fermentation was set up to have a chemostat system for phytase production. Various dilution rates between 0.03 to 0.13 h^{-1} were evaluated for phytase activity. Fermentation was started as a batch until late log phase, then the system was switched to continuous fermentation by turning on the inlet and outlet pumps at the specified dilution rates. Samples were collected at steady-state conditions and analyzed for phytase activity.

11.3.6. Phytase Activity Analysis

Samples were centrifuged at 5,200 x *g* for 15 min (Galaxy 5D, VWR, Radnor, PA) to remove the biomass, then the supernatant was used for the phytase activity analyses. The enzyme assay was performed under the temperature and pH conditions as described by Kim et al. (1998) with minor modifications. Briefly, cell-free broth (0.125 ml) was mixed with 0.125 ml of a 1.5 mM Na-phytate in 0.1 M sodium acetate solution and mixtures were incubated in a water bath at 55ºC for 30 min. After incubation, the reaction was stopped by adding 0.25 ml of 15% tricholoroacetic acid solution into the tubes. Then, 2 ml of color regent was added, which was prepared freshly with; 2:1:1:1 ratio of water: 2.5 % ammonium molybdate: 6 N H_2SO_4: 10 % ascorbic acid and tubes were incubated at 55ºC for 30 min. After coolingto room temperature, absorbance was measured at 700 nm by using a spectrophotometer (Beckman Coulter, Fullerton, CA). Uninoculated fermentation medium was used as the blank for the measurement. Thedata was used to calculate the activity phytase (U/ml), which was defined as the μmole of phosphorus liberated from 1.5 mM phytate per min under the set assay conditions.

11.3.7. Glucose Analysis

Glucose concentrations were measured using high pressure liquid chromatography (HPLC) with a refractive index detector (Waters, Milford, MA). An Aminex HPX-87H column (Bio-Rad, Richmond, CA) was used with a 0.8 ml/min isocratic flow of 0.012 N sulfuric acid. The detector and column temperature were maintained at as 35 and 65ºC, respectively. The cell-free samples were filtered by using 13 mm diameter, 0.2 μm pore sized nylon filters (PALL Life Sciences, Port Washington, NY).

11.3.8. Microbial Imaging

Biomass samples were collected from the bioreactors at 72 h of fermentation before fed-batch additions and were washed with 0.9% NaCl solution to remove excessive microparticles and medium from the pellets. Thereafter, selected average sizes of pellets from the each sample were visually analyzed using a light microscope (1242MM, Van Guard, Kirkland, WA).

11.4. Results and Discussion

11.4.1. Effect of Talcum Microparticles on Phytase Activity in Fed-batch Fermentations

Figure 11.1 shows the effect of glucose and phytate addition on *A. ficuum* phytase activity without addition of talcum. It was shown that addition of glucose and phytate increased the phytase activity in all fed-batch fermentations. The maximum phytase activity was almost obtained at 96 h of fermentation and slightly increased till 120 h of fermentation in 48 h added fermentations. Similarly, maximum phytase activities were determined at 120 h of fermentation for control and 72 h added fermentations. However, the maximum phytase activities were measured at 144 h of fermentation for 96 and 120 h added fed-batch fermentations. The highest phytase activity was measured as 4.912 U/ml in 96 h of addition reactor among all fed-batch fermentations without talcum addition. Additionally, phytase productivity increased to 0.054 from 0.035 U/ml/h in 96 h addition reactor compared to the control fermentation. Furthermore, it was calculated that addition of glucose and phytate in early stages, increased phytase activities higher compared to late stages. Phytase activities increased by 281, 186, 78, and 26% after 24 h of addition in 48, 72, 96, and 120 h of fed-batch fermentations, respectively.

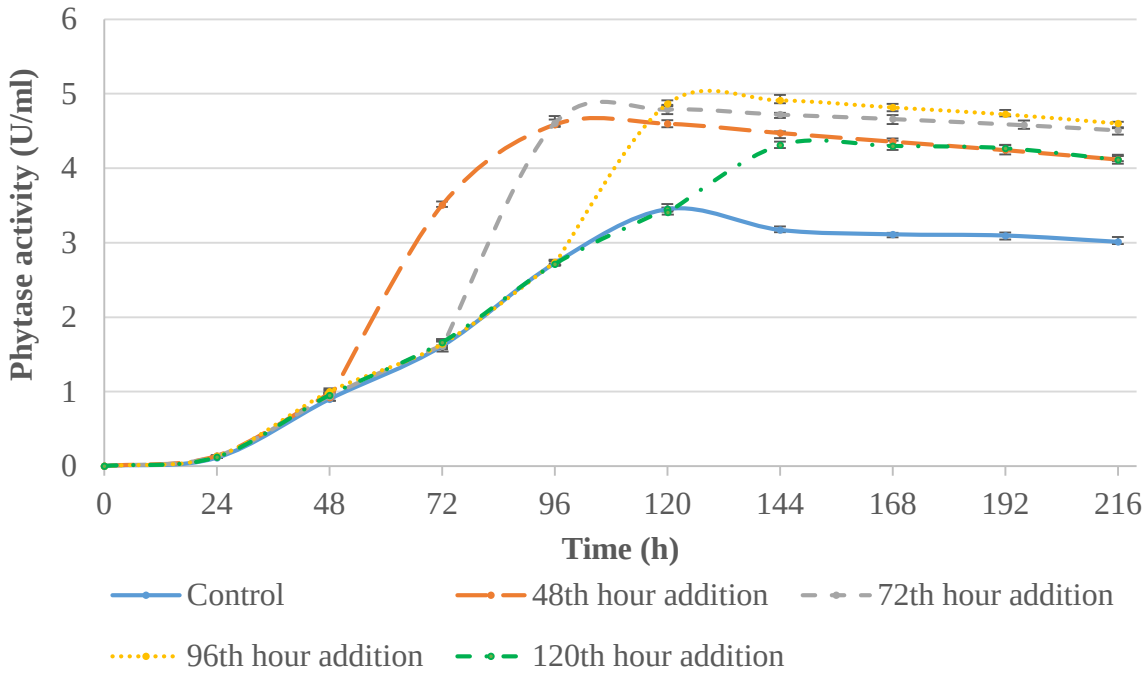

Figure 11.1. Effect of fed-batch glucose and phytate addition on phytase activity without talcum addition.

On the other hand, addition of 15 g of talcum further increased phytase activity in fed-batch bioreactors (Figure 11.2). The maximum phytase activities were measured at 120 h of fermentation for control, 48, and 72 h added fed-batch fermentations, whereas the maximum phytase activities were determined at 144 h of the fermentation for 96 and 120 h added fermentations. The highest phytase activity was measured as 9.587 U/ml in 72 h of addition fed-batch reactors, which is 95% higher than the maximum phytase activity in not talcum added fed-batch fermentations.

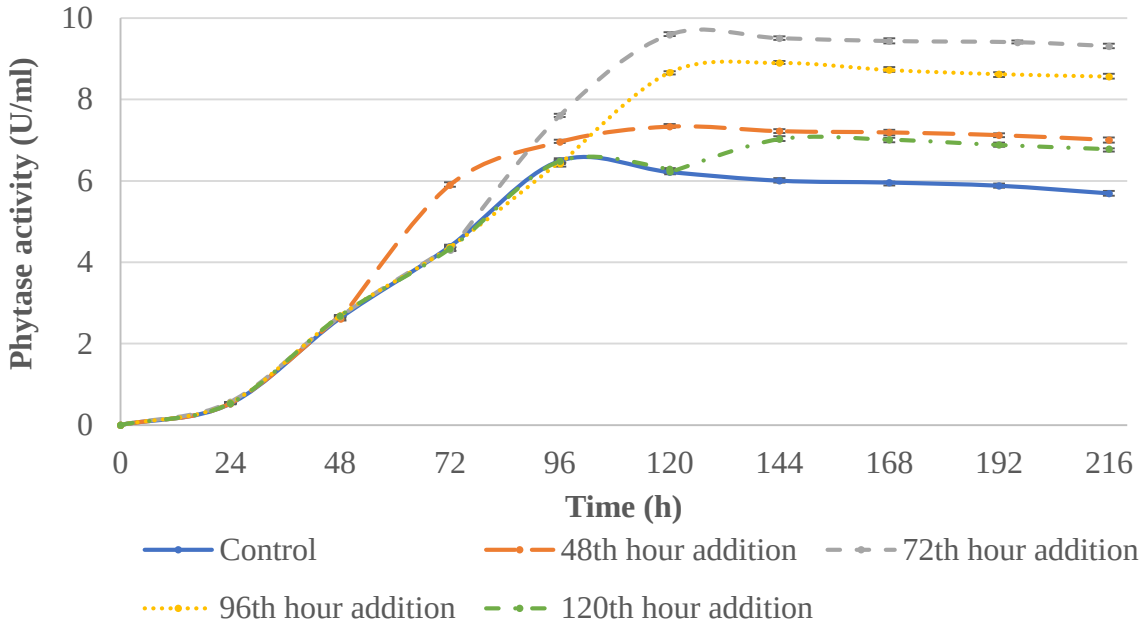

Figure 11.2. Effect of fed-batch glucose and phytate addition on phytase activity with talcum addition.

Additionally, it was observed that the maximum phytase activity was measured at 96 h of fermentation in control fermentations, whereas the maximum phytase activity was obtained at 120 h of fermentation, when talcum was not used. This can be explained by that talcum addition provided smaller pellet structures and better mass transfer in the fermentation compared to control fermentation (Figure 11.3). Tukey test also showed that 15 g/L talcum addition significantly (p-value<0.05) increased phytase activity in fed-batch fermentations (Table 11.1).

Table 11.1 One-way ANOVA and Tukey's statistical method evaluation of microparticle addition on fed-batch phytase fermentation.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factors	1	32.6433	32.6433	318471.59	0.000
Error	4	0.0004	0.0001		
Total	5	32.6437			

S	R-sq	R-sq(adj)	R-sq(pred)
0.0101242	100.00%	100.00%	100.00%

Factors	N	Mean	Grouping
15 g/L talcum added fed-batch fermentations	3	9.577	A
Fed-batch fermentations without talcum addition	3	4.912	B

Talcum addition also increased phytase productivity to 0.109 U/ml/h, compared to control fermentation (0.083 U/ml/h). Furthermore, increases in phytase activities after 24 h of addition were calculated as 126, 76, 35, and 13% in 48, 72, 96, and 120 h of fed-batch addition reactors, respectively. It was shown that early stage addition of glucose and phytate increased phytase activity more compared to late stage additions. This can be explained by higher microbial activity in earlier stage of fermentation compared to late stage. Our previous study also showed that phytase activity reaches the maximum level at 120 h of fermentations in batch fermentations and activity decreases in further time of fermentations because of microorganisms go into death phase (Chapter 8). Also, it was shown that talcum addition decreased the phytase activity lose after reaching the highest point. It was observed that phytase activity decreased by 6.31% after 72 h of reaching the maximum value in 96 h addition bioreactor without talcum addition, whereas this value was observed as 1.92% in 72 h of fed-batch fermentation with talcum addition.

11.4.2. Effect of Addition of Talcum on Fungal Morphology in Bioreactors

Effect of addition of 15 g/L talcum on fungal pellet morphology was shown in Figure 11.3. Figures 11.3A and 11.3B demonstrate the fungal pellet size without and with 15 g/L talcum added bioreactors at 72 h of fermentation, respectively. Average pellet radius length was measured as 500 µm, when talcum was not used (Figure 11.3A). However, addition 15 g/L of talcum into the medium decreased average pellet radius length around 100 µm (Figure 11.3B).

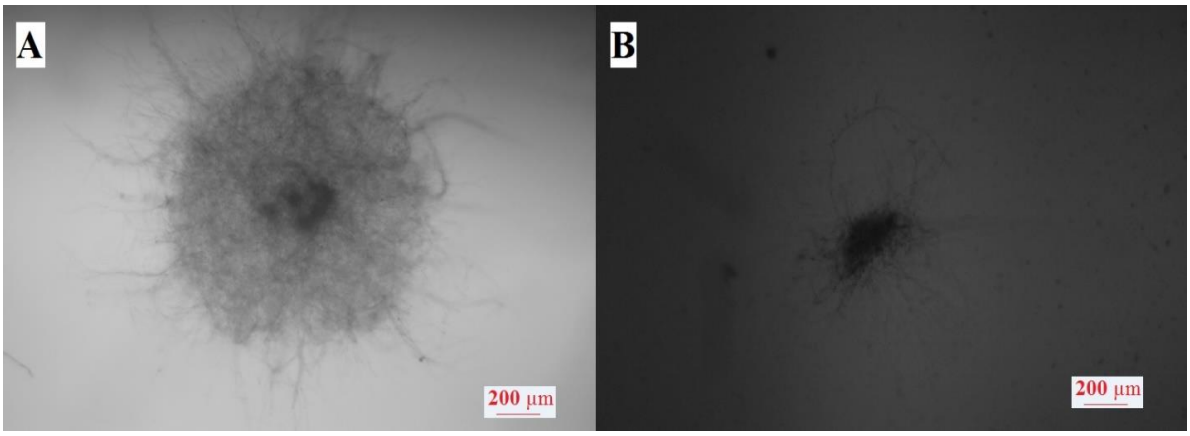

Figure 11.3. A: Fungal pellet structure at 72 h of fermentation without talcum addition. B: Fungal pellet structure at 72 h of fermentation with 15 g/L talcum addition.

It was also found that thefermentation medium was more homogenous in talcum added bioreactors compared to the control fermentation in further fermentation hours. The microbial growth was homogenously dispersed in the fermentation medium when talcum was added reactors, whereas a clumpy fungal growth accumulation was observed, when talcum was not used. It was also determined that addition of 15 g/L talcum resulted in smaller fungal pellet structures in bioreactors, compared to shake-flask experiments in the previous study (Chapter 10). This can be explained by the effective aeration and mechanical agitation in the bioreactor providing more interaction between the microorganism and the talcum particles, which resulted in smaller fungal structures.

11.4.3. Effect of Talcum on Phytase Activity in Continuous Fermentations

Continuous fermentations, which did not include talcum were run under various dilution rates. Figure 11.4 shows the process conditions under various dilution rates.

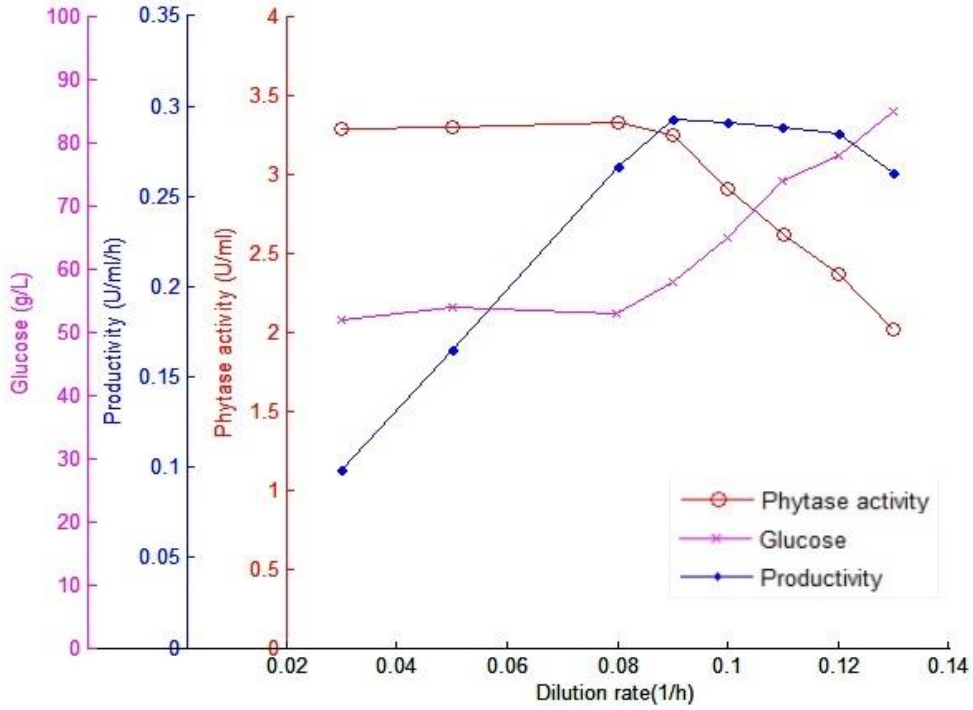

Figure 11.4. Production of *A. ficuum* phytase in continuous fermentation.

Phytase activity and glucose concentrations remained almost the same around 3.3 U/ml and 53 g/L, when dilution rates were between 0.03-0.08 h^{-1}, respectively. However, at higher dilution rates, phytase activity decreased dramatically and glucose concentration increased because of the washout effect. Additionally, the highest phytase productivity was obtained at 0.09 h^{-1} dilution rate as 0.293 U/ml/h, which is 8 fold higher compared to batch fermentations.

Similar to fed-batch results, talcum addition also enhanced phytase activity in continuous fermentations too. Figure 11.5 shows the continuous production of *A. ficuum* phytase under various dilution rates.

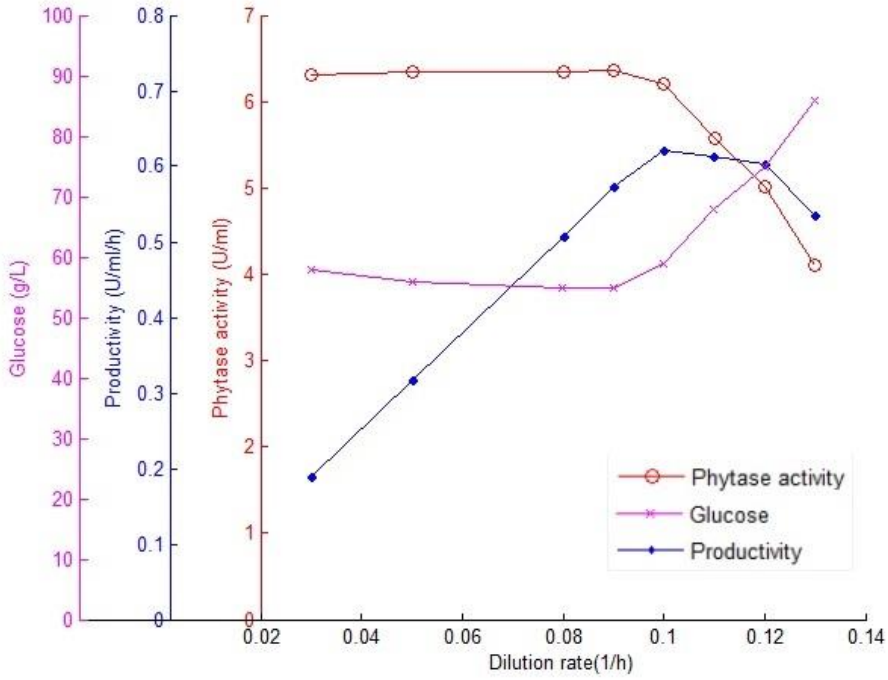

Figure 11.5. Production of *A. ficuum* phytase in continuous fermentation with talcum addition.

Phytase activity remained around 6.3 U/ml until 0.09 h^{-1} dilution rates and decreased significantly at higher dilution rates. Similarly, glucose concentration increased in the fermentation broth at higher dilution rates than 0.09 h^{-1}. These dilution rates were also reported for an *Aspergillus* related fermentation by Hidayat et al. (2006). They reported that acid phosphatase production with *A. niger* N402A can be performed between 0.07 and 0.13 h^{-1} dilution rates in continuous fermentations. Talcum addition enhanced the maximum phytase activity by 90% compared to continuous fermentations without talcum. Furthermore, phytase productivity increased to 0.621 U/ml/h, which is around 6 and 18 times higher compared to fed-batch and batch fermentations, respectively.

11.5. Conclusions

In this study, the effects of talcum microparticle addition on fed-batch and continuous *Aspergillus ficuum* phytase production were studied. It was shown that addition of 15 g/L talcum um enhanced phytase activity both in fed-batch and continuous fermentations remarkably.

Phytase activity increased from 4.912 to 9.587 U/ml and from 3.3 to 6.3 U/ml in fed-batch and continuous fermentations, respectively with addition of 15 g/L talcum in the fermentation medium. It was also found out that addition of talcum in the fermentation medium decreased fungal pellet size and time to reach the maximum phytase activity in the bioreactor. Furthermore, phytase productivities increased from 0.054 to 0.109 U/ml/h and from 0.293 U/ml/h to 0.621 U/ml/h in fed-batch and continuous fermentations, respectively. Also, addition of talcum increased the optimum dilution rate from 0.09 to 0.1 h^{-1} in continuous fermentations. Overall, this study showed that the addition of talcum microparticles can be used to enhance phytase production with *A. ficuum* in bioreactors, which is an encouraging step forward for the commercial production of phytase by submerge fermentation.

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CHAPTER 12

MODELING OF PHYTASE FERMENTATION WITH *ASPERGILLUS FICUUM*

12.1. Abstract

Phytase, is an important feed and food additive, which is both used in animal and human diets. Phytase has been used to increase the absorption of several divalent ions, amino acids, and proteins in the bodies and to decrease the excessive phosphorus release in the manure to prevent negative effects on the environment. In our previous study, 3.45 U/ml phytase was produced with *Aspergillus ficuum* after optimization of growth and medium parameters in the batch fermentation. Mathematical models of phytase production and glucose consumption can provide the kinetic-metabolic nature of the process. In this study, the modified Gompertz model and modified logistic model were used to predict phytase production and glucose consumption by *Aspergillus ficuum*. Goodness of fit was measured by calculation R^2, slope, MAE and RMSE values. It was shown that the modified Gompertz model provided slightly smaller RMSE and MAE values compared to the modified logistic model for phytase prediction. However, higher MAE and RMSE values were obtained from the modified Gompertz model (8.158 and 9.693) for the glucose consumption prediction compared to the modified logistic model (4.648 and 8.870). Overall, this study demonstrated that modeling of phytase production can provide better understanding of fermentation kinetics and sets the stage for scale up to industrial production.

12.2. Introduction

Phytate, which is the major phosphate source in plants can cause several negative effects on animal and human health. Phytate is a very strong chelating agent, which binds proteins, amino acids, and divalent ions such as Ca^{+2}, Mg^{+2}, Zn^{+2}, Cu^{+2}, Fe^{+2}, and Mn^{+2} and decreases the absorption in vivo (Haefner et al., 2005). Therefore, phytatein the diet may cause some several health problems such as iron deficiency, bone weakness, tooth decay, and digestion problems (Hurrel et al., 2003; Sanson et al., 1981). Additionally, monogastric animals such as chickens and pigs are not able to break down and utilize phytate. Therefore accumulation of excessive

phosphorus in the manure causes environmental problems such as water pollution, algal blooms, fish kills, and changing of fauna and flora (Mullaney et al., 2000). Application of phytase into the diets can overcome these problems. Nelson et al. (1968) reported that application of phytase into broilers diets increased phosphorus availability by 60% and reduced in manure by 50%. Phytase addition also helped to improve body weight of male and female broilers by 13.2 and 5.8%, respectively after 21 days on a diet supplemented with phytase (Nelson et al. 1968). Furthermore, Sebastian et al. (1996) reported that phytase supplementation increased the relative retention of total P^{-3}, Ca^{+2}, Cu^{+2}, and Zn^{+2} by 12.5, 12.2., 19.3, and 62.3%, respectively, in broilers.

Several studied are available in the literature about phytase productions with various microorganisms. Mittal et al. (2011) reported production of phytase using orange peels as phytate source with *Klebsiella sp*. DB3. They reported that phytase activity increased from 0.6 to 3.15 U/ml, when 2% orange peel bran was used in the fermentation medium. Similarly, Lan et al. (2002) used rice bran as phytate source in the fermentation medium. They reported that increase the amount of rice bran in the fermentation medium from 5 to 20% doubled phytase activity. In another study, Sasirekha et al. (2012) studied effect of several carbon sources on phytase production by *Pseudomonas* sp. They reported 0.727, 0.739, 0.724, and 0.704 U/ml phytase activity, when glucose, sucrose, and maltose was used as carbon source in the fermentation medium, respectively. In our previous study, *Aspergillus ficuum* phytase activity was obtained as 1.02 U/ml in shake-flask production. Phytase activity was increased to 2.27 U/ml by optimizing the growth parameters in 1-L working volume bioreactors (Chapter 7). Further, optimization of the fermentation medium and fed-batch fermentation studies increased phytase activity to 3.45 and 4.82 U/ml, respectively (Chapters 8 and 9).

Mathematical models are important tools to provide information about product formation and substrate consumption to facilitate the control of the fermentation processes. The modified Gompertz and modified logistic models are the most commonused in the literature to predict microbial biomass and production formation. Dufosse et al. (2001) reported that the Gompertz model successfully predicted the effect of nitrogenous substrates on microbial growth. Also, Zhao et al. (2014) studied the modeling of the effect of various temperatures on *Proteus mirabilis* growth. They reported that the Gompertz and logistic models predicted the experimental data successfully with low mean error square values (MSE). Similarly, the Gompertz model and logistic model were used by Pongtharangkul et al. (2008) and Lv et al. (2005) for nisin production. Currently, there is no study available in the literature modeling phytase productions

for submerged fermentation. Therefore, this study has been undertaken to model phytase production and glucose consumption by *A. ficuum* in bioreactors.

12.3. Materials and Methods

12.3.1. Microorganisms and Inoculum Preparation

Aspergillus ficuum (NRRL 3135) was used in this study as suggested by our previous study (Chapter 7). *A. ficuum* was obtained from Agricultural Research Service Culture Collection (Peoria, IL) and grown on potato dextrose agar (PDA) (Difco, Sparks, MD) slants for 6 days at 30^{o}C and stored at 4^{o}C as the working culture. In order to maintain viability, *A. ficuum* was regularly transferred to sterile fresh agar slant biweekly. Inoculum preparation was done by growing *A. ficuum* spores on 25 PDA plates for 6 days at 30°C. After incubation, spores were suspended by adding 7 ml of sterile 0.1% peptone water to each plate and the resulting solution (~10^6 spores/ml) was collected and used as the inoculum.

12.3.2. Batch Fermentation in Bioreactors

Batch fermentation was run in a Sartorius Biostat B Plus bioreactor (Allentown, PA) equipped with a 2-L vessel with 1-L working volume. The reactor was inoculated with 3% prepared inoculum andfermentation runs were performed at 33^{o}C, 4.5 pH and 0.9 vvm aeration conditions (Chapter 7). As suggested by our previous study, the medium consists of 126 g of glucose, 0.5 g of KCl, 0.1 g of $FeSO_4(7H_2O)$, 0.5 g of $MgSO_4(7H_2O)$, 0.01 g of $MnSO_4(7H_2O)$, 8.6 g of $NaNO_3$, 3 g $(NH_4)_2SO_4$, 1.1 g $CaSO_4$, and 14 g of Na-phytate (A&Z Food Additives Co. Ltd., Zhejiang, China) per litter of deionized water (Chapter 8). Agitation was maintained at 300 rpm for all fermentation runs.

12.3.3. Analysis

Samples were centrifuged at 5,200 x *g* for 15 min to remove the biomass. Then, the supernatant was used for phytase activity and glucose concentration analyses.

12.3.3.1. Phytase Activity

The enzyme assay was performed under theoptimum temperature and pH as described in the literature with minor modifications (Kim et al., 1998). Briefly, cell-free broth (0.125 ml) was mixed with 0.125 ml of 1.5 mM Na-phytate in 0.1 M sodium acetate solution and mixtures were incubated in a water bath at 55ºC for 30 min. After incubation, the reaction was stopped by adding 0.25 ml of 15% tricholoroacetic acid solution into the tubes. Then, 2 ml of color regent was added, which was prepared freshly with; 2:1:1:1 ratio of water: 2.5 % ammonium molybdate: 6 N H_2SO_4: 10 % ascorbic acid and tubes were incubated at 55ºC for 30 min. After cooling to room temperature, absorbances were measured at 700 nm using a spectrophotometer (Beckman Coulter, Fullerton, CA). Uninoculated fermentation medium was used as the blank for the measurement fed-batch glucose addition fermentations. The data obtained was used to calculate the activity unit of phytase (U/ml), which was defined as μmole of phosphorus liberated from 1.5 mM phytate per min under the set assay conditions.

12.3.3.2. Glucose Concentration

Glucose concentrations were measured using high pressure liquid chromatography (HPLC) with a refractive index detector (Waters, Milford, MA) and an Aminex HPX-87H column (Bio-Rad, Richmond, CA) with a 0.8 ml/min of 0.012 N sulfuric acid. The detector and column temperature were maintained at as 35 and 65ºC, respectively. The cell-free samples were filtered using a 13 mm diameter, 0.2 μm pore sized filter (PALL Life Sciences, Port Washington, NY).

12.3.3.3. Mathematical Models

Phytase is a primary metabolite, which usually exhibits a sigmoid production pattern with a lag, exponential, and stationary phase. Therefore, the modified Gompertz (Equation 12.1) and modified logistic models (Equation 12.2) were used to predict phytase production and glucose consumption during the fermentation. Zwietering et al. (1990) reported the modified Gompertz and modified logistic equations as below.

$$A_t = A_m \exp\left\{-\exp\left[\frac{B_m e}{A_m}(\lambda - t) + 1\right]\right\}$$ .....**Equation 12.1**

$$A_t = \frac{A_m}{\left\{1+\exp\left[\frac{4B_m}{A_m}(\lambda - t)+2\right]\right\}}$$ .....**Equation 12.2**

where A_t= The concentration of the studied factor at "t" time

A_m= The maximum concentration of the studied factor

B_m= The production or consumption rate of the studied factor

λ= Duration of lag phase (h)

t= Time of the sampling

All these parameters were determined from the batch fermentation, which was run under the optimum conditions as described in Chapter 8. Maximum phytase activity at the end of the log phase and maximum glucose concentration at the beginning of lag phase were assigned A_m. B_m values were obtained by the measurement of the maximum production or consumption rate of the factors. λ was calculated by the measurement of the time to reach the log phase.

12.3.3.4. Validation of the Mathematical Models

Predicted data from the modified Gompertz and modified logistic models were compared with the experimental data by calculation of root mean square error (RMSE), mean absolute error (MAE). Also, the R^2 and slope were evaluated by plotting predicted versus experimental data.

12.4. Results and Discussion

12.4.1. Modeling of Phytase Production

Experimental phytase production with *A. ficuum* was modeled using the modified Gompertz and modified logistic models (Figure 12.1). Both models predicted phytase production better in lag and stationary phases compared to exponential phase (Figure 12.1). However, the

modified logistic model showed higher over-prediction compared to modified Gompertz model in the first 36 h of fermentation. Predictions of both modified Gompertz model and modified logistic model were higher that experimental data for all exponential phase. Both models under-predicted the experimental phytase data in only the stationary phase (120 h of fermentation). Maximum phytase activity (3.45 U/ml) was predicted as 3.27 and 3.37 U/ml for modified Gompertz and modified logistic models, respectively.

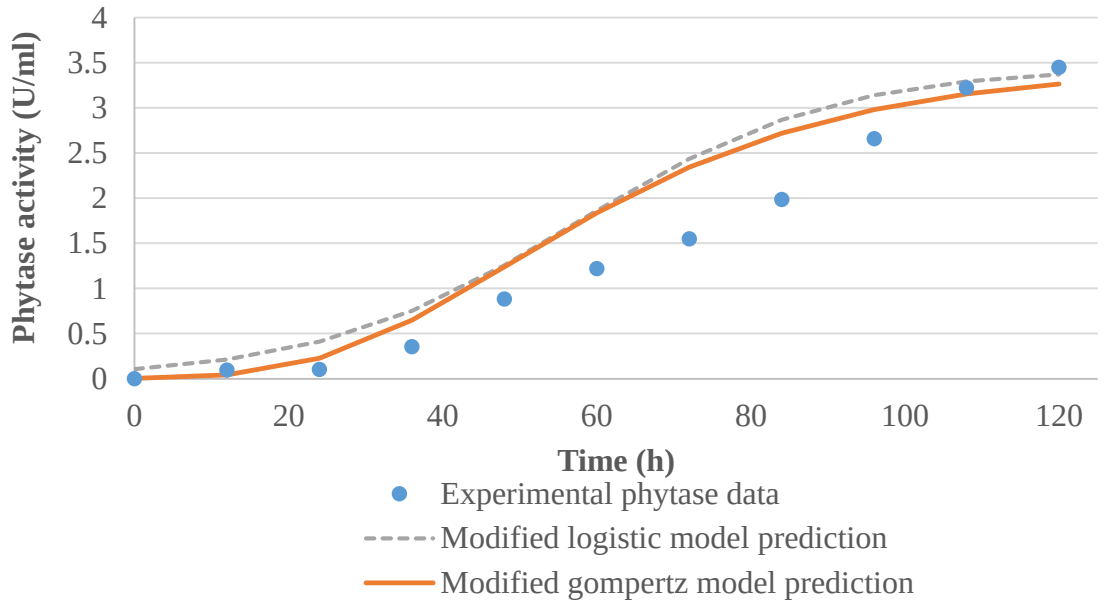

Figure 12.1. Phytase production with *A. ficuum* fitted with the modified Gompertz model and modified logistic model.

The values for RMSE, MAE, R^2, and slope from models are shown in Table 12.1. All good of fitness evaluations were close to each other for the both models. However, based on the error values, it can be seen that modified Gompertz model predicted *A. ficuum* phytase production slightly better compared to modified logistic model. RMSE and MAE values were calculated as 0.488 and 0.379 for modified logistic model, whereas they were 0.417 and 0.265 for modified Gompertz model, respectively.

Table 12.1. RMSE, MAE, R^2, and slope values about phytase production prediction for modified logistic and modified Gompertz models.

Model	RMSE (U/ml)	MAE (U/ml)	R^2	Slope
Modified logistic	0.488	0.379	0.9378	0.9850
Modified Gompertz	0.417	0.265	0.9323	0.9833

12.4.2. Modeling of Glucose Consumption

Glucose consumption with *A. ficuum* was also modeled using the modified Gompertz and modified logistic models (Figure 12.2). The modified logistic model predicted experimental glucose data low for the first 24 h of the fermentation then over-predicted the rest experimental data. On the other hand, the modified Gompertz model predicted the experimental glucose data low for the first 12 h of fermentation and then over-predicted rest of the experimental results. Both models gave very close predictions to each other after 72 h of the fermentation data. The initial glucose concentration (125.6 g/L) was better predicted by the modified Gompertz model (123 g/L) compared to modified logistic model (116 g/L). However, both models predicted the final glucose concentration as 60 g/L at 120 h of the fermentation, whereas the experimental data was 47 g/L.

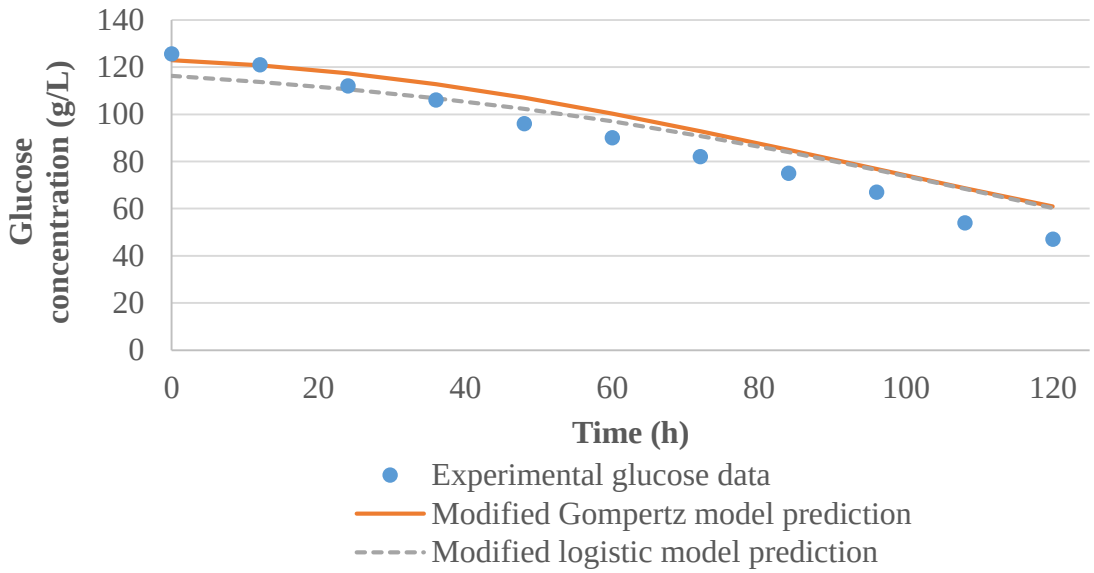

Figure 12.2. Glucose consumption fitted with the modified Gompertz model and modified logistic model.

These goodness of fit parameters for glucose consumption from both models are shown in Table 12.2. Higher RMSE and MAE values were obtained with modified Gompertz model (9.693 and 8.158) compared modified logistic model (8.868 and 4.648), respectively. On the other hand, higher R^2 and slope values were calculated for the modified Gompertz model compared to modified logistic model.

Table 12.2. RMSE, MAE, R^2, and slope values about glucose consumption prediction for modified logistic and modified Gompertz models.

Model	**RMSE (g/L)**	**MAE (g/L)**	**R^2**	**Slope**
Modified logistic	8.868	4.648	0.9833	0.714
Modified Gompertz	9.693	8.158	0.9866	0.815

12.5. Conclusions

In this study, phytase production and glucose consumption were predicted with the modified Gompertz and modified logistic models. It was determined that the modified Gompertz model predicted phytase activity slightly more successfully compared to modified logistic model. Although lower MAE and RMSE values were obtained for glucoseprediction by the modified logistic model, higher R^2 and slope values were obtained with the modified Gompertz model. Overall, these models can be used for further development of phytase production and scale-up of fermentations.

Nomenclature

Parameters used in the models	Symbols	Value	Unit
Maximum specific phytase production rate	B_m	0.0517	U/L/h
Maximum specific glucose consumption rate	B_m	0.68	g/L/h
Maximum phytase activity	A_m	3.45	U/ml
Maximum glucose concentration	A_m	125.6	g/L
Duration of lag phase	λ	24	h
e		2.71	

12.6. References

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CHAPTER 13

CONCLUSIONS AND SCOPE FOR FUTURE RESEARCH

Feed and food additives are important compounds in industry to increase quality of diets. They are used for several applications such as controlling decomposition and deterioration, nutritional losses, loss of functional properties and aesthetic value (Branen et al., 2002). In this study, microbial production of PPA and phytase were studied. Significant improvements were achieved in the concentration of PPA and phytase activity in each step of this study (Figures 13.1 and 13.2, respectively).

PPA production studies started with a microbial selection to identify the best yield microorganism for PPA production. Among the four evaluated microorganisms, *P. vulgaris* demonstrated the highest PPA production in shake-flask fermentations. Optimization of growth parameters for PPA and biomass production was studied in batch fermentation. Optimum conditions were determined as 34.5°C, pH 5.12, and 0.5 vvm aeration for PPA production and 36.9°C, pH 6.87, and 0.96 vvm aeration for biomass production. Under these specific conditions, 1054 mg/L PPA and 3.25 g/L biomass productions achieved in bioreactors (Chapter 3). All growth factors were assigned as significant for biomass production, whereas aeration was not a significant factor for PPA production (p-value>0.05).

Additionally, optimization of fermentation medium composition was studied in the bioreactors. Optimum medium conditions for PPA production were determined as 119.4 g/L of glucose, 3.68 g/L of yeast extract, and 14.85 g/L of phenylalanine, whereas 163.8 g/L glucose, 10.75 g/L yeast extract, and 9.84 g/L phenylalanine for biomass production. Under the specific optimum conditions maximum PPA and biomass concentrations were 1349 mg/L and 4.35 g/L, respectively (Chapter 4). All factors were significantly effective on PPA production, however phenylalanine concentration was insignificant for biomass production (p-value >0.05).

Furthermore, fed-batch and continuous fermentations were evaluated to improve PPA production in the bioreactor. Maximum PPA concentration was increased to 2958 mg/L, when 4 g of phenylalanine was added into the reactor at 30 h of fermentation. Additionally, PPA productivity increased more than 5 times in continuous fermentations (259 mg/L/h) compared to batch fermentations (48 mg/L/h) (Chapter 5). Figure 13.1 depicts these improvements for each step.

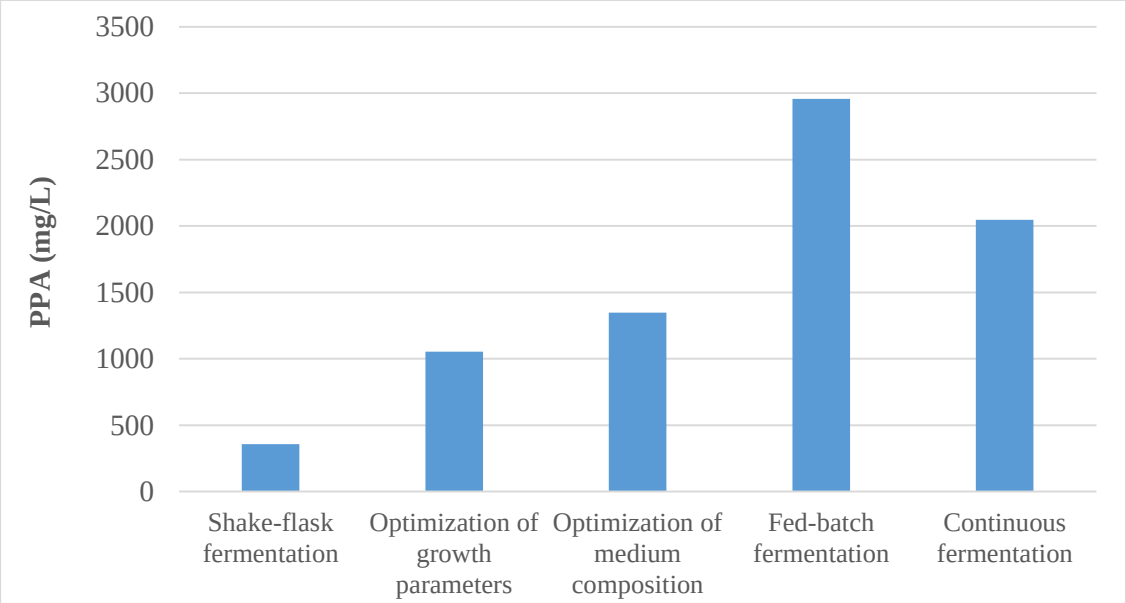

Figure 13.1. Comparison of PPA production for various fermentation methods.

PPA productions and substrate consumptions in batch fermentation were also modeled using the modified Gompertz model and modified logistic model (Chapter 6). It was determined that modified Gompertz model provided better predictions compared to modified logistic model for all cases. MAE values were obtained as 0.317, 91.958, -0.624, -0.844 for biomass, PPA, phenylalanine, and glucose prediction, respectively, for modified Gompertz model. On the other hand, MAE values were obtained as 0.448, 131.709, -1.181, and -4.527 for biomass, PPA, phenylalanine, and glucose prediction, respectively, for modified logistic model.

Similar to objectives applied for PPA production, phytase production was also studied with various strategies. *A. ficuum* was identified as the most productive strain for phytase production among *A. ficuum, L. plantarum, L. acidophilus,* and *L. amylovorus.* The highest phytase activity was measured as 1.02 U/ml in shake-flask fermentation. However, maximum phytase activity increased to 2.27 U/ml by optimization of growth parameters in the bioreactors. Optimum growth conditions for phytase production were determined as 33°C, 4.5 pH, and 0.9 vvm aeration (Chapter 7). All growth factors were assigned as significant for phytase production with *A. ficuum.*

In the subsequent study, optimum pH and temperature were determined as 5.5 and 55°C, respectively for *A. ficuum* phytase. Also, phytase activity increased to 3.45 U/ml by optimization

of the fermentation medium with 126 g/L of glucose, 14 g/L of Na-phytate, and 1.1 g/L of $CaSO_4$ (Chapter 8). All evaluated medium parameters were significant for phytase production with *A. ficuum.* Additionally, it was shown that 4^{o}C was the best for storage when less than 1 week for *A. ficuum* phytase.

Additionally, the effects of glucose and phytate additions on phytase activity were also studied in fed-batch fermentation. Maximum phytase activities were measured as 3.84, 4.63, and 4.83 U/ml, when 45 g of glucose, 5 g of Na-phytate, and 10 g of Na-phytate was added to the bioreactors, respectively at 96 of the fermentations (Chapter 9). Also, continuous fermentation was evaluated for phytase fermentation, for which phytase activity was measured as 3.33 U/ml in continuous fermentations. Maximum dilution rate was determined as 0.08 h^{-1} in continuous fermentations for *A. ficuum* (Chapter 11).

The effects of aluminum oxide and talcum were studied on phytase production. It was determined that phytase activity increased to 2.01 and 2.93 U/ml, respectively by addition of 15 g/L of aluminum oxide and talcum in shake-flask fermentation. Effect of microparticle addition on fungal pellet structure was also measured that 15 g/L talcum or aluminum oxide decreased average fungal pellet diameter from 800 µm to 200 and 500 µm, respectively, in shake-flask fermentations. Moreover, the highest phytase activity was measured as 6.49 U/ml by addition of 15 g/L of talcum in bioreactors at 96 h of fermentation (Chapter 10).

Phytase activity was further increased by addition of talcum in fed-batch fermentations. Activity increased from 4.912 to 9.587 U/ml in fed-batch by the addition of 15 g/L talcum. Moreover, phytase productivity increased from 0.293 U/ml/h to 0.621 U/ml/h in continuous fermentations by 15 g/L talcum addition (Chapter 11). Figure 13.2 depits these improvements for each step.

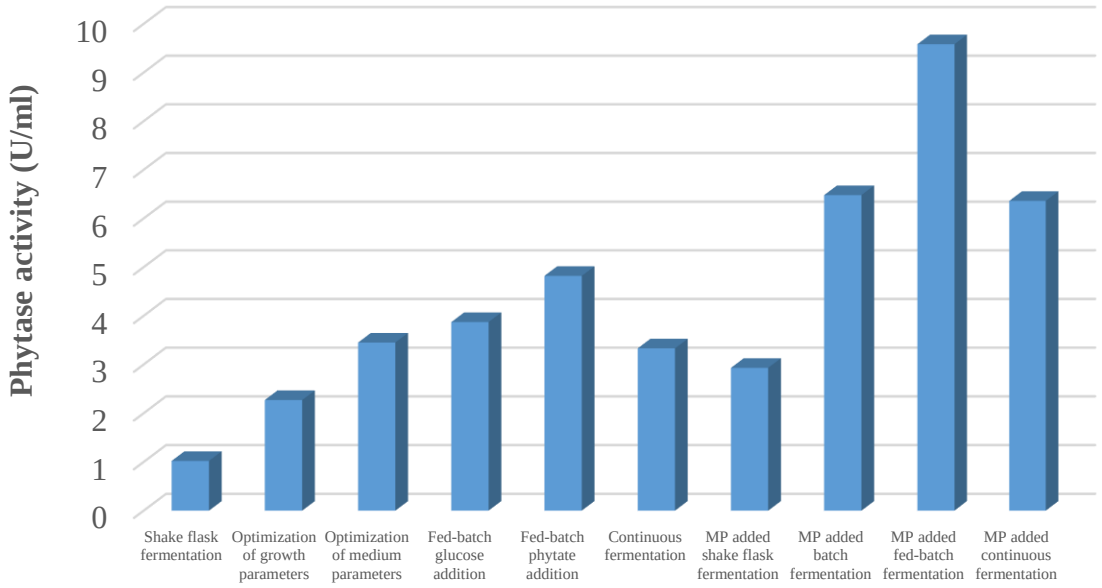

Figure 13.2. Comparison of phytase production for various fermentation methods (MP: Microparticle).

Phytase production and glucose consumption were modeled using the modified Gompertz model and modified logistic model. The modified Gompertz model provided smaller RMSE and MAE values compared to the modified logistic model for phytase production. Higher MAE and RMSE values were obtained by modified Gompertz model compared to modified logistic model for glucose consumption (Chapter 12).

In conclusion, these studies demonstrated that PPA and phytase productions significantly increased in the bench-top bioreactors by optimization studies and using novel fermentation techniques.

As the recommendations for the future research, for PPA studies, it was observed that concentration decreases rapidly and continuously after obtaining the highest value in the fermentations. There may be several factors caused this situation. Microorganisms may be using PPA for their metabolism as nutrition. Additionally, deaminase enzyme may lose its activity by the time in the fermentation medium and PPA production rate decreases. Also, PPA may be degraded by other by-products such as hydrogen peroxide. In order to overcome these problems, microorganisms can be removed from the broth at the end of the log phase and catalase may be added in order to eliminate microbial and hydrogen peroxide effects, respectively. In this way,

phenylalanine can be added into the reaction medium, which includes only conversion enzyme, but not microorganism.

Moreover, there are several microorganisms that they are capable to produce L-phenylalanine (Ikeda et al., 1993). These microorganisms can be used to produce L-phenylalanine and final product can be converted to PPA by *P. vulgaris* deaminase enzyme to create a more cost-effective fermentation. For example, phenylalanine can be produced by microorganisms such as *C. glutamicum* and then can be converted to PPA with *P. vulgaris.*

Advance downstream processes can be also performed to increase the purity of the PPA. Additionally, purified PPA can be replaced instead of phenylalanine in the broilers diet to study the effect of PPA on bone health and nitrogen accumulaton in the manure.

Following can be suggested for phytase fermentations as the future research topics. Firstly, high phytate content agricultural wastes such as rice bran and corn cob can be used to as the phytate source in the fermentation medium to reduce the fermentation medium cost. These wastes are abundant in the nature and easy to be used as substrate after simple pre-treatments.

Additionally, genetical modifications can be studied to over-express phytase expression gene to increase phytase activity in the fermenations. In this way, phytase production gene of *A. ficuum* can be transferred to faster growing bacteria such as *E. coli* and phytase production can be achieved in shorter fermentation time. Similarly, phytase 1-3 and 1-6 can be produced simultaneously by performing genetic modifications of coculture fermentations.

In terms of microparticles, effect of other microparticles such as titanium oxide on phytase activity can be studied. Moreover, several novel methods can be used to evaluate the biological and chemical interactions between microparticles and fungi. Additionally, the effect of micronutrients on mass transfer during the fungal fermentations can be analyzed. Furhermore, effects of different size ranges and nanoparticles can be also evaluated for fungal phytase fermentation.

Phytase can be also purified and use in chicken diets to study the effect of phytase addition on mineral absorption, phosphorus accumulation in the manure and enhancement on the meat quality.

Increase in the demand to these products causes a need for scale-up work. Therefore, several scale-up strategies such as maintaining mixing time, Reynold’s number, and mass transfer coefficient can be studied for both productions in order to convert laboratory scale to the industial.

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EDUCATION

2011-2014	Ph.D., Agricultural and Biological Engineering, The Pennsylvania State University (CGPA: 3.88/4.00)
2007-2009	M.Sc., Bioengineering, Ege University, Turkey (CGPA: 92/100).
2004-2005	Erasmus Student, Biology Department, University of Crete, Greece (Grade: A).
2001-2006	B.S., Bioengineering, Ege University, Turkey (CGPA: 80.28/100).

RESEARCH EXPERIENCE

Research assistant at The Pennsylvania State University (2011-present).

- Conversion of phenylalanine to phenylpyruvic acid by microbial fermentation (2011-2015).
- Production of microbial phytase in submerged fermentations (2012-2015).
- Electrolyzed oxidizing water as a novel cleaning in place approach for milking system (2012-2013).

Research assistant at Ege University (2007-2009).

- Microbial lipase production from olive mill waste water (2007-2009).
- Production of chitin from shrimp shells by microbial fermentation (2007-2008).

Undergrad thesis research at Ege University (2006).

- In vitro immunization for monoclonal antibody production (2006).

INTERNSHIPS

- Bioprocessing laboratory in Bioengineering Department at Ege University. 2004.
- Animal cell culture laboratory in Bioengineering Department at Ege University. 2004.
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