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Production of Phytase and Reduction of Phytic Acid Content in Canola Meal by Solid State Fermentation

by

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A thesis submitted to
the School of Graduate Studies and Research
in partial fulfillment of the requirements for the
degree of Master of Applied Science
in
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DEPARTMENT OF CHEMICAL ENGINEERING
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Dedication

This work is dedicated to my dear son,

Emmanuel Keffie Takow (Jr.)

Abstract

A static state technique for fermentation was applied using *Aspergillus ficuum* NRRL 3135 on canola meal for the production of phytase and for the reduction of the phytic acid content in the meal. *Aspergillus ficuum* was chosen as a result of its high phytase producing capacity. In the study of the effects of physical and nutritional factors on the enzyme production and the phytic acid content reduction, it was found that moisture content was a critical factor and 64% water in the medium was found to be the optimum moisture content for both enzyme production and phytic acid content reduction. Increasing time of homogenization of inoculum up to 240 seconds improved enzyme production and phytic acid reduction. Increase in both inoculum concentration (biomass) and inoculum age up to 7–days old increased enzyme production and rate of phytic acid content reduction. With increase in initial pH of medium of up to 5.7, increased enzyme production and rate of phytic acid reduction were achieved.

In the addition of surfactants to medium, sodium oleate was found to significantly increase enzyme production and the rate of phytic acid content reduction while Triton X-100 gave a negative effect. The addition of 1 mg phosphate remarkably increased the enzyme production and phytic acid content reduction; though a negative effect was obtained for systems containing combined portions of oleate and

phosphate. Biomass in the solid culture was found to increase during fermentation up to 144 h of incubation and the protein content of culture also increased to about 18% after 96 h of incubation; hence this method of fermentation could be used to improve the nutritional quality of the meal for animal feed.

Acknowledgement

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And unto Him (God Almighty) who is able to do abundantly above all that we could ever ask or think of, I say 'Thank You' for His steadfast love and faithfulness.

Notation

Various symbols, superscripts, subscripts, and abbreviations used frequently in this work are summarized below. Each notation is fully defined where it first arises in the text.

Symbols

E	enzyme activity (units/ml)
K_m	Michaelis constant (mM)
K_i	Inhibition constant (mM)
V_{max}	limiting (maximum) velocity (units/h)
k	exponential rate constant for decay of activity (h^{-1})
Y	yield coefficient (mg dry wt biomass/mg glucose produced)
k_s	saturation constant (mg)
s	substrate (mg)
x	biomass (mg dry wt/ml) ;(mg dry wt/g culture)
D	dilution rate (t^{-1})
R	pellet radius (m)
t	time (sec, min, h)
g	acceleration due to gravity (m/s^2)

Greek Symbols

μ	specific growth rate (h^{-1})
μ_{max}	maximum specific growth rate (h^{-1})
γ	as defined in Equation 3.9

Abbreviations

SSF	solid state fermentation
IP6	phytic acid
EDTA	ethylenediaminetetra–acetic acid
RF	rapeseed flour
RPC	rapeseed protein flour
Pi	inorganic phosphate (PO_4)
rpm	revolutions per minute
TCA	trichloro–acetic acid
IUPAC–IUB	International Union of Pure and Applied Chemistry-International Union of Biochemistry

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Chapter 1

Introduction

Rapeseed ranks fifth among the world's oilseeds. Its protein has a well balanced amino acid composition and it is especially rich in sulphur-containing amino acids. These facts explain the current interest in rapeseed as a new source for the manufacture of food quality protein in products.

Rapeseed has become far more useful today than when it was brought into Canada, 1942 when its only use then known was as a source of lubricant. Rapeseed, in particular canola varieties of the genus *Brassica* are the only oilseeds that can be grown on a commercial scale in the cooler areas of the world such as Canada, China, Northern Europe, and the Indian Sub-continent.

The meal which is obtained when the edible oil has been extracted from the rapeseed is now widely used as animal feed. Undoubtedly, the most important fact that contributed to the expansion of usage of rapeseed meal in feeds for swine and poultry in recent years has been the development of low glucosinolate, low erucic acid type rapeseed by Canadian and European plant breeders; and in particular the 'canola seed' which is a new cultivar of rapeseed with very low levels of cholesterol and saturated fatty acids developed by the Canadian scientists has become the most

important oilseed in the world economy. Canola is now defined as containing 2% or less erucic acid (Harris, 1988).

The protein content of canola meal varies depending on the cultivar from which the meal is produced; but on the average canola meal made from a mixture of low glucosinolate cultivars can be expected to contain 37–40% protein.

Generally speaking, canola meal is a richer source of minerals than soybean meal which is also another widely-used animal feed. Although not generally looked upon as a major source of vitamins, canola meal does contain a good number of vitamins. Canola meal thus has a high nutritional value; it is available and has a competitive price compared to other protein supplements. It is definitely a more suitable feedstuff for livestock and poultry than the moderately high glucosinolate rapeseed meal formerly produced in Canada, or many still being produced in other parts of the world. These nutritional and economic benefits and the fact that other protein concentrates are in relatively low supply could all lead to canola meal's increased usage in livestock rations.

However, there are some pertinent problems associated with the use of these rapeseed meals in poultry and livestock rations. It has been shown (Mothes et al., 1990) that phytic acid in these meals reduces the availability of protein and minerals. Canola meal is known to contain about 3–7% of phytic acid. It is therefore absolutely necessary that methods be developed to reduce or completely eliminate this acid from the meals in order to obtain their complete nutritional benefits.

Chemical techniques have been applied to the meals to reduce the phytic acid content; but owing to the extensiveness, expensiveness, and in most cases loss of nutritional constituents by the chemical techniques, other applications have been,

and are still being developed to achieve this aim.

Besides other techniques, solid state fermentation which has been in use for decades for food treatment has been found not only to be effective but also profitable when properly applied. This type of fermentation strictly relates to the growth of microorganisms on solid substrates without the presence of any free liquid.

Some of the results of the preparation or treatment of foods by this method can be summarized as follows: 1) produces desirable enzymes; 2) destroys undesirable constituents and odours; 3) adds flavour; 4) preserves food; 5) synthesizes desirable constituents, such as vitamins and proteins; 6) increases digestibility; 7) changes physical state.

The solid state fermentation technique is chosen for this study as a result of a number of additional advantages it provides as stated in section 2.3, and also because it has been successfully applied for the production of enzymes and for the reduction of phytic acid in products.

The conversion of phytic acid (myo–inositol–hexaphosphate) to myo–inositol–penta, -tetra, -tri, -di, -mono phosphate is a very important metabolic process involving enzymatic steps.

Extracellular phytases have been found to be produced by *Aspergillus* species, and a survey of sources designated a specific strain of *Aspergillus ficuum* NRRL 3135 to be the highest producer of active phytase (shieh and Ware, 1968).

The objectives of this work are:

1. to study the production of phytase on canola meal by solid state fermentation using *Aspergillus ficuum* NRRL 3135.

2. to study the effects of certain parameters on the reduction of phytic acid

content in canola meal by solid state fermentation using *Aspergillus ficuum* NRRL 3135.

Chapter 2

Literature Review

This chapter discusses the product canola meal which is used as the substrate for the phytase production and hence the reduction of its phytic acid content. The chapter also describes the structure and nomenclature of phytic acid, its occurrence, its applications, nutritional significance, and the various methods by which researchers have attempted to eliminate the presence of this acid from different products

This chapter also defines and describes a solid state fermentation and the various areas in which it has been applied in biotechnology. The chapter discusses the enzyme phytase which hydrolyses the phytic acid. It describes some characteristics of this enzyme, its production, and application in phytate removal. The growth kinetics of solid state fermentation is also discussed.

2.1 Canola Oil and Canola Meal

The oil processed from Canola seed is much lower in harmful materials, such as erucic acid and glucosinolate than other rapeseed oil and consequently its production has increased by over 300% in the last decade (Vaisey-Genser and Harry, 1987); with

Canada being the world's largest producer of the seeds, and the largest exporter of the oil and seeds (Harris, 1988). The oil is mainly used for producing margarine, salad oil and shortening.

Canola meal is a product obtained from the seed after the extraction of its oil. Besides containing about 37–40% protein, 28–32% carbohydrates, 11–15% crude fibre, and 3–7% phytic acid; Canola meal also contains a number of minerals, such as calcium, iron, manganese, phosphorus, selenium and zinc. Canloa meal is also a good source of vitamins, containing appreciable quantities of biotin, folic acid, niacin, riboflavin and thiamine.

2.2 Phytic Acid

This section discusses the structure, occurrence, nutritional significance, and the removal of phytates, phytin and phytic acid from substrates.

2.2.1 Nomenclature and Structure

Phytates represent a complex class of naturally occurring compounds that can significantly influence functional and nutritional properties of food. Although their presence in plant seeds and roots of tubers, serving as storage form of phosphorus has been known for over a century; their structure and role are not yet completely understood and thus represent areas of active research.

Numerous polyphosphorylated inositols can be found in nature and depending on the complex formed, a wide variety of compounds can exist. This perhaps leads to some of the confusion in terminology concerning the nomenclature of these compounds with terms such as phytin, phytate, and phytic acid being prevalent in

literature.

It is generally believed that the compound phytic acid can be commonly called myo-inositol hexaphosphoric acid, or scientifically as 1,2,3,4,5,6-hexakis(dihydrogen phosphate) myo-inositol (IUPAC - IUB, 1968). This acid is supposed to have three strongly bound water molecules. The term phytin implies a calcium-magnesium salt of phytic acid; whereas phytate would mean the mono to dodeca anion of phytic acid (Maga, 1982).

The structure of phytic acid has not been clearly demonstrated though some structures have been proposed. $C_6H_{24}O_{27}P_6$, with eighteen acid hydrogens about an inositol phosphate nucleus has been proposed by Neuberg; while Anderson proposes $C_6H_{18}O_{24}P_6$–containing twelve acid hydrogens. Various studies of the chemistry of this acid have produced results that support both proposals. Which ever of the two structures one chooses, it remains, that, myo-inositol hexakis(phosphoric acid) or phytic acid (designated as IP6) exhibits six acidic phosphate groups regularly bound around a myo-inositol ring. However, several additional structures have been proposed after Neuberg and Anderson. Evidence using nuclear magnetic resonance and X-ray crystallography leave little doubt that the structure proposed by Anderson (Fig. 2.1) is in fact the one found in plant seeds. According to Cosgrove (1966), soil phytate is a mixture of polyphosphates of several isomers of inositol.

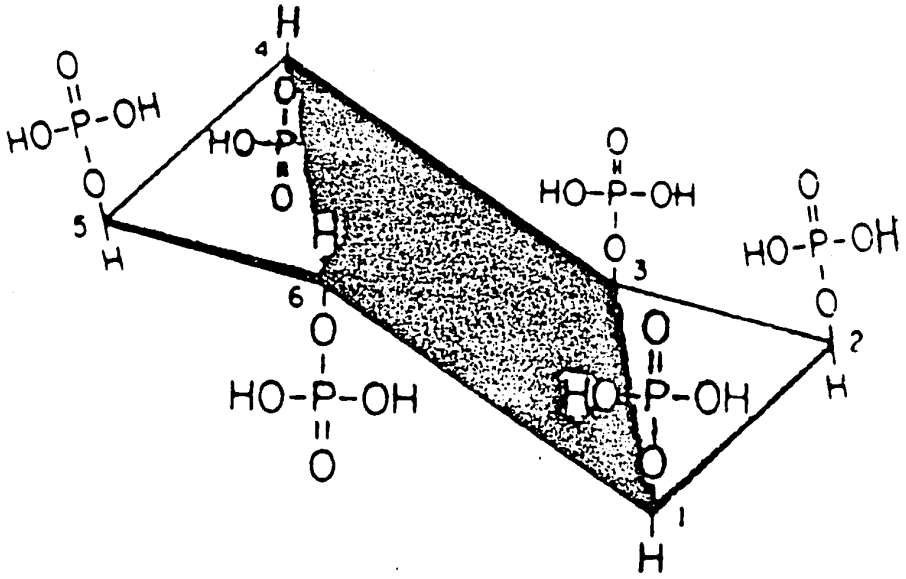

Figure 2.1: Myo-inositol 1,2,3,4,5,6-Hexakis(dihydrogen phosphate) Oberlas, 1969

2.2.2 Occurrence

Phytates in soy seem to be unique in that although associated with protein bodies, they appear to have no specific side localization (Tombs,1967).

Utilizing scanning electron microscopy, Pomerez (1973) observed that in the case of barley phytate is in the form of potassium and magnesium salts instead of the calcium-magnesium complex normally thought to be present in most other cereals. Phytic acid levels in 18 varieties of barley were found to range from 0.97 to 1.08% dry weight (Lolas et al., 1976).

Lolas and Markakis, (1975) measured phytic acid level of 50 cultivated varieties of *Phaesolus vulgaris* grown over a 2-year period and found a range of 0.54–1.58%. They also found a high degree of correlation between total phosphorus content and phytic acid level. In addition a protein-phytate complex was isolated; and it was noted that over 99% of the total phytic acid was in a water soluble form which could serve as a means of removing or lowering phytic acid levels in the products.

Lolas et al., (1976) reported that the phytic acid content of 15 soybean varieties, ranged from 1.00 to 1.47% dry weight which was represented by 51.4 to 57.1% of the total phosphorus.

2.2.3 Applications

The structural characteristics in conjunction with the ionization possibilities of phytic acid confer uncommon complexation properties to this molecule.

The fact that insoluble protein-phytic acid complexes are formed at pHs' below the isoelectric points of proteins enables phytic acid to be used for the precipitation of rapeseed proteins.

According to Graf and Eaton, (1985) phytic acid might exert beneficial effects on coronary heart diseases, renal calculi and colon cancer.

The ability of phytic acid to chelate metallic cations also gives rise to a variety of food, industrial and biological applications. These include its use as a preservative and anti-oxidant in food (Graf et al., 1987); or as an anti-corrosion agent in various coating materials (Graf, 1983).

In the biological area, technitium-99 m complexed by phytate produces a clinically useful radiopharmaceutical (Graf and Eaton, 1985). In the particular case of haemoglobin, phytic acid is known to alter the haemoglobin-oxygen affinity. It could therefore be incorporated into erythrocytes and used in the treatment of acute and chronic vital ischemia (Graf and Eaton, 1985).

Phytic acid is also used to promote commercial fermentation processes for the production of antibiotics, yeasts, lactic acid, and enzymes (Graf, 1986).

2.2.4 Nutritional Significance

It has been well established that phytic acid can adversely affect the bioavailability of dietary polyvalent cations through the formation of insoluble complexes at intestinal pH values 7–8 (Oberlas, 1983). Polyvalent cation binding sites on phytic acid molecule have only been determined for Mn^{+2} (EgbeWatt and Dill, 1987). Phytic acid also binds proteins at intestinal pH values as supported by ultrafiltration and dialysis experiments. It has been postulated that divalent cations mediate an interaction between protein and phytic acid at pH values above the isoelectric point of the protein. The suggested protein binding sites for the ternary protein-cation-phytate complexes are the imidazole group of histidine and ionized carboxyl

groups (Cheryan, 1980). If such ternary complexes exist, the possibility arises that digestion of phytate-containing food proteins in the small intestines could result in the formation of amino acid/ small peptide-cation-phytate complexes.

2.2.5 Phytate Removal

Nearly all seeds contain phytic acid chiefly as salts of calcium, magnesium, and potassium (Mayer and Poljakoff-Mayer, 1963). For seeds that have inherent phytase, this enzyme is activated during germination in the seeds to catalyze hydrolysis of the phytic acid in the seeds, into releasing inorganic phosphates.

However, there are certain seeds that though contain considerable quantities of phytic acid do not have inherent phytase; hence other means of their phytic acid content reduction or complete removal are necessary. Canola meal has been shown to contain no phytase (Stone et al., 1984).

Apparently phytates are fairly stable to heat. In the case of soy isolate, Rackis (1974) reported that autoclaving for 4 h at 115°C is required to destroy most of the phytic acid; which from a nutritional standpoint would be unacceptable due to amino acid destruction. Under similar time/temperature conditions, only 20% of the phytate associated with sesame meal was destroyed; thus indicating that structural differences are apparent among products. Autoclaving for 30 min resulted in little phytate loss. However, after 2 h of autoclaving approximately 70% of soy phytate was lost; whereas, rice, wheat, and sesame had only lost 5–25%.

Numerous studies have been done in order to reduce the phytate content in the protein products of cereal and legume origin, especially in soybean and rapeseed products (Okubo et al., 1975).

Boland et al., (1975) reported that heat destruction of phytic acid in cereal grains was species specific. Dry heat (i.e autoclaving without water in the substrate) destroyed little phytate but also increased the rate of enzymatic hydrolysis in the heated sample.

The extent of phytate-protein binding depends on pH and/or the content of divalent ions. At acid pH the protein possesses a net positive charge while phytic acid is negatively charged; thus a binary protein-phytate complex forms. Excess calcium ions is liberated to dissociate this complex by competing for phytate with the cationic groups of protein (Cheryan, 1980). At alkaline pH both protein and phytic acid are negatively charged; thus the interaction is mediated by multivalent cations to form a ternary protein-mineral-phytate complex. Disruption of this complex requires the removal of cations from the system by use of chelating agents such as ethylenediaminetetra-acetic acid (EDTA) to which some cations will bind more preferentially than to phytate. Soluble cation-EDTA complexes are formed thus preventing the formation of the protein-phytate complex.

Serriano and Thompson, (1984) studied the effects of pH adjustment on the protein-phytate complex, hence on the disruption and removal of phytic acid from the rapeseed flour (RF) and protein concentrate (RPC) in a dialysis system. Phytic acid removal from RF and RPC dispersions was also tried by using various amounts of EDTA (0.25–0.75) at pH 9.0; Calcium chloride (0.25–0.75) at pH 3.5, or phytase (3%) at pH 5.15. Dispersions of RF and RPC were dialyzed against distilled water for 7 days. While greatest removal of phytic acid (100%) with the least loss of nitrogen was observed with phytase treatment, adjustment of RPC to pH 3.5 and RF to pH 5.15 resulted in 73% and 88% losses of phytic acid, respectively. Dialysis

of RF dispersion without pH adjustment, i.e at pH 5.8, removed up to 67% of the phytic acid.

Phytates have been eliminated using physical and/or chemical methods. Some of these treatment are complicated and may result in some changes in the physico-chemical properties of the protein (De Rham and Jost, 1979), which in turn influence their functionality (Chen and Morr, 1985).

The use of enzyme treatment in phytate removal is achieved by the hydrolysis of the phytate thereby eliminating the available binding sites.

Han (1988) studied the removal of phytic acid from soybean cottonseed meals by using treatments with enzyme, heat, and chemicals. He observed that in samples which were treated with phytase alone, 37% of the phytate was hydrolyzed during 3 h-incubation at 37°C. The extent of phytate hydrolysis was further increased by co-application of cellulase with phytase. Almost half of the cottonseed phytate was hydrolyzed by the endogenous phytase upon incubation for 16 h at 60°C. Since most of the cationic agents tested did not significantly increase the amount of water-soluble phytate, or reduce water-insoluble phytate, treatment of soybean meals with metal solutions did not appear practical.

Recently, a new enzymatic method has been developed (Vaara and Simell, 1989) for decomposing the phytate in cereal or legume materials under relatively mild conditions. The phytate free isolate does not contain any inositol hexa, -penta, -tetra, or triphosphates. This phytic acid hydrolysis method decreased more effectively the phytate content of the soy isolate than did the pilot scale ion-exchange method used by and Chen and Morr, (1985). The enzymatic hydrolysis method eliminated phytate totally; whereas the ion-exchange method removed approximately 75% of

the phytate content.

The use of enzymes from microorganisms has been very effectively and successfully used by many researchers in the reduction of phytic acid content in products. In particular, the solid state fermentation technique was used by Nair et al., (1990) to reduce phytic acid content in canola meal. The acid was completely eliminated from the meal, thus rendering the commodity more suitable for animal feed.

2.3 Solid State Fermentation

According to Hellestine (1972) and other researchers, solid state fermentation (SSF) strictly refers to the growth of microorganisms on solid substrate in the absence of any free water. The term SSF cannot be applied to a fermentation on solid materials suspended in a liquid phase.

SSF was therefore defined as a system with solid matrix particles, a liquid phase bound with them, and a gaseous phase entrapped in the particles. The physical properties of the system, such as water potential, water holding capacity, which can be an index of aeration and bulk density which predicts the volume of pore space, help define the conditions of this type of fermentation.

Solid fermentation has been used for decades, mainly for food treatments, long before understanding the underlying microbiological or biochemical process involved. SSF is also presently used for a number of other applications.

In recent years attention has been directed to the production of protein by microscopic (filamentous) fungi exhibiting a high growth rate, big protein content biomass, high protein digestibility, and interesting amino acid composition; the production of high protein content feed of agricultural surplus or residues is of

particular interest.

Raimbault et al., (1985) studied protein enrichment of cassava by solid state fermentation using molds isolated from traditional foods. They found that Aspergillus spp. strains gave the best results under the standard conditions studied.

Oriol et al., (1988) studied the solid state fermentation of cassava starch by *Aspergillus niger*. Good growth and substrate conversion to biomass were obtained. They observed from their study that water availalibilty was a critical factor in the SSF of starch substrates.

Mathot and Brakel, (1990) were able to raise the protein content of barley, wheat, and dehydrated beet pulps to 30% by SSF. These results were obtained without any previous sterilization nor acid-alkaline pretreatment of the substrate (crushing for cereal, only).

The solid state technique is also being used for the production of enzymes. Han et al., (1987) studied phytase production by *Aspergillus ficuum* using solid state cultivation of several cereal grains and legume seeds. The microbial phytase was used to hydrolyze the phytate in the soybean meal and cottonseed meal. They observed that the level of phytase production was higher than that obtained by submerged fermentation.

Gattinger et al., (1990) studied the use of canola meal as a substrate for xylanase production by *Trichoderma reesi*. They found that better yields of these enzyme were produced from canola meal than from solka-floc,xylan, or glucose. This enzyme system produced using canola meal also contained a higher proportion of acetyl-xylan esterase, cellulase, and xylosidase activities.

Rivera-Munoz (1991) grew several filamentous fungi on solid state fermentation

systems for the production of microbial lipases. He also did a comparison between kinetics on solid and submerged fermentation using *Penicillium candidum.* He obtained highest titers and a stable production in the solid fermentation than in the submerged.

The solid state technique has also found great use in the production of other valuable materials besides protein and enzymes, and also in the degradation and/or elimination of some undesired constituents in substrates.

Rossi et al., (1988) used some agricultural wastes and surplus (orange peel, tapioca, potatoes, olive husk, beet pulps, tobacco and topinambur fibre, grape stalks), for the production of citric acid by solid state fermentation using *Aspergillus niger.* The highest production (46 g/kg) was obtained on lyophilized orange peel powder. Citric acid production was also improved by adding glycerol to the substrate under tray fermentation conditions.

Reid (1989) used the white-rot fungus *Phlebia tremellosa* in solid state fermentation to delignify aspen wood and increase the accessibility of its polysaccharides to enzymatic hydrolysis.

SSF is also used in phytate removal as described in section (2.2.5)

In all the studies done using solid state fermentation, there are a number of outstanding advantages of the technique as well as a few, but pertinent drawbacks. Some of the advantages include:

1. The medium is relatively simple, for instance whole grain with water just sufficient to moisten the substrate.

2. Aeration is easily obtained since there are air spaces between each particle of the substrate.

3. The desired product may be easily extracted by addition of solvent directly into the vessel.

4. Contamination is less, due to reduced moisture content as opposed to submerged culture.

5. Products obtained are concentrated.

6. The conditions under which the fungus (used in most SSF) grows are more like the conditions in its natural habitat.

7. Usually the substrates contain all or most of the nutrients needed for growth and product production, hence needs little or no additive.

8. Most processes do not require pretreatment.

Growth kinetics and mathematical modelling of microbial growth in solid state fermentation have received little attention due to difficulties associated with its study. Some of these difficulties include:

1. The systems are geometrically complex due to non-homogenous spatial distribution of the components.

2. Growth is largely limited to the surface of the particles. This leads to the formation of concentration gradients which are virtually impossible to measure experimentally.

3. Solid substrates are usually relatively unprocessed byproducts of agriculture and may be structurally or nutritionally heterogeneous. In addition, significant variations can occur between batches.

4. Important parameters may be difficult or impossible to measure. The critical problem is the penetration of fungal hyphae in the solid substrates, which prevents direct recovery and determination of biomass. Studies of SSF rely on indirect methods of biomass determination, the accuracy of which has not yet been conclusively proven. Another parameter which is difficult to measure and control is pH, since conventional glass bulb electrodes cannot be used directly in the medium.

5. Mixing of the system in order to obtain good representative samples for analyses, highly damages the mycelia of the microorganism. This greatly lowers the rate of cell growth of the microorganism.

2.4 Phytase

This section describes the occurrence, production and characteristics of phytase.

2.4.1 Occurrence and Production

Phytase is a phosphomonoesterase capable of hydrolyzing phytic acid (IP6) to yield inorganic orthophosphate (Pi) and a series of lower phosphoric esters of myo-inositol and ultimately, in some cases, free myo-inositol (Irving and Cosgrove, 1972). IUPAC-IUB recognizes two phytases. One is 3-phytase (EC 3.1.3.8), which hydrolyzes IP6 to produce D-myo-inositol 1,2,4,5,6-pentakisphosphate and Pi; another is 6-phytase (EC 3.1.3.26) , which hydrolyzes IP6 to produce L-myo-inositol 1,2,3,4,5-penta-kis phosphate and Pi (Cosgrove, 1980).

This enzyme phytase is widely distributed in plant and animal tissues in many species of fungi and in certain bacteria (Cosgrove, 1966). The distribution and

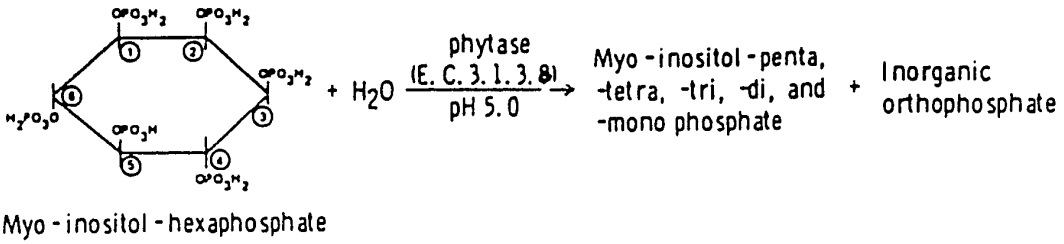

Figure 2.2: Enzymatic (phytase) hydrolysis of phytate.

quantity of phytase are not proportional to the phytic acid content and they are not correlated with glycerophytase and pyrophosphatase activities in plant tissues (Courtois and Perez, 1948a).

Han and Gallinger, (1987) while studying the effects of nutritional and cultural conditions on cell growth and phosphatase production by *Aspergillus ficuum* in a submerged culture found that the organism produced a non-specific acid phosphomonoesterase rather than a phytin specific phosphatase. The enzyme hydrolyzed a variety of phosphates and produced orthophosphate. The enzyme activity in the growth medium increased continually during a one-month growth period. For a high level of phosphatase production, low levels (1–5 mg) of initial phosphorus were necessary and the addition of surfactants, such as polyoxyethylene ethers and sodium oleate to fungal culture medium markedly increased the level of the phosphatase production.

Han et al., (1987) studied phytase production using *Aspergillus ficuum* by solid state cultivation on several grains of legume seeds. Wheat bran, soybean meal, cottonseed meal, and corn meal supported good fungal growth and yielded a high level of phytase when an adequate amount of moisture was present. The level of phytase production on solid substrate was higher than that obtained by submerged liquid fermentation. Higher levels of phosphorus (more than 10 mg/100 g substrate) in the growth medium (static culture) inhibited phytase synthesis, and the degree of phosphorus inhibition was less apparent in semi-solid medium than in liquid medium. A static cultivation on semi-solid substrate produced a higher level of phytase (2–20 fold) than that obtained by agitated cultivation. The minimal amount of water required for growth and enzyme production on the substrates was about 15%, while the optimum level for phytase production was between 25 and 35%, and that for cell growth was above 50%. Optimum pH for phytase production was between 4 and 6. *A. ficuum* grew well on raw (unheated) substrate containing minimal amount of water and produced as much phytase as on heated substrate.

2.4.2 Phytase Characteristics

Irving and Cosgrove, (1972) studied two enzymes produced by *Aspergillus ficuum* NRRL 3135. They found that the pathways of dephosphorylation of myo-inositol hexaphosphate by these two enzymes differed from that of wheat bran phytase which forms L-myo-inositol 1,2,3,4,5-pentaphosphate; while the non-specific orthosphoric monoester phosphohydrolase at pH 2.0 produced a single pentaphosphate, D-myo-inositol 1,2,4,5,6-pentaphosphate; and the phytase, at both pH 2.0 and 5.5, produced a mixture of two pentaphosphates.

Irving and Cosgrove, (1974) partially purified samples of the acid phosphatase and phytase from *A. ficuum* by gel filtration and ion-exchange dextran chromatography. In studying the characteristics and kinetics of these enzymes, they found the pH optimum in 0.02 M phthalate buffer of the acid phosphatase to be 2.2; while in the same buffer the phytase was found to have at least two optima at pH 2.5 and 5.3, with the suggestion of a third one at pH 2.8. The K_m value of the acid phosphatase was 0.127 mM; while K_m of phytase at pH 2.5 was 0.0244 mM and at pH 5.3, it was 0.0129 mM.

Ullah (1988) studied the characteristic of purified phytase of *A. ficuum* NRRL 3135, using sodium phytate as substrate. He observed that phytate concentration in excess of 1250 μM inhibited enzyme activity at both pH 5.0 and 2.5. The inhibition being more prominent at pH 2.5 and 5.0. At a substrate concentration of 4 mM the phytase activity was totally inhibited at pH 2.5; but at pH 5.0 only 13% less catalytic activity was observed. He found that *A. ficuum* phytase performed maximally at a substrate concentration of 1.0–2.0 mM at pH 5.0; but under identical conditions at pH 2.5 the enzyme showed rapid loss of activity. He obtained K_i and K_m values of 1.9 mM and 42.5 μM, respectively, for the phytase.

Ghareub (1990) produced phytase from *Aspergillus carneus* in a culture filtrate. Using crude and purified phytase he studied the characteristics of the enzyme. He observed that maximum activity of the crude enzyme occurred at 35°C and pH 5.6. The pure enzyme was found to be stable between pH 5.6–6.2, and about 68% of enzyme activity was lost by heating at 45°C for 60 min. The pure phytase retained its activity for over a long period when stored at 4°C.

Nair et al., (1990) studied some characteristics of phytase produced from *Aspergillus ficuum* NRRL 3135. The enzyme system was saturated with the substrate when its concentration was 2.0 mM. They obtained K_m and V_{max} values of 0.27 mM and 0.46 units/h^{-1}, respectively, for the enzyme. Optimum pH and temperature values were found to be 5.0 and 60°C, respectively. Preincubation of the enzyme at 60°C for half an hour did not cause any significant reduction in the enzyme activity; however 70% of the enzyme activity was lost after 3 h of incubation; and preincubation at 50°C for 3 h resulted in only 5% loss of activity. When the enzyme was stored at 4°C for 5 weeks, a total loss of activity of only 15% was noted.

2.5 Growth Kinetics of Solid State Fermentation (SSF)

This section discusses growth models proposed for solid state fermentations using mold or other filamentous organisms.

Okazaki et al., (1980) proposed a logistic mathematical growth model from the study of a surface culture using *koji* mold. The model can be rewritten (to be consistent with notation used in other sections) as

$$\frac{dx}{dt} = \mu_m.x.[1 - \frac{x}{x_m}] \tag{2.1}$$

which on integration gives

$$x = \frac{x_m}{1 + [\frac{x_m}{x_0} - 1]e^{\mu_m t}} \tag{2.2}$$

x: biomass (mg dry wt/cm^2)

x_m: maximum biomass (mg dry wt/cm^2)

t: time of fermentation (h)

where μ_m is the maximum specific growth rate (h^{-1}).

Mitchell et al., (1991) proposed a semimechanistic model which he developed from a study of an SSF using *Rhizopus oligosporus*. The equations were presented for the release of glucoamylase, the diffusion of glucoamylase, the hydrolysis of starch, the generation and diffusion of glucose, and the uptake of glucose and conversion into new biomass. Based on the assumption of a direct relationship between the biomass production and enzyme production. i.e

$$\frac{dx}{dt} = Y.E \tag{2.3}$$

x: biomass concentration (mg dry wt/cm^2)

t: time of fermentation (h)

Y: yield coefficient (mg dry wt biomass/mg glucose produced)

E: glucosamylase activity (U/cm^2, where 1 U = 1 mg glucose produced/h)

The glucosamylase activity in the figure was approximated empirically as:

$$E = r.t \qquad ;0 < t < t_1 \tag{2.4}$$

$$E = (r.t_1)e^{-k(t-t_1)} \;; t \geq t_1 \tag{2.5}$$

r: the rate of increase in glucosamylase activity (U/cm^2/h) until the maximum activity ($r.t_1$) is attained at time t_1

k: the exponential rate constant for decay of activity after t_1 (h^{-1})

The following equations were thus obtained for biomass production:

$$\frac{dx}{dt} = Y.r.t \qquad ;0 < t < t_1 \tag{2.6}$$

$$\frac{dx}{dt} = Y(r.t_1)e^{-k(t-t_1)} \; ; t \geq t_1 \tag{2.7}$$

which on integration gives:

$$x = x_0 + \frac{Y.r.t^2}{2} \quad ; 0 < t < t_1 \tag{2.8}$$

$$x = [x_0 + \frac{Y.r.(t_1)^2}{2}] + \frac{Y.r.t_1}{k}.[1 - e^{-k(t-t_1)}] \; ; t \geq t_1 \tag{2.9}$$

where x_0 is the initial biomass concentration (mg dry wt/cm^2)

The applications of predictions of both models agreed closely with their experimental profiles, indicating that both the logistic and empirical models represented good approximations of the growth kinetics.

Chapter 3

Theory

This chapter discusses the kinetics of substrate utilization, product formation, and biomass production in cell cultures (taken from Metz and Kossen, 1977; and Bailey and Ollis, 1986).

3.1 Kinetics of Balanced Growth

Associated with cell growth are two other processes: uptake of some material from the cell's environment and release of metabolic end products into the surroundings.

In the first attempt to model and understand cell population kinetics, unstructured models in which only cell mass or cell number concentrations are employed to characterize the biophase. The net rate of cell mass growth, r, is often written as μx where x is the cell mass per unit culture volume and μ, which has the units of reciprocal time, is the specific growth rate of the cells. Therefore in a submerged culture, in a steady–state CSTR material balance for cell mass gives

$$Dx_f = (D - \mu)x \tag{3.1}$$

where D is the dilution rate.

The general goal in making a medium is to support good growth and/or high rates of product synthesis. Contrary to intuitive expectation, this does not necessarily mean that all nutrients should be supplied in great excess. For one thing, excessive concentration of a nutrient can inhibit or even poison cell growth. Moreover, if the cells grow too extensively, their accumulated metabolic end products will often disrupt the normal biochemical processes of the cells. Consequently, it is common practice to limit total growth by limiting the amount of one nutrient in the medium.

If the concentration of essential medium constituent is varied while the concentrations of all other medium components are kept constant, the growth rate typically changes in a hyperbolic fashion. A functional relationship between the specific growth rate μ and an essential compound's concentration was proposed by Monod in 1942. The Monod equation states that

$$\mu = \frac{\mu_{max} s}{k_s + s} \tag{3.2}$$

Here μ_{max} is the maximum growth rate achievable when $s >> k_s$ and the concentration of all other essential nutrients are unchanged. k_s is that value of the limiting nutrient concentration at which the specific growth rate is half its maximum value; roughly speaking, it is the division between the lower concentration range, where μ is strongly (linearly) dependent on s, and the higher range, where μ becomes independent of s.

3.2 Transient Growth Kinetics

This section describes growth–cycle phases for batch cultivation, growth kinetics for unstructured batch growth models, and growth kinetics for batch growth models

of molds and other filamentous organisms.

During certain intervals in batch cultivation or during startups and disturbances to continuous flow reactors, cell populations grow in a transient state in which more complicated kinetic behaviour may be observed; and different types of kinetic models may be required to describe different transient growth situations.

3.2.1 Growth–Cycle Phases for Batch Cultivation

In a typical batch process the number of living cells varies with time. After a *lag phase*, where no increase in cell numbers is evident, a period of rapid growth ensues, during which the cell numbers increase exponentially with time. Although this stage of batch culture is often called the *logarithmic phase*, a more descriptive term *exponential growth*, is generally used. Naturally in a closed vessel the cells cannot multiply indefinitely, and a *stationary phase* follows the period of exponential growth. Eventually a decline in cell numbers occurs during the *death phase*. Here an exponential decrease in the number of living individuals is often observed.

Each phase is of potential importance in biochemical processes; for the general objective of a good design may be to minimize the length of the lag phase and to maximize the rate and length of the exponential phase, the last objective being achieved by slowing the onset of the transition to stationary growth. Reaching the largest possible cell density at the end of the process is frequently very important.

Many intracellular enzymes require activation by small molecules (vitamins, cofactors) or ions (activators) which may have appreciable permeability through the cell membrane. Transfer of a small culture volume of medium causes outward diffusion of these requisites for catalysis into the bulk medium if the new medium is

lacking in these species or differs appreciably in ionic strength. The rate of growth will fall, corresponding to the lower concentrations of such species inside the cell, and again a lag phase will appear while a new machinery to generate such activities is assembled.

The growth stage of the inoculum exerts a strong influence on the length of lag phase; a young cell population shows no lag upon transfer into a medium rich in metabolic intermediates such as amino acids; while the older cultures, at the time of transfer, their original medium may already contain a reasonable bulk concentration of intermediates, and the dilution on transfer will have a lesser effect. Transfer of an old culture, thus results in a longer lag.

3.2.2 Unstructured Batch Growth Models in Submerged Cultures

In the simplest approach to modelling batch culture, it is supposed that the rate of increase in cell mass is a function of the cell mass only. Thus

$$\frac{dx}{dt} = f(x) \tag{3.3}$$

One of the simplest models, using this supposition is *Malthus' law*, which uses

$$f(x) = \mu x \tag{3.4}$$

with μ as a constant, being the growth rate characteristic of exponential growth. Malthus' prediction of death phase resulting from unrestrained population growth has not yet been realized, and transition to a stationary population is generally observed for microbial populations.

3.2.3 Growth of Filamentous Organisms

Unlike the analysis of microbial populations in which increase in biomass is accomplished by an increase in the number of cells is the case of molds and other filamentous organisms, where the mass and the morphology of the mold pellet or pulp varies as growth proceeds. Several experimental studies of batch submerged culture have indicated that biomass increases at a slower than exponential rate with proportionality to (time)^3 providing a reasonable representation of the data. Such a growth pattern can be rationalized from observations of one– and two–dimensional mold cultures. In the first instance, the rate of increase in the colony length is constant, while radius of the mold colony increases at a constant rate in surface culture (two– dimensional). Extrapolating to a spherical pellet growing in submerged culture, it is assumed that

$$\frac{dR}{dt} = k_g = constant \tag{3.5}$$

where R denotes the pellet radius. Since the biomass x is

$$x = \rho\frac{4}{3}\pi R^3 \tag{3.6}$$

From eqs.(3.5) and (3.6)

$$\frac{dx}{dt} = \rho 4\pi R^2 \frac{dR}{dt} = k_g 4\pi R^2 \rho \tag{3.7}$$

Eliminating R from (3.7) using (3.6) leaves

$$\frac{dx}{dt} = \gamma x^{\frac{2}{3}} \tag{3.8}$$

$$where \qquad \gamma = k_g[36\pi\rho]^{\frac{1}{3}} \tag{3.9}$$

Integrating eq. (3.8) with an initial biomass of x_0 yields

$$x = (x_0^{\frac{1}{3}} + \frac{\gamma t}{3})^3 \tag{3.10}$$

Since x_0 is usually quite small relative to x, eq. (3.10) gives the cubic dependance of x on t.

A complete analysis of filamentous organism growth should also consider the kinetics of pellet formation. This is due to agglomeration of spores and subsequent growth or by outgrowth from an individual spore. Research to date has shown that many properties of the organism and its growth environment interact to influence pellet formation. Because the mechanisms are complicated and not sufficiently well understood or documented, general kinetic models for pellet formation have not yet been developed.

When considering growth of existing pellets, the model (Fig. 3.1) here outlined must be viewed as a crude approximation of reality. Pellet size, morphology, and internal structure, all of which are expected to influence pellet kinetics, are determined by interactions among agitation intensity, pellet concentration, organism properties, and medium composition (Metz and Kossen, 1977). Overall kinetics of pellets often depend upon diffusion–chemical reaction interactions.

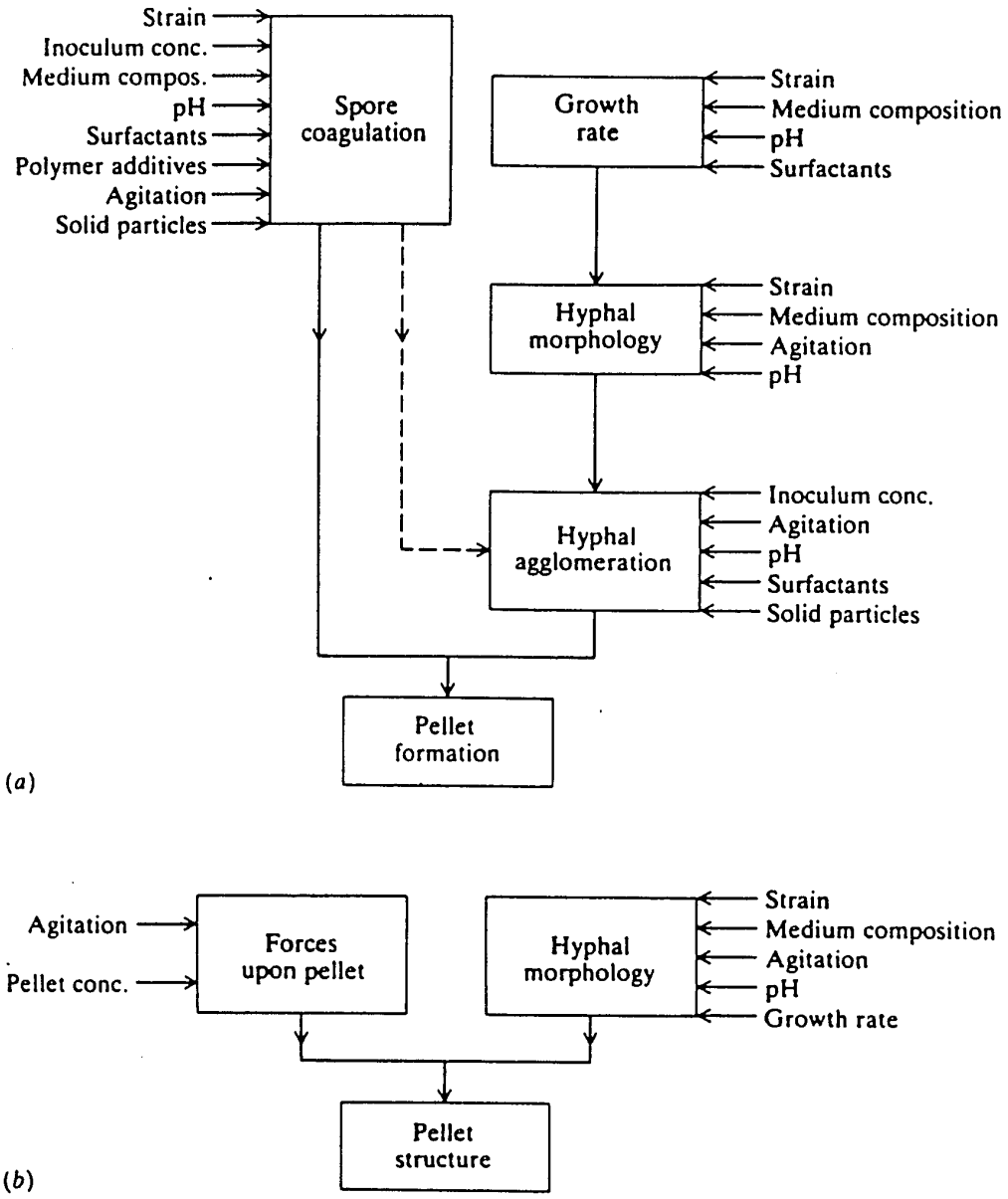

Figure 3.1: Summary of factors which interact to determine a) pellet formation and b) pellet structure during cultivation of mycelial organism. (Metz and Koseen, 1977)

3.2.4 Growth Kinetics for SSF

There is yet no concept, in other words generally accepted models for solid state fermentation, in particular for molds or other filamentous organisms, for reasons stated in section 2.3. However, two proposals have been presented so far, one by Okazaki et al., (1980); and the other by Mitchell et al., (1991). Both of these models have been discussed in section 2.5.

Chapter 4

Materials and Methods

This section describes the microorganism used in this study, the compositions and preparation of the cultivation media, the analyses of the various relevant components of the substrate (canola meal), namely: phytic acid, available carbohydrates, and crude protein. pH of the solid medium, phytase assay, and the determination of biomass in both liquid and solid medium are also discussed.

4.1 Microorganism

Aspergillus ficuum NRRL 3135 was used throughout this work based on conclusions drawn by certain researchers. Shieh and Ware, (1968) after having considered a number of strains of Aspergillus species, concluded that *A. ficuum* NRRL 3135 strain produced the highest amount of phytase; ten times the amount produced by *A. niger* and five times the amount produced by *A. carbonarius*. Nair and Duvnjak, (1991) using a number of microorganisms (Rhizopus oligosporus NRRL 2990, Aspergillus niger NRC 5765 and 401121, a wild Saccharomyces cerevisiae strain, and Aspergillus ficuum NRRL 3135), found that all these microorganisms could be used for the reduction of phytic acid content in canola meal; and that *A. ficuum* was

the most efficient because it completely hydrolyzed the phytic acid in 48 h, while the others either could not completely hydrolyze the phytic acid or needed longer periods of time.

4.1.1 Preparation of Slants and Liquid Culture

A. ficuum NRRL 3135 was grown on slants at 30°C (Table 4.1). The microorganism appeared as a thin white mass (mycelia) on the slant after the first day of incubation; and then the colour changed into dark brown as it began to sporulate. By the third or fourth day spores were completely formed. The slants were stored at 4°C for further use.

Aspergillus ficuum NRRL 3135 was found to grow well in the liquid medium (Table 4.1), in the form of pellets; the size of which was smaller (but greater number of pellets) with higher concentrations of spore suspensions used for inoculation. The liquid culture was grown on a rotary shaker (200 rpm) at 30°C.

4.1.2 Preparation of Inoculum for SSF

As mentioned in the above paragraph (section 4.1.1), the microorganism grows in the liquid medium in the form of pellets. It was difficult or almost impossible to transfer consistent amounts of inoculum into the solid medium for each inoculation without incurring contamination, in particular when parallel batches were to be run. It was therefore neccessary that the inoculum be homogenized before being used for inoculation. The time of homogenization varied between 30–120 seconds, depending on the nature and size of the pellets the microorganism formed during its growth. Usually, homogenization was done to give a free-flowing culture into and out of the 10 ml pipette; in other words the microorganism did not adhere unto

Table 4.1: Growth media for microorganism.

Component (%)	Solid	Liquid
Malt agar	4.5	—
Glucose	0.5	0.5
Yeast extract	0.5	0.5
Nutrient broth	—	0.8

the inside walls of the pipette after it had been emptied.

4.1.3 Preparation of Solid Medium

50 g of canola meal and 50 ml of distilled water (unless otherwise stated) were put into a 500 ml Erlenmeyer flask and autoclaved at 121°C for 45 min. The flask was then allowed to cool, and inoculated with homogenized inoculum and incubated at 30°C. This medium was used as the control in the fermentation batches. The phytase which was produced by the microorganism during incubation, reduced the phytic acid content in the canola meal.

4.2 Analysis of Samples

This section describes the methods used in analyzing the different parameters evaluated in this study.

4.2.1 Phytic Acid

The method adopted for phytic acid determination was according to Haug and Lantzsch (1983), for rapid determination of phytic acid by measuring the residual iron concentration.

Approximately 3 g of samples were taken into 150 ml Erlenmeyer flasks and the

phytic acid was extracted using 50 ml of 2.4% HCl under continuous shaking (200 rpm) for 1 h. The samples were then centrifuged (6000 × g, 15 min) and 5 ml of the supernatant was mixed with 10 ml of ferric solution (0.2 g $(NH_4Fe(SO_4)_2.12H_2O)$ in 100 ml of 2N HCl and made up to 1000 ml with distilled water). The solution was boiled for 30 min and then cooled in ice water for 15 min. It was removed from the ice water and allowed to reach room temperature. The solution was again centrifuged (3000 × g, 30 min). 2 ml of the supernatant was treated with 3 ml of bipyridine-thioglycollic acid (10 g bipyridine and 10 ml thioglycollic acid in distilled water and made up to 1000 ml). The absorbance was read at 519 nm, using distilled water as reference.

4.2.2 Protein

The method described by Lowry et al., (1951) was adopted for protein determination. Reagents used for the analysis were:

1. 2% sodium carbonate (Na_2CO_3) in 0.1N sodium hydroxide (NaOH).
2. 0.5% copper sulphate ($CuSO_4.5H_2O$) in 1% sodium tartrate.
3. 50 ml of (1) + 1 ml of (2)
4. 1:2 phenol reagent (Folin & Ciocalteu).

Approximately 3 g samples and 50 ml 1N NaOH were taken into 150 ml Erlenmeyer flasks and boiled for 10 min. The samples were cooled and centrifuged (6000 × g, 15 min). 1 ml of diluted supernatant (25–125 μg/ml) was mixed with 5 ml of reagent 3. The solution was allowed to stand for 15 min; after which 0.5 ml of reagent 4 was added to the solution and mixed thoroughly by vortexing. The

resulting solution was kept for 45 min, then absorbance was read at 750 nm against water similarly treated. The protein concentration was then calculated from the standard curve similarly prepared using albumin.

4.2.3 Carbohydrates

Weiner's method (1978) for carbohydrate determination was adopted and the extraction procedure was taken from the Official methods of Analysis (AOAC, 1975). Using 50 ml of distilled water and 10 ml of conc. HCl, carbohydrates were extracted from approximately 3 g samples by boiling for 2 h. 3 ml of diluted supernatant was treated with 10 ml of anthrone reagent (1 g anthrone reagent in 1 L of 85% v/v H_2SO_4, and 10 g thiourea), and again boiled for 20 min. Solution was rapidly cooled to 20 $\pm$ 1°C in an ice bath; and absorbance read at 625 nm against water similarly treated. The carbohydrate content was calculated from the standard curve similarly prepared using sucrose.

4.2.4 Phosphorus

The method adopted for phosphorus measurement was that described by Harland and Harland (1980). This method required the use of Taussky-Schoor reagent which was prepared as follows: 10 g of ammonium molybdate ($(NH_4)_6Mo_7O_{24}.4H_2O$) was placed in a 100 ml flask and made up to volume with 10N H_2SO_4. 5 g ferrous sulphate heptahydrate ($FeSO_4.7H_2O$) was placed in another 100 ml flask, and 10 ml of the ammonium molybdate solution was added to it. The resulting solution was then diluted to volume with deionized water.

To determine the amount of phosphorus, 5 ml of supernatant was mixed with 5 ml of Taussky-Schoor reagent and absorbance read at 660 nm immediately using

water similarly treated as reference. Phosphorus concentration was calculated from standard curve prepared using potassium dihydrogen phosphate (KH_2PO_4).

4.2.5 pH Measurement

Approximately 2 g samples were taken and mixed thoroughly with 20 ml distilled water. The mixtures were allowed to stand for 45–60 min. pH was measured using Fisher Accumet pH meter (model 805 MP).

4.2.6 Biomass from Liquid Culture

Known volumes of samples were centrifuged at 15000 × g for 15 min. The supernatant was drained off and the biomass washed with distilled water. This process was repeated twice for the same sample; the biomass was then dried at 105°C for 24 h. The amount of biomass was expressed in mg or g dry wt/ml.

4.2.7 Glucosamine

In the case of the liquid culture, biomass could be determined directly as described above (section 4.2.6). But in the solid culture, because of the penetration of the fungal hyphae in the solid substrate, it is impossible to obtain direct total recovery of biomass. As a result of this, an indirect method was adopted.

Glucosamine content of cell has been found to be an indicator which is consistent throughout fungal growth and remains the same under different cultivation conditions (Desgrandes et al., 1991); so glucosamine content of cell was chosen as the indicator for determining the biomass during the solid state fermentation. It was therefore necessary to have a direct relationship between the glucosamine content and the biomass of this microorganism in a liquid medium (as described in

section 4.4), in order to enable one determine the biomass in the solid medium.

Blix's method (1948) for the determination of glucosamine was adopted. The range of glucosamine concentration in solution was 0.03–0.14 mg/2 ml. 2 ml of acetylacetone solution (1.5 ml acetylacetone + 50 ml 1.25N Na_2CO_3— made fresh everyday) was added to 2 ml of glucosamine solution. The mixture was heated at 96°C in a closed tube for 60 min in a water bath. It was cooled to room temperature in cold water, and 20 ml of 96% ethyl alcohol was added to it and thoroughly mixed. 2 ml of Ehrlich's reagent (1.6 g p-dimethylamino benzaldehyde + 30 ml conc. HCl and 30 ml 96% alcohol) was also added. The resulting solution was allowed to stand for 45–60 min; and absorbance was read at 530 nm using water similarly treated as reference

4.3 Enzyme Preparation and Assay Procedures

Enzyme activity and amount of enzyme produced were determined in the crude enzyme extracted from the canola meal culture during the fermentation process. The crude enzyme was extracted from the meal using 2% aqueous solution of $CaCl_2.2H_2O$. The meal to extractant ratio was 1:5 (unless otherwise stated). After properly mixing the solid culture (with sterilized spatula) to obtain representative samples for analysis, the enzyme was extracted from 1 g samples using 5 ml of the extractant by continuous shaking on a rotary shaker (200 rpm) for 1 h. The liquid was squeezed out and filtered through a double layer cheese cloth and centrifuged (5000 × g, 15 min; 4°C). The clear supernatant was designated as the crude enzyme.

Enzyme activity was assayed by measuring the amount of phosphorus released by using sodium phytate as substrate. A reaction mixture consisted of 0.2M acetate

buffer (pH 4.7), 1 ml of 1.5 mM sodium phytate and 0.1 ml of the crude enzyme. The reactions were carried out at 60°C for 10 min, and were stopped by adding 5 ml of 10% TCA. Spectrophotometric determination of the released phosphorus was done by the Taussky-Schoor reagent as described by Harland and Harland (1980).

One unit of enzyme activity was defined as the amount of enzyme required to release 1 mg of phosphorus from 1 ml of 1.5 mM sodium phytate solution per hour at the given temperature and pH.

4.4 Biomass (from solid culture) Assay Procedures

Biomass in the solid culture was measured by determining the glucosamine content in the culture; and the method of biomass hydrolysis and neutralization was adopted from Sakurai et al., (1976). About 0.5 g samples were taken into test tubes. The samples were immersed in 60% H_2SO_4 (final concentration, counting the moisture in the sample), and held for 24 h at 25°C. The mixture was then diluted with distilled water (18.3 times dilution by volume) into 1N of H_2SO_4 in 100 ml Erlenmeyer flask, and the top of the flask was sealed with four layer of polyvinylidene wrap and rubber band. The flask was autoclaved at 101.325 kPa (121°C) for 1 h. The mixture was neutralized with 1N NaOH to about pH 7, and diluted to a definite volume (an adequate extent was in 100 to 250 ml). The glucosamine liberated was estimated colorimetrically by Blix's method (1948). The amount of biomass was determined from the graph of biomass versus glucosamine (Appendix B).

The relationship between glucosamine and biomass was established as follows:

The microorganism was grown in 500 ml Erlenmeyer flasks containing 150 ml of liquid medium. The dry biomass and glucosamine contents of the medium were determined at intervals of 24 h; and the relationship between the two variables obtained (Appendix B).

Chapter 5

Results and Discussions

This chapter gives the quantities of some relevant components of canola meal; discusses some characteristics of the phytase enzyme. The chapter also discusses the effects of some physical and chemical factors, namely: inoculum concentration, inoculum age, time of homogenization, initial moisture content, and initial pH on phytase production and phytic acid content reduction.

Some nutritional factors like added glucose, surfactants and added phosphate are also discussed as regards to their effects on phytase production and phytic acid content reduction.

5.1 Composition of Canola Meal

Solid state fermentations were carried out with *A. ficuum* NRRL 3135 for the production of phytase and for the reduction of phytic acid content in canola meal by the enzyme produced during the incubation period. The commercially available meal was used in this work. As a result of this a number of the meal components relevant to this work were determined prior to further use of the meal, and are here compared with other reported values (Table 5.1).

Table 5.1: Some components of Canola meal.

components	This work (%)	Literature values (%)	References
Moisture	8	8–11	[1,4,6]
Crude Protein	33	34–38	[1,4,2,10]
Available Carbohydrates	13.5	9–15	[8,3,11]
Phytic Acid	6	3–7	[1,4,2,5,6,7,9,12]

5.2 Enzyme Characteristics

Some characteristics of phytase were studied in order to determine the optimum conditions under which the enzyme assay had to be done. The length of incubation time and the effect of enzyme and substrate concentrations of the reaction mixture were considered.

The effect of incubation time on enzyme activity showed that for a period of incubation of 30 min, the relationship between the time of incubation and the enzyme activity was linear. The maximum activity of the enzyme was obtained at an incubation period of 40 min. After that period the activity leveled off. From these results, a period of 10 min was chosen for subsequent enzyme assay (Fig.5.1).

The graph illustrating the effect of enzyme concentration on its activity shows that an almost linear relationship existed between the amount of enzyme and its activity up to 1 ml of the enzyme in the system (Fig.5.2). With further increase in the enzyme concentration, the activity increased at a much lower rate. 0.1 ml of enzyme was used for subsequent enzyme assay.

Fig.5.3 shows the effect of substrate concentration on the enzyme activity. Sodium phytate was used as substrate. The Lineweaver-Burk plot of the results

(Fig.5.4), yielded a K_m value of 0.39 mM and a V_{max} of 5.7 units/h for this enzyme. Shieh et al. (1969) reported the K_m value of A. ficuum phytase to be 1.25 mM, using calcium phytate as substrate; Irving and Cosgrove, (1972; 1974) reported the K_m value of A. ficuum to be 0.013 mM, using sodium phytate as substrate; while Nair et al., (1990) reported a K_m value of 0.27 mM for A. ficuum, also using sodium phytate as substrate. My results are well within the reported range.

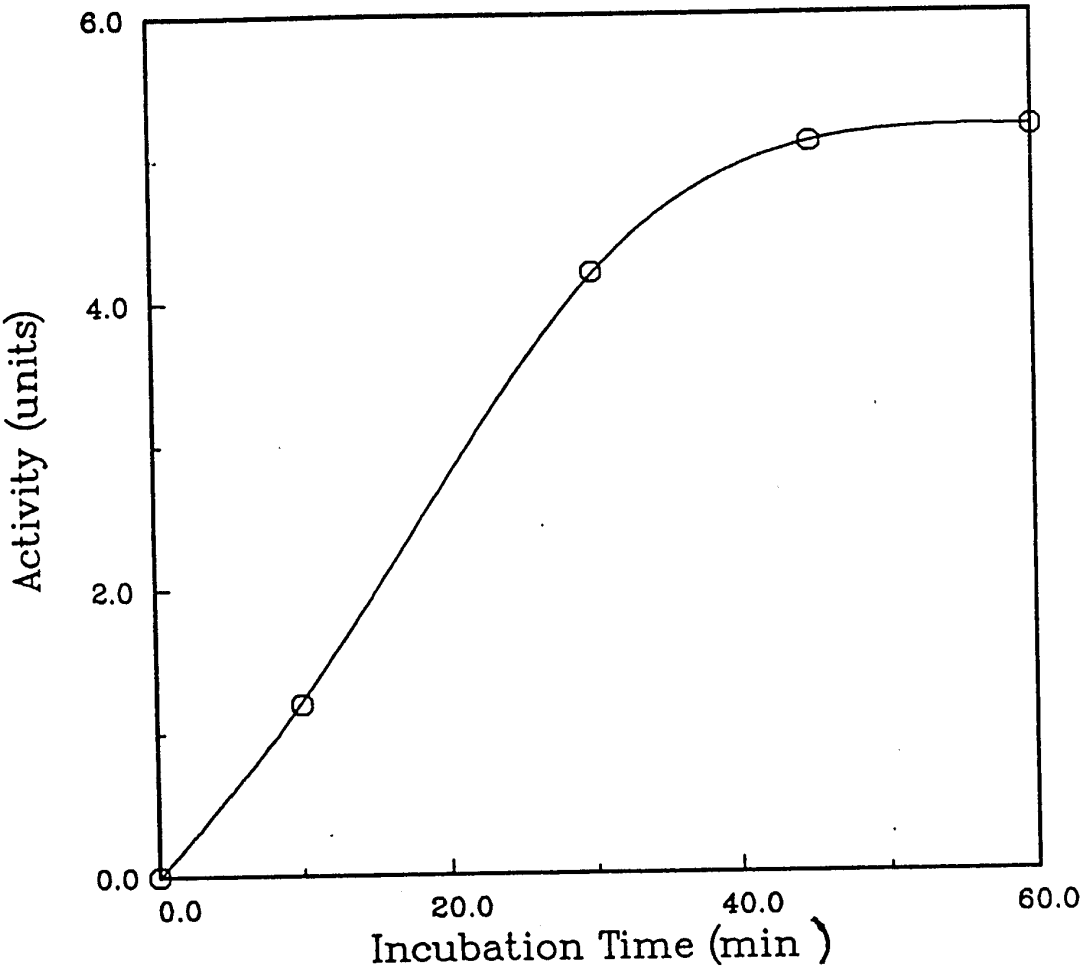

Figure 5.1: Effect of incubation time on enzyme activity at 60°C and pH 4.8

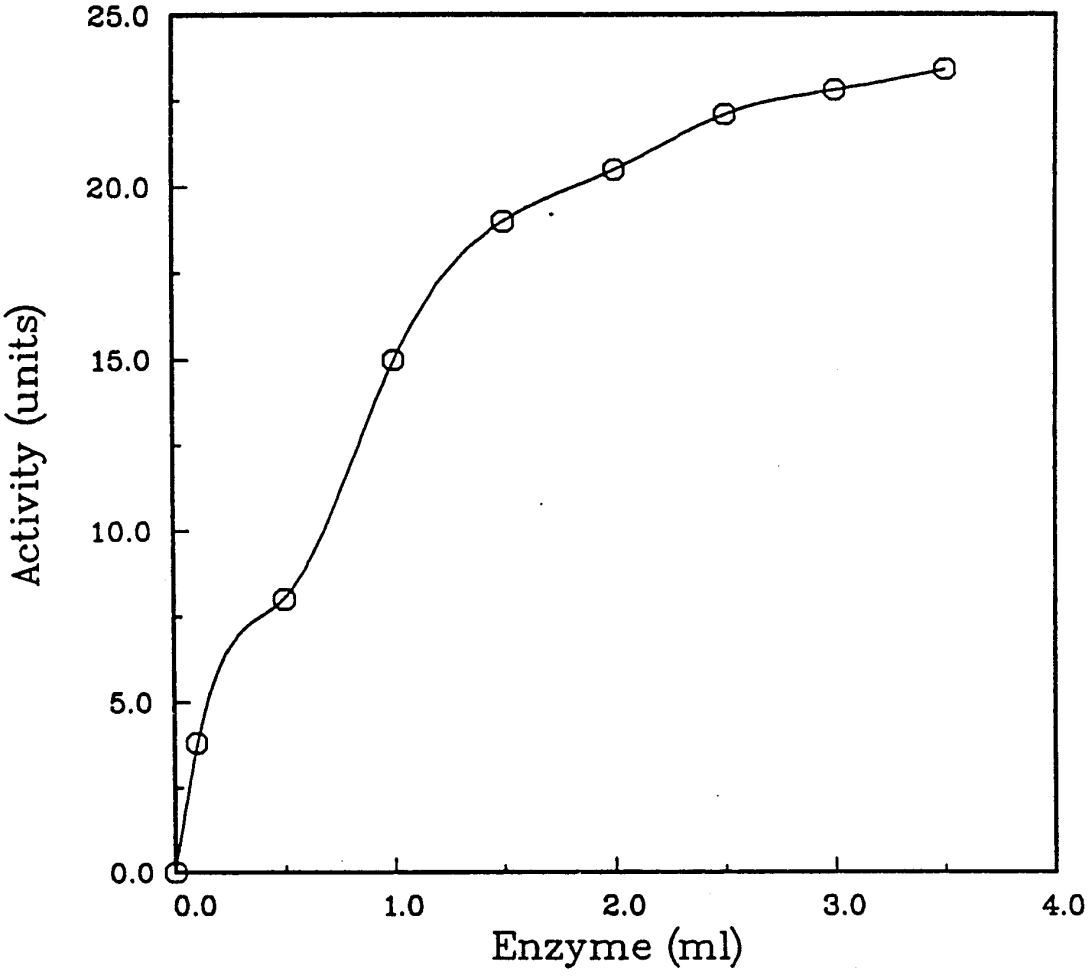

Figure 5.2: Effect of enzyme concentration on its activity at 60°C and pH 4.8

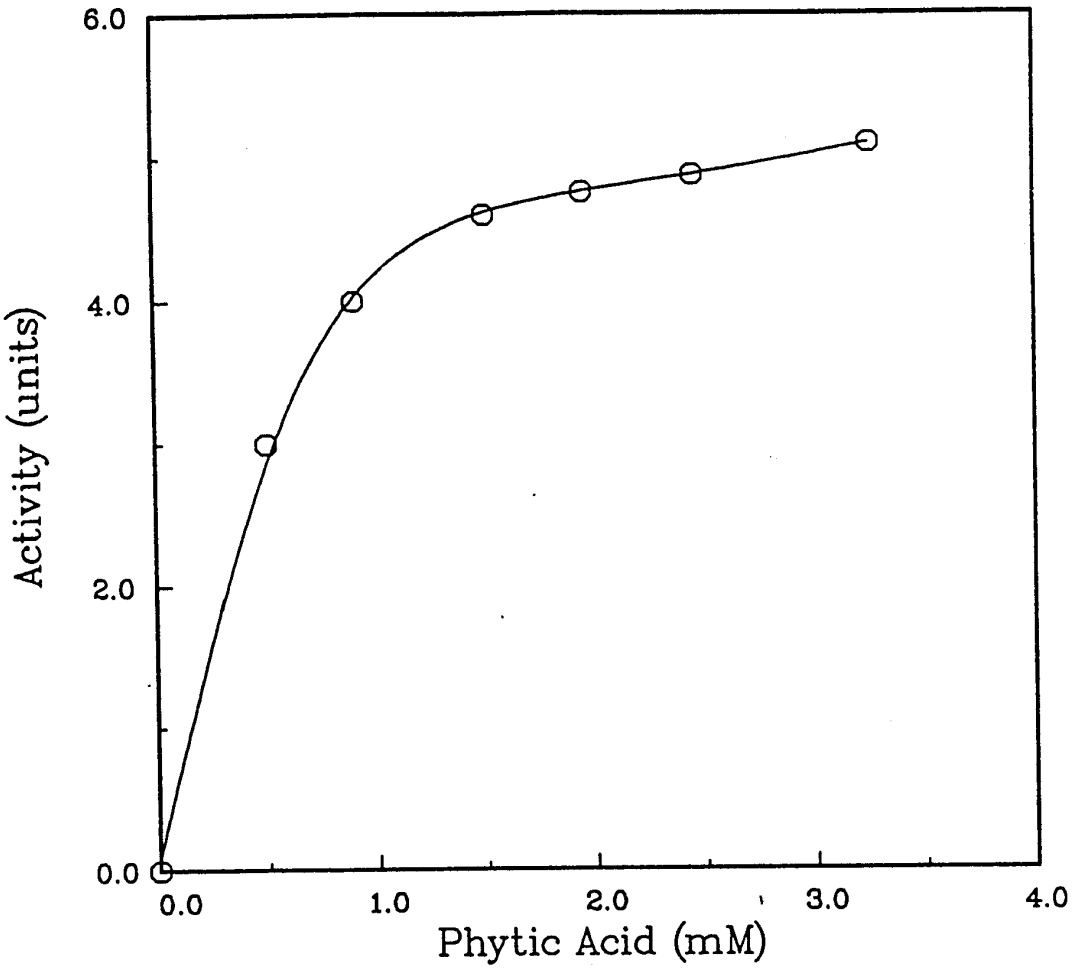

Figure 5.3: Effect of phytic acid concentration on enzyme activity at 60°C and pH 4.8

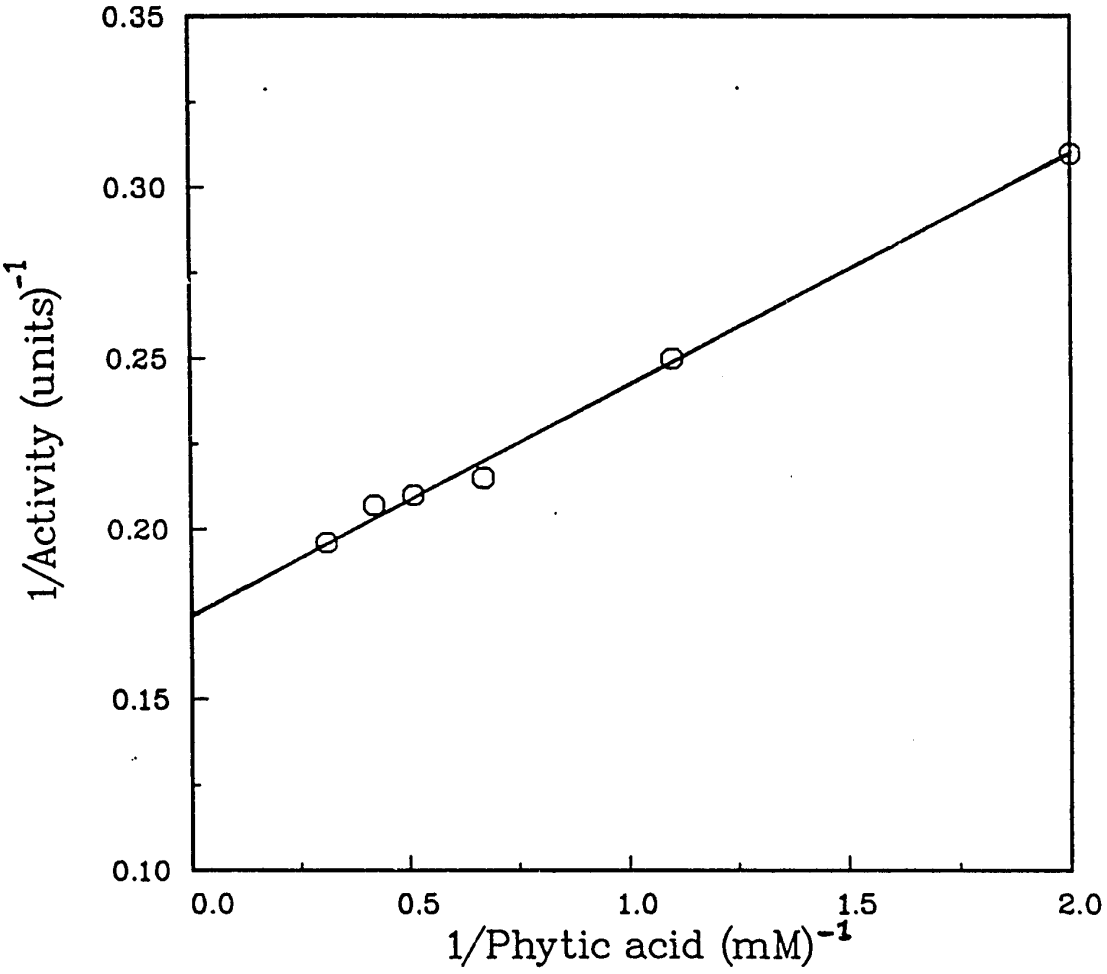

Figure 5.4: Determining K_m and V_{max} values of the enzyme

5.3 Physical and Chemical Factors

In this section, the effects of inoculum concentration, inoculum age, time of homogenization, initial moisture content, and initial pH of medium on enzyme production and phytic acid reduction are discussed.

5.3.1 Effect of Inoculum Concentration

It was necessary to determine the effect of inoculum concentration in order to minimize wastage of inoculum and also to optimize enzyme production and increase the rate of phytic acid content reduction. Fig.5.5 shows the effect of inoculum concentration on enzyme production. More enzyme was produced in medium having greater inoculum concentration. The buffered system was slower in enzyme production but by 96 h of incubation had produced as much enzyme as that of the unbuffered system at 72 h of incubation.

The effect of inoculum concentration on the reduction of phytic acid content in the solid medium is shown in Fig.5.6. The phytic acid content of the medium was completely eliminated in each of the systems regardless of the inoculum concentration; but the rates of the process differed. The medium having inoculum which contained 130 mg biomass required 62 h for complete phytic acid hydrolysis; while that with inoculum containing 170 mg biomass needed only 54 h for complete phytic acid hydrolysis. The buffered medium with 130 mg biomass required the longest period of time to achieve complete phytic acid elimination. Hence with increase in inoculum concentration of up to 170 mg/solid medium, improved enzyme production and increased rate of phytic acid reduction were obtained for unbuffered systems. Nair, V., 1991 also found a similar trend in a submerged culture for phytic

acid content reduction.

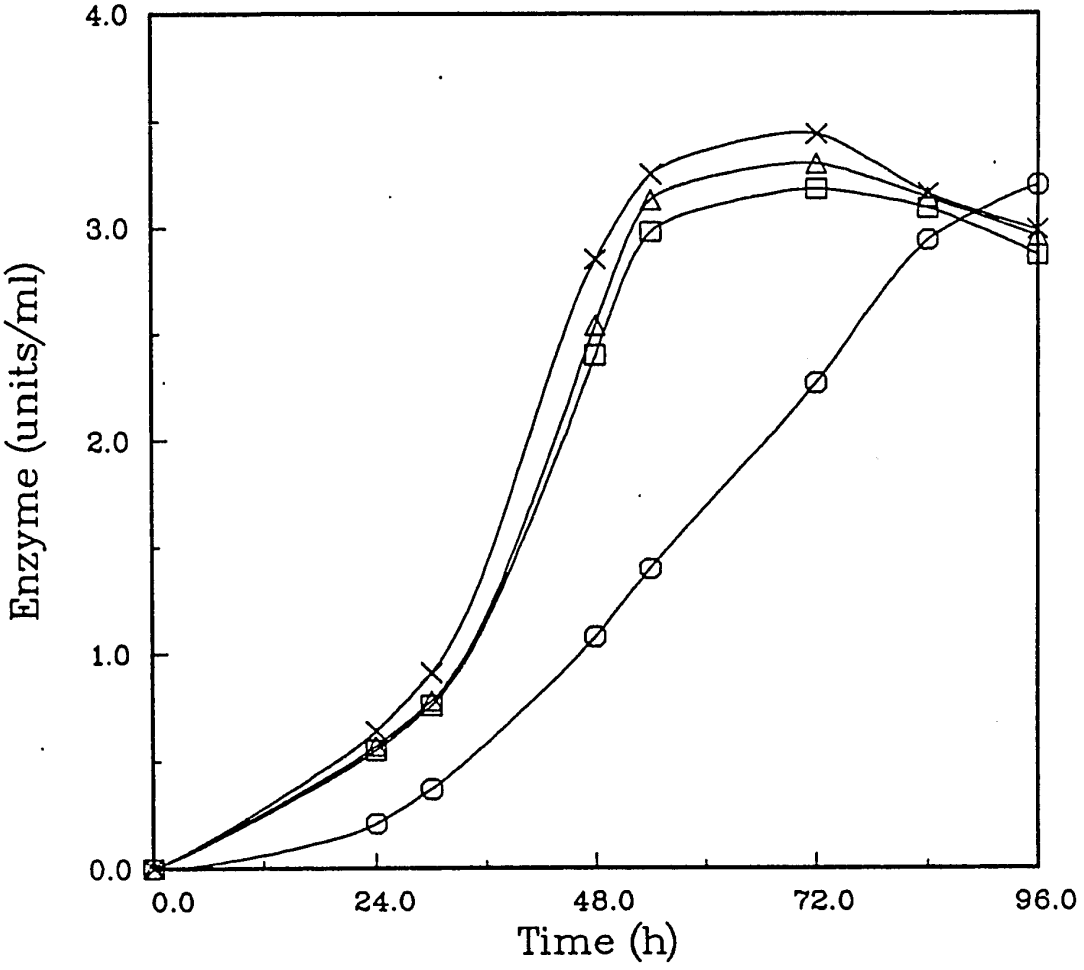

Figure 5.5: Effect of inoculum concentration enzyme production. Inoculum (mg) (□–130, ○–130 (in buffer), △–150, ×–170)

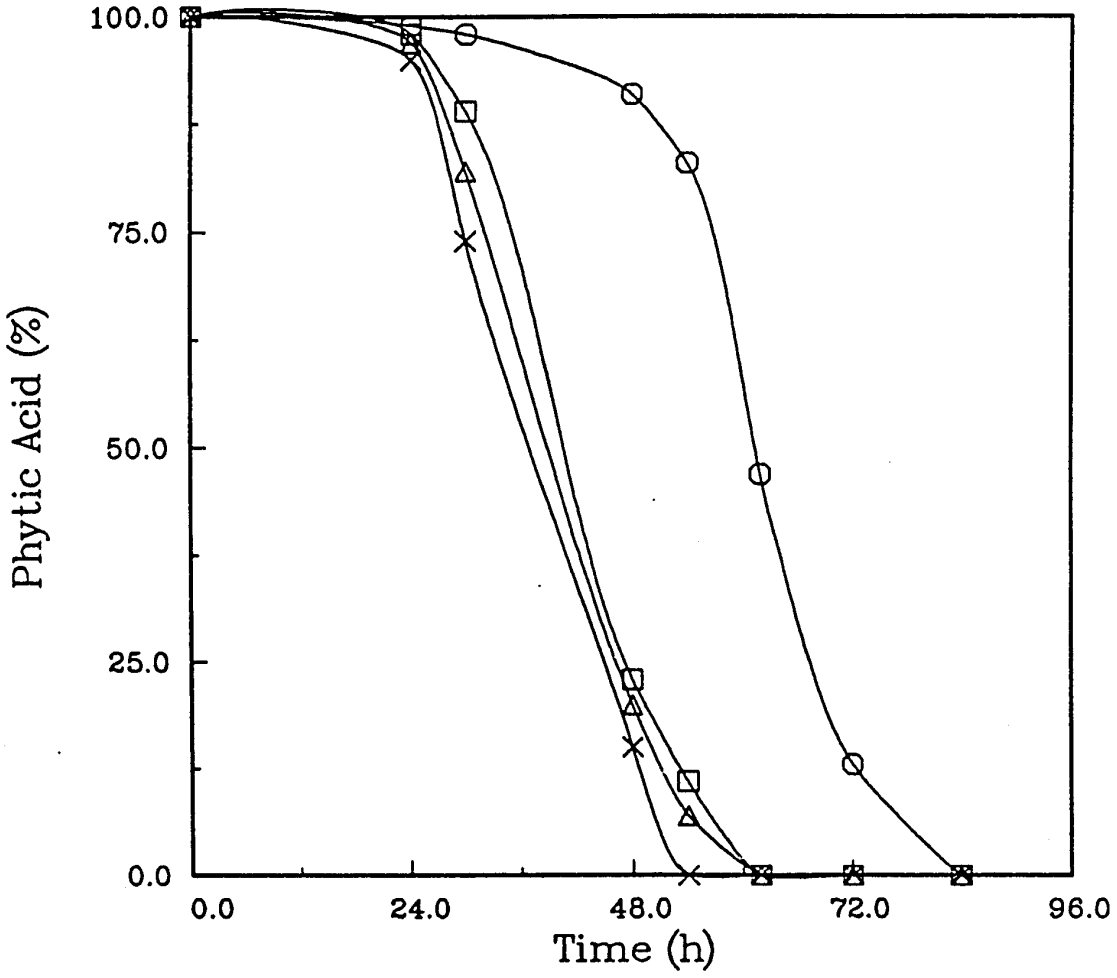

Figure 5.6: Effect of inoculum concentration on phytic acid content reduction. Inoculum (mg) (□–130, ○–130 (in buffer), △–150, ×–170)

5.3.2 Effect of Inoculum Age

Microorganisms undergo different stages during their growth cycle. The possibility of these various stages influencing enzyme production and phytic acid content reduction were taken into consideration by using inoculum of different ages for inoculation. The age range of 2–7 days were considered.

The age of inoculum used in the solid state culture was found to affect the amount of enzyme produced in the culture. The oldest inoculum (7–days) produced the largest amount of enzyme; while the youngest produced the least. The lag phase in Fig 5.7 could be explained as due to the breaking away of the cells after the third day of cultivation; hence a period of reconstruction of cell structure was required after the inoculum had been introduced into the solid medium. But the breaking away of cells in the liquid medium caused it to be better distributed in the solid medium and also produced more growth centres; hence the high enzyme production exhibited by these cultures.

The rate of phytic acid content reduction was highest for the oldest inoculum (7–days), and the lowest rate was obtained for the youngest inoculum (2–days old) (Fig.5.8). These results were obtained for the same reasons as given for enzyme production.

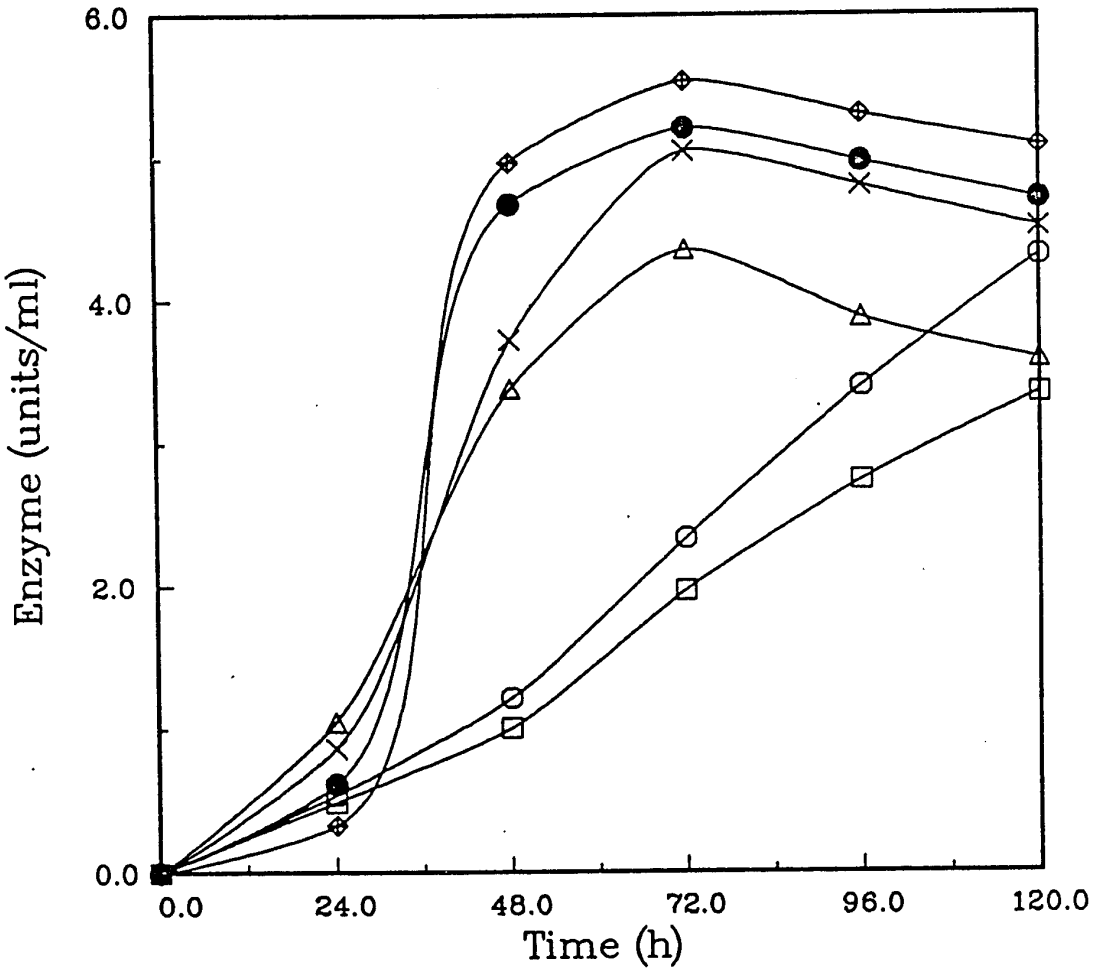

Figure 5.7: Effect of inoculum age on enzyme production. Inoculum age (days) (□–2, ◠ –3, △–4, ×–5, •–6, ⊕–7)

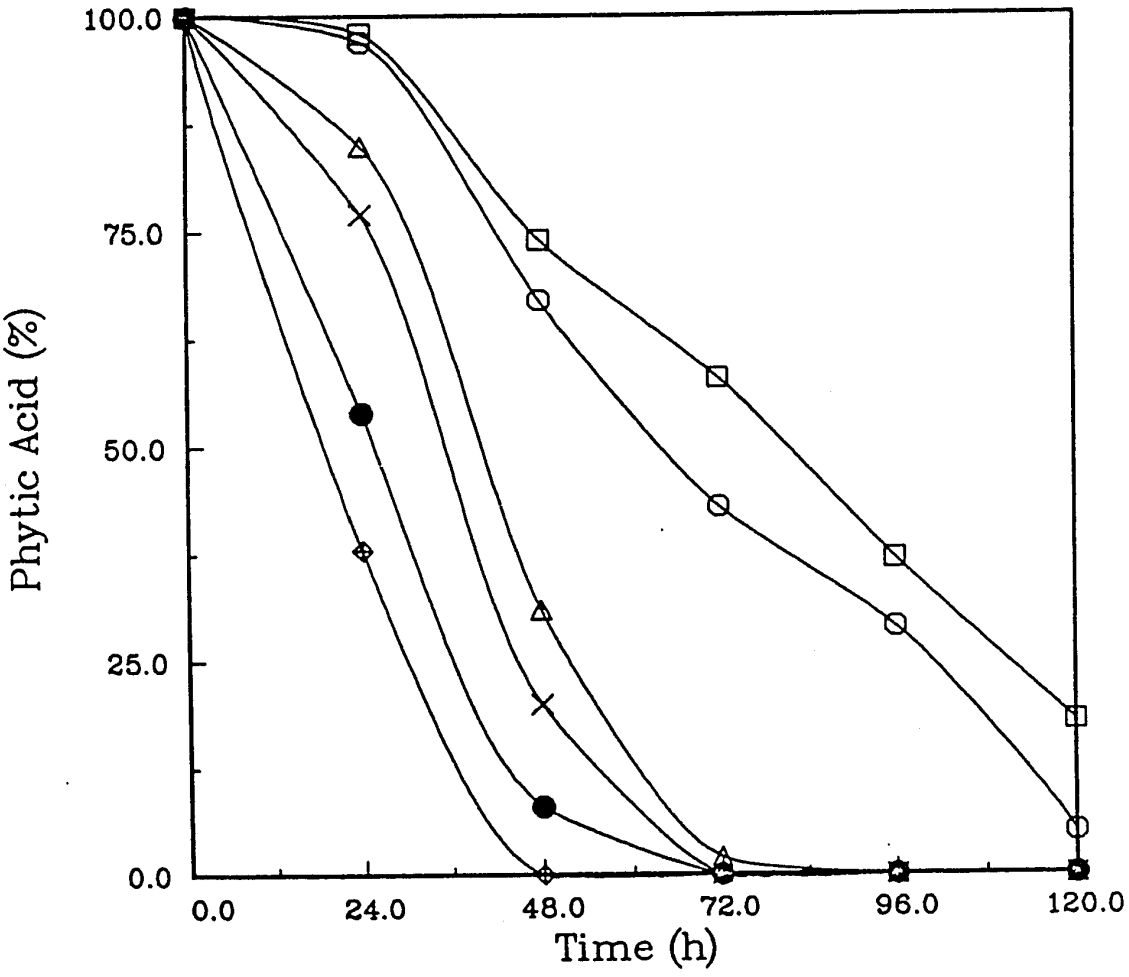

Figure 5.8: Effect of inoculum age on phytic acid content reduction. Inoculum age (days) (□–2, ○ –3, △–4, ×–5, •–6, ⊕–7)

5.3.3 Effect of Time of Homogenization

As explained in section 4.1.2, homogenization of inoculum was necessary. There was therefore the need to determine the effect of various times of homogenization of inoculum on the production of enzyme and the reduction of phytic acid content.

Homogenization destroyed the mycelia of the microorganism which therefore required a period for reconstruction before the production of enzymes in the solid medium. Hence the inoculum which was homogenized for the shortest length of time had least destruction of mycelia and could therefore begin faster production of enzymes; while the inoculum homogenized for a longer period of time showed an initial lag phase wherein the mycelia were being reconstructed. Fig 5.9 suggests 36 hours to be the length of time required for cell reconstruction. But due to the fact that inoculum homogenized for longer periods had better distribution in the solid medium and also had more growth centres, they showed better enzyme production after the lag phase.

Fig. 5.10 shows the enzyme production at particular times of fermentation in medium inoculated with inoculum homogenized for various times. At 24 h of incubation, the inoculum homogenized for 10 s had produced more enzyme than that homogenized for 240 s, as a result of the mycelia destruction in the latter case; but after 72 h, the system with inoculum homogenized for 240 s had produced almost twice as much enzyme as that produced by the system containing the inoculum homogenized for 10 s. Again, this could be explained by the better distribution in the nutrient source (the meal), and the presence of more growth centres of the inoculum homogenized for longer times.

The effect of time of homogenization of inoculum on the phytic acid content

reduction is shown in Fig. 5.11. The results show that the phytic acid content was reduced to zero in all of the systems but differed in their rates. The results show that the longer the time of homogenization the higher the rate of phytic acid content reduction. It could be suggested that homogenizing the inoculum freed the intracellular enzymes; hence the longer the homogenization time, the more enzymes the inoculum contained. Homogenization of the inoculum also caused it to be better distributed in the solid medium.

The phytic acid content reduction at particular times of fermentation in medium inoculated with inoculum homogenized for various times, is shown in Fig. 5.12. At 24 h of incubation the inoculum homogenized for 240 s had reduced the phytic acid content to 71%, while the inoculum homogenized for 10 s had reduced the phytic acid content to 86%. At 48 h the inoculum homogenized for 240 s had obtained complete reduction of the phytic acid content; while the system containing inoculum homogenized for 10 s still had 11% of the phytic acid content present in the meal.

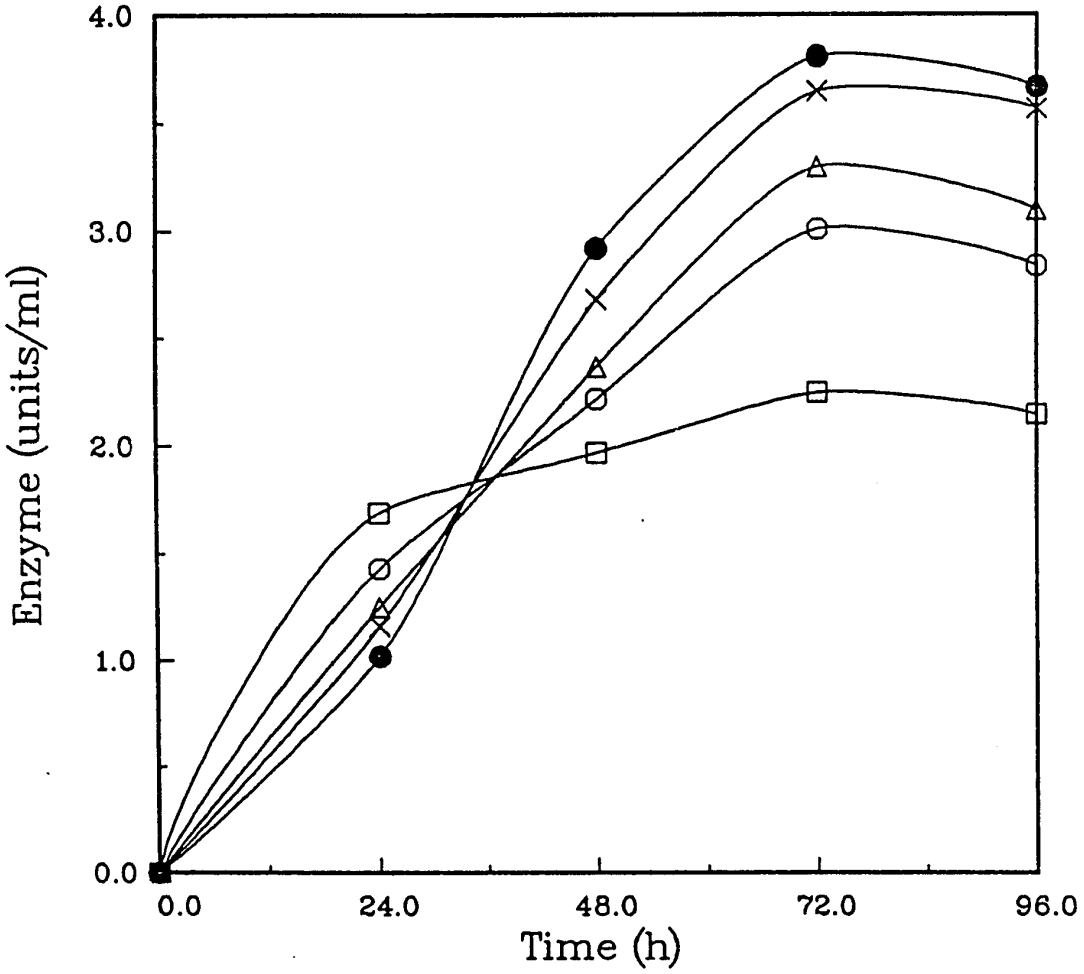

Figure 5.9: Effect of time of homogenization of inoculum on enzyme production. Time (seconds) (□–10, ○–30, △–60, ×–120, •–240)

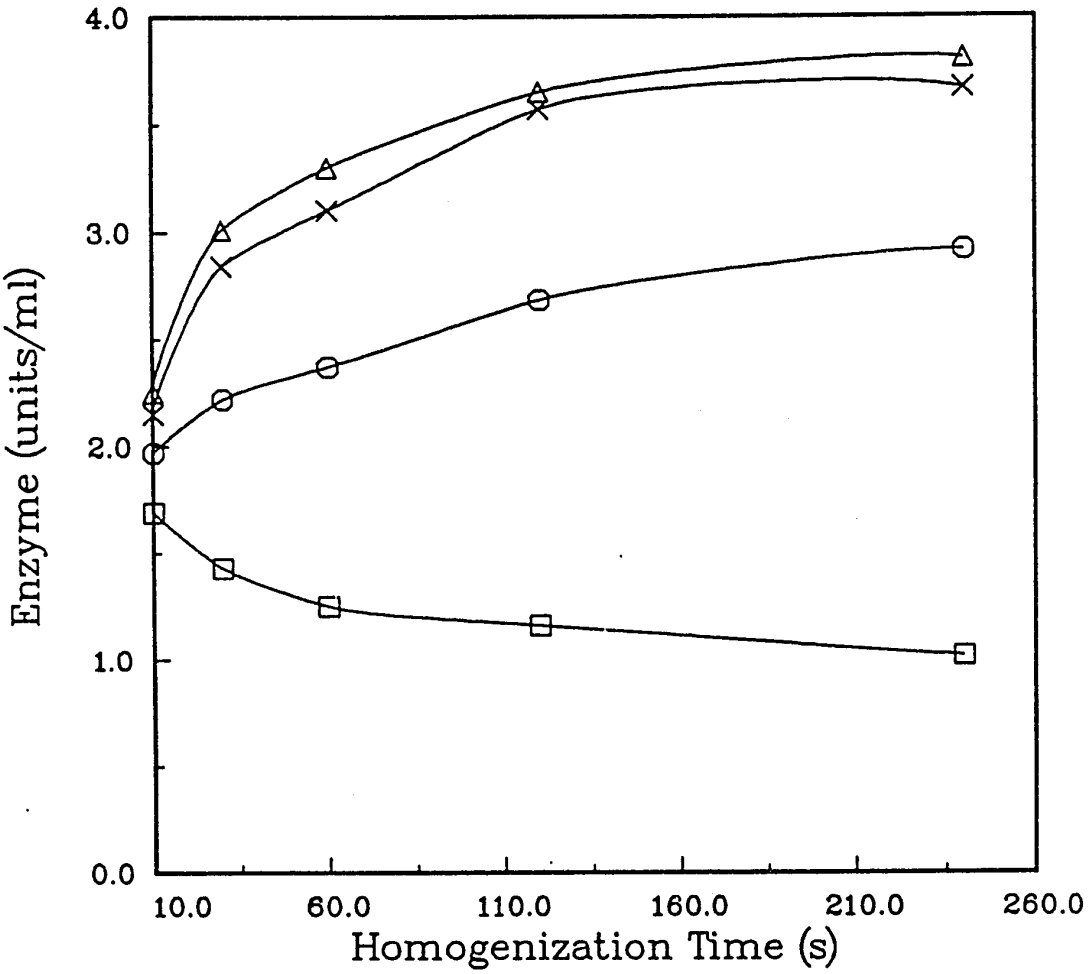

Figure 5.10: Enzyme production at particular times of fermentation in medium inoculated with inoculum homogenized for various length of time. Time of fermentation (h) (□–24, ○–48, △– 72, ×–96)

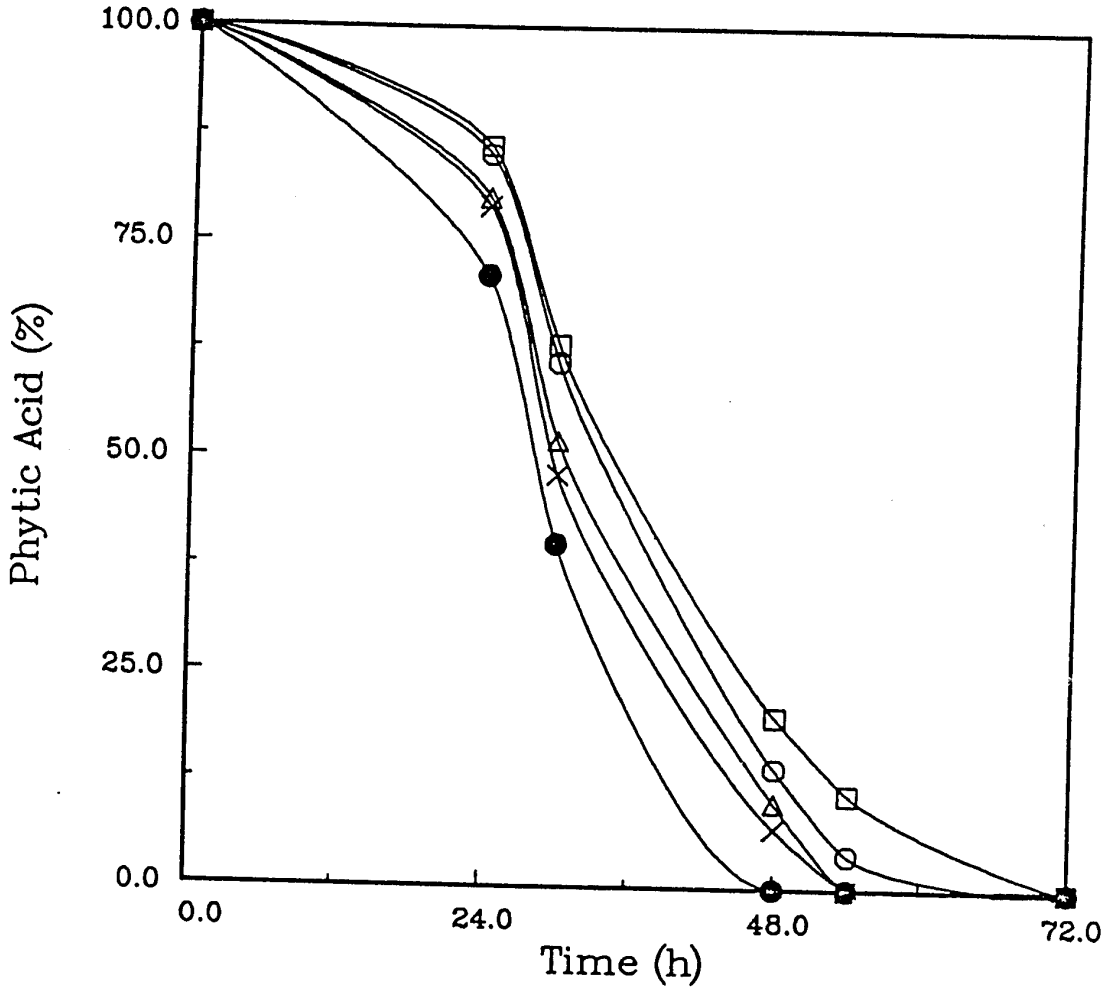

Figure 5.11: Effect of time of homogenization of inoculum on phytic acid content reduction. Time (seconds) (□–10, ○–30, △–60, ×– 120, •–240)

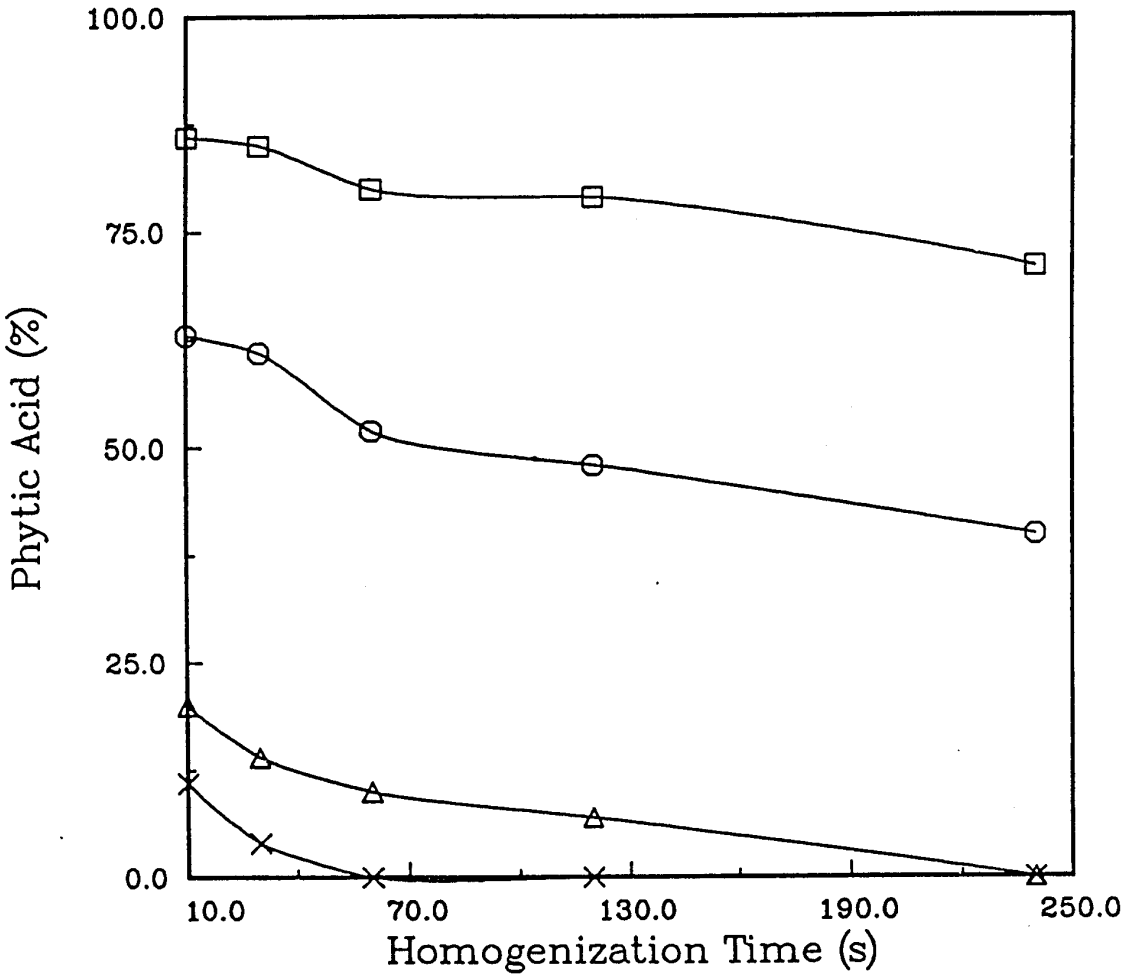

Figure 5.12: Phytic acid content reduction at particular times of fermentation in medium inoculated with inoculum homogenized for various length of time. Time of fermentation (h) (□–24, ○–30, △– 48, ×–54)

5.3.4 Effect of Initial Moisture Content

The water content of substrate plays an important role in both cell growth and enzyme production in a solid state fermentation. Addition of water causes swelling of the substrate and facilitates its utilization by the microorganisms. However, the optimal amount of water required varies and must be determined for each system and microorganism (Cannel and Moo-Young, 1980).

The results in Fig.5.13 show that the amount of enzyme produced increased as the moisture content increased from 21 to 50%, and since 50% moisture content gave improved enzyme production and could not bring about complete reduction of phytic acid content, higher moisture contents of medium were considered.

The phytic acid content in the medium having 50% moisture content had been reduced to about 20% after 120 h of incubation; while the system which had 31% moisture content still contained 75% phytic acid and that with 21% moisture content showed no apparent phytic acid content reduction (Fig.5.14).

Fig.5.15 shows that when the moisture content was increased, even further improved enzyme production was obtained. The system with the highest moisture content of 68% produced less enzyme than the 50% moisture content medium. The optimum moisture content for enzyme production was found to be 64%.

The rate of phytic acid content reduction in the higher moisture content systems showed that complete phytic acid content reduction was obtained at 96 h of incubation in the medium having 64% moisture content. The system with a higher moisture content of 68% was still found to contain 5% of the phytic acid after 96 h (Fig.5.16). A similar trend of results were obtained by Han et al., (1987). They studied phytase production by *Aspergillus ficuum* using solid state cultivation on

several cereal grains and legume seeds. They found that higher moisture content yielded low enzyme as a result of substrate adhering to the fermentor wall, and hence preventing the access of air to the substrate. The texture of the substrate became soggy as the fermentation proceeded, perhaps as a result of microbial metabolism.

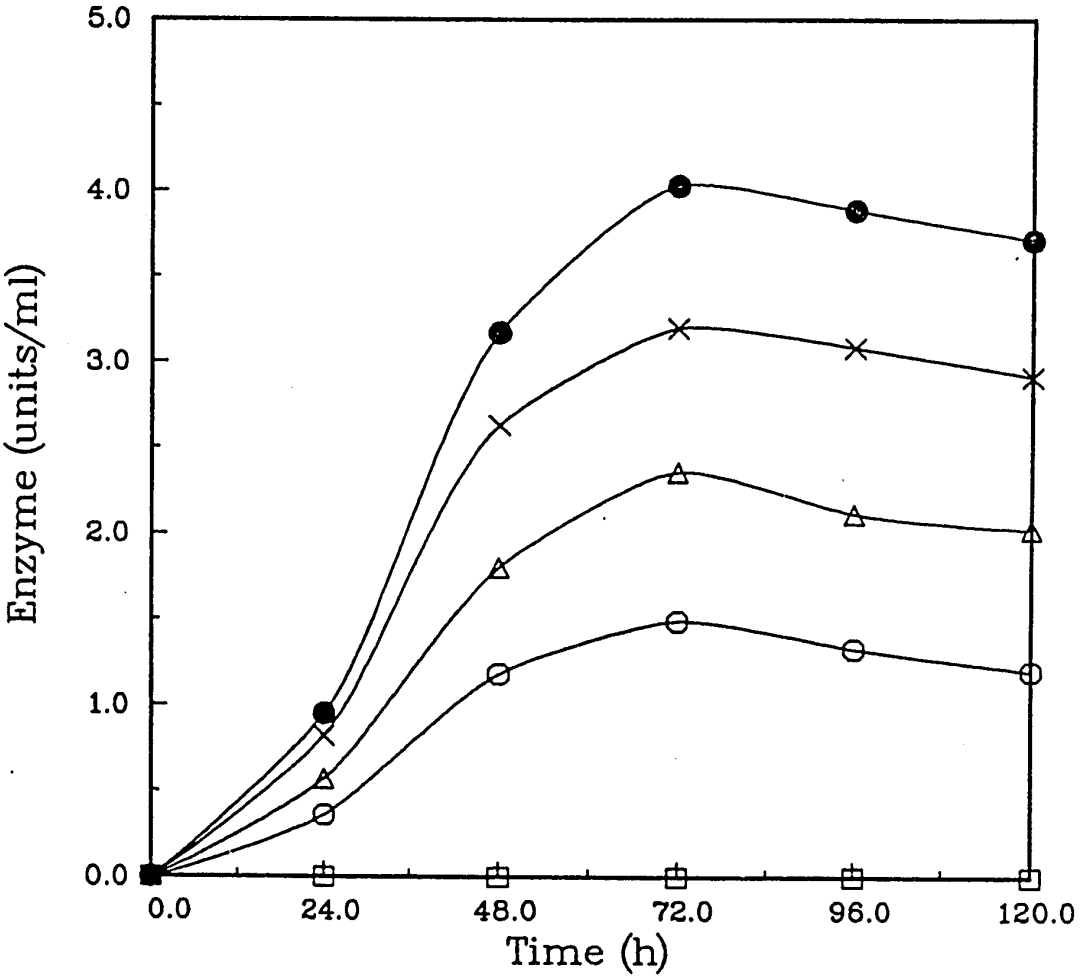

Figure 5.13: Effect of initial moisture content on enzyme production. Moisture content (% w/w) (□–21, ○–31, △- -39, ×–45, •–50)

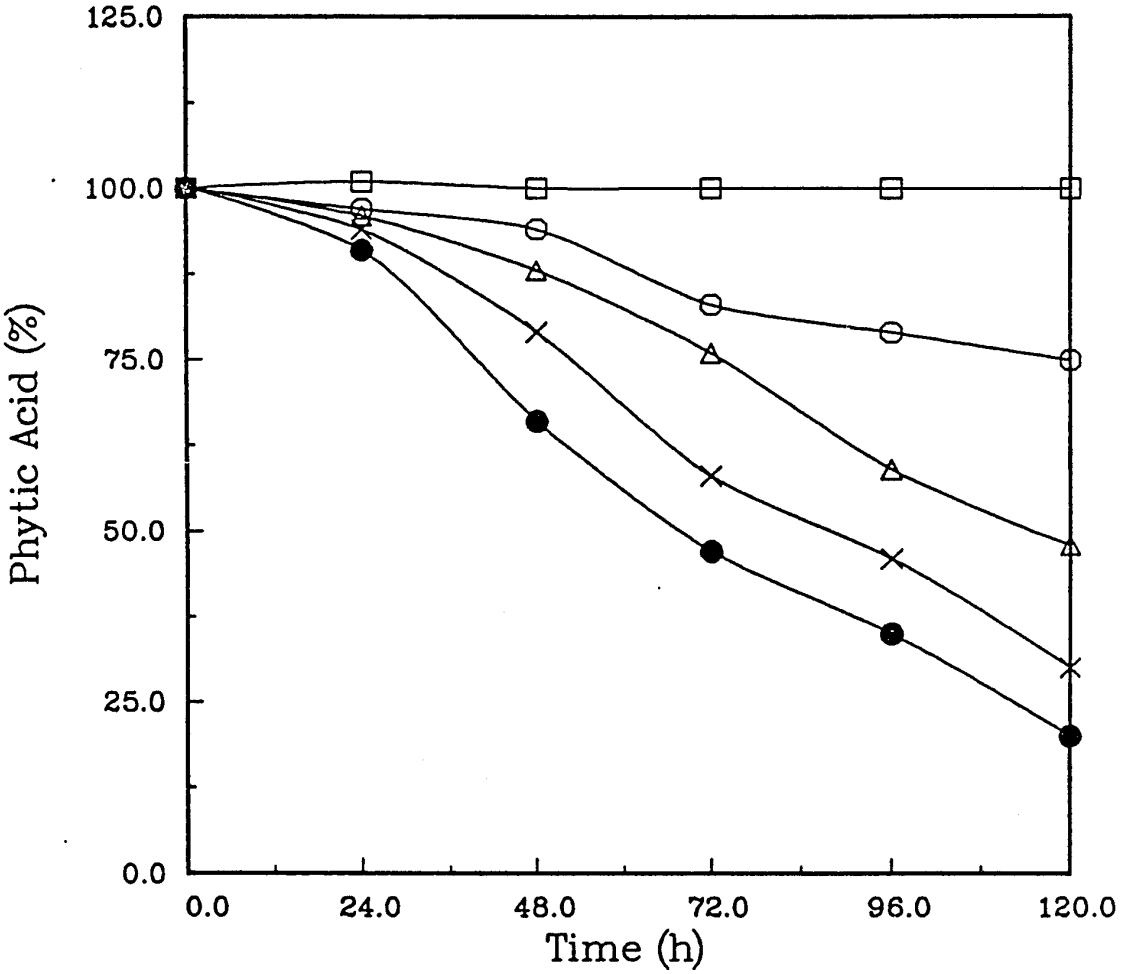

Figure 5.14: Effect of initial moisture content on phytic acid content reduction. Moisture content (% w/w) (□–21, ○–31, △–39, ×–45, •–50)

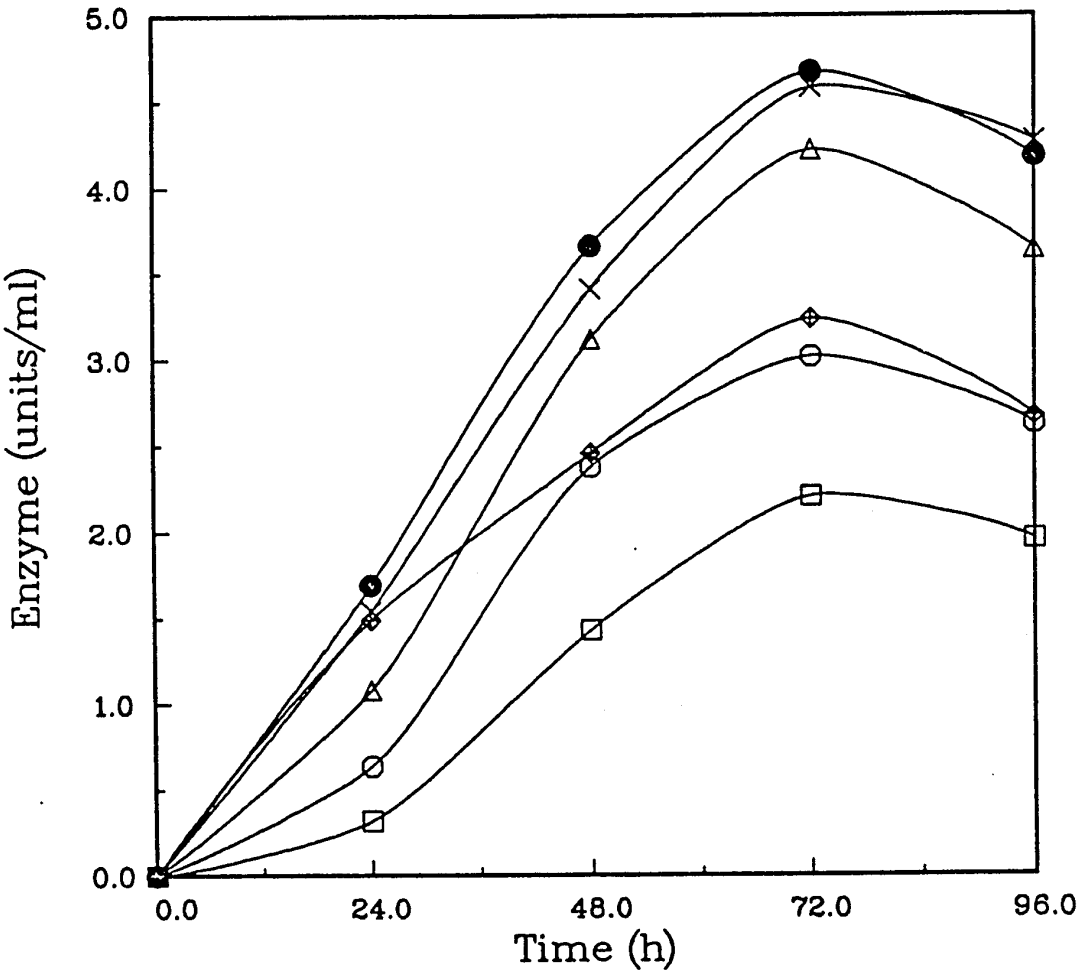

Figure 5.15: Effect of higher initial moisture content on enzyme production. Moisture content (% w/w) (□–31, ○ –39, △- -50, ×–60, •–64, ◈–68)

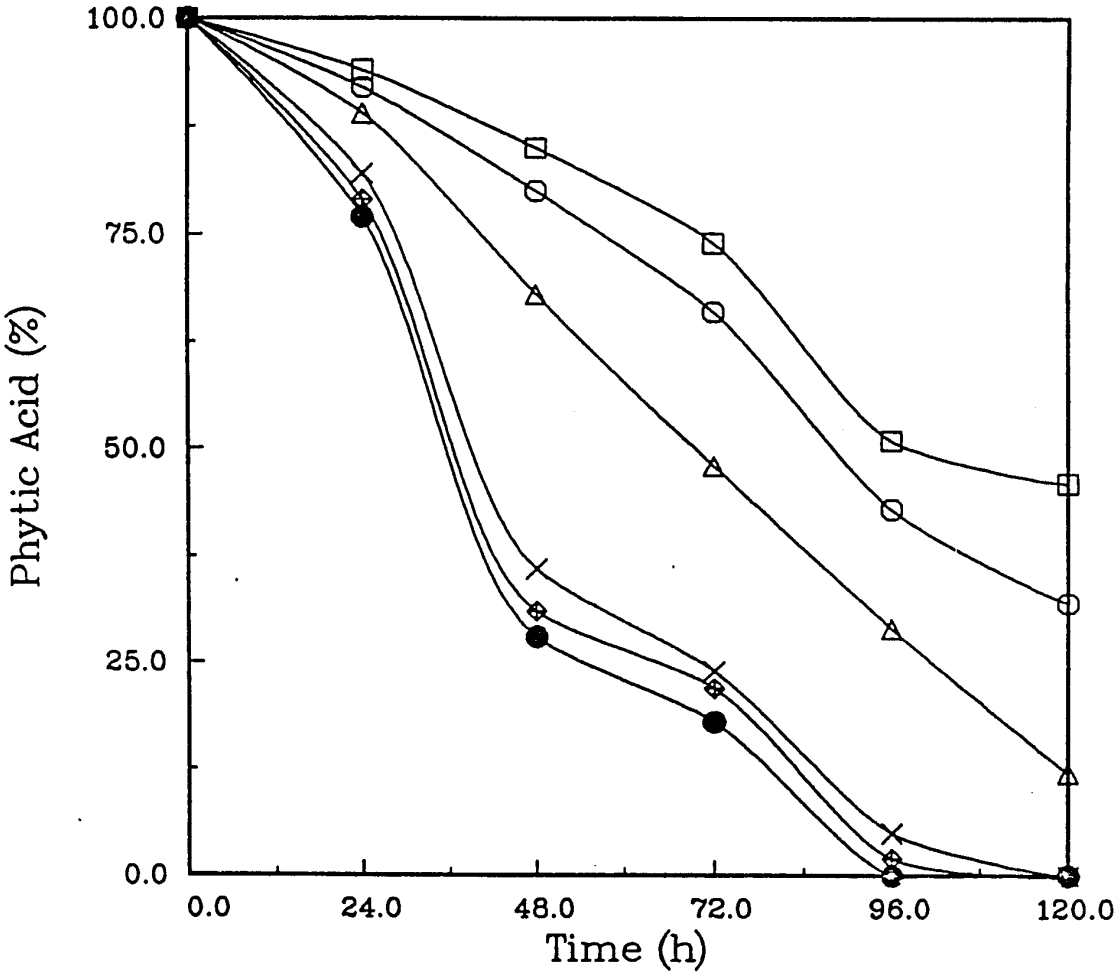

Figure 5.16: Effect of higher initial moisture content on phytic acid content reduction. Moisture content (% w/w) (□-31, ○-39, △- -50, ×-60, •-64, ⊕-68)

5.3.5 Effect of Initial pH

pH has been found to greatly influence phytase activity. Various researchers have found different optimum values based on the overall conditions of the systems used. Irving and Cosgrove, 1974 and Ullah, 1988, using partially and highly purified phytase of *Aspergillus ficuum* respectively found the optimum pH to be 5.3 and 5.0. But an average pH range of 4.6–5.8 has been found to be optimum for phytase activity. It is necessary to determine an optimum pH value or range for growth of microorganism and production of enzymes. In this study, a pH range of 5–5.7 was considered.

The level of enzyme production was found to be dependent on the initial pH of the medium. Fig.5.17 shows that the enzyme production in both systems with pH 5.7 increased steadily to a peak during 72 h of incubation; while those with lower pH values exhibited a lag in the first 48 hours of their incubation, and then increased steadily to the 96 h; the system with pH 5.0, producing the least amount of enzymes.

The effect of initial pH of medium on phytic acid content reduction is shown in Fig.5.18. The rate of the phytic acid content reduction was found to increase with increase in initial pH of medium of 5.0 to 5.7. The systems with initial pH 5.7 (one with distilled water, and another with buffer) had complete reduction in the phytic acid content after the 96 h of incubation; while the system with pH 5.4 still had 12% of the phytic acid content; that with pH 5.2 had 31%; and the pH 5.0 system had 36% of the phytic acid still present in the culture.

Since the systems containing distilled water produced the largest amount of enzyme and also gave the fastest rate of phytic acid content reduction, distilled

water could be preferably used instead of buffer to achieve these goals in order to reduce cost.

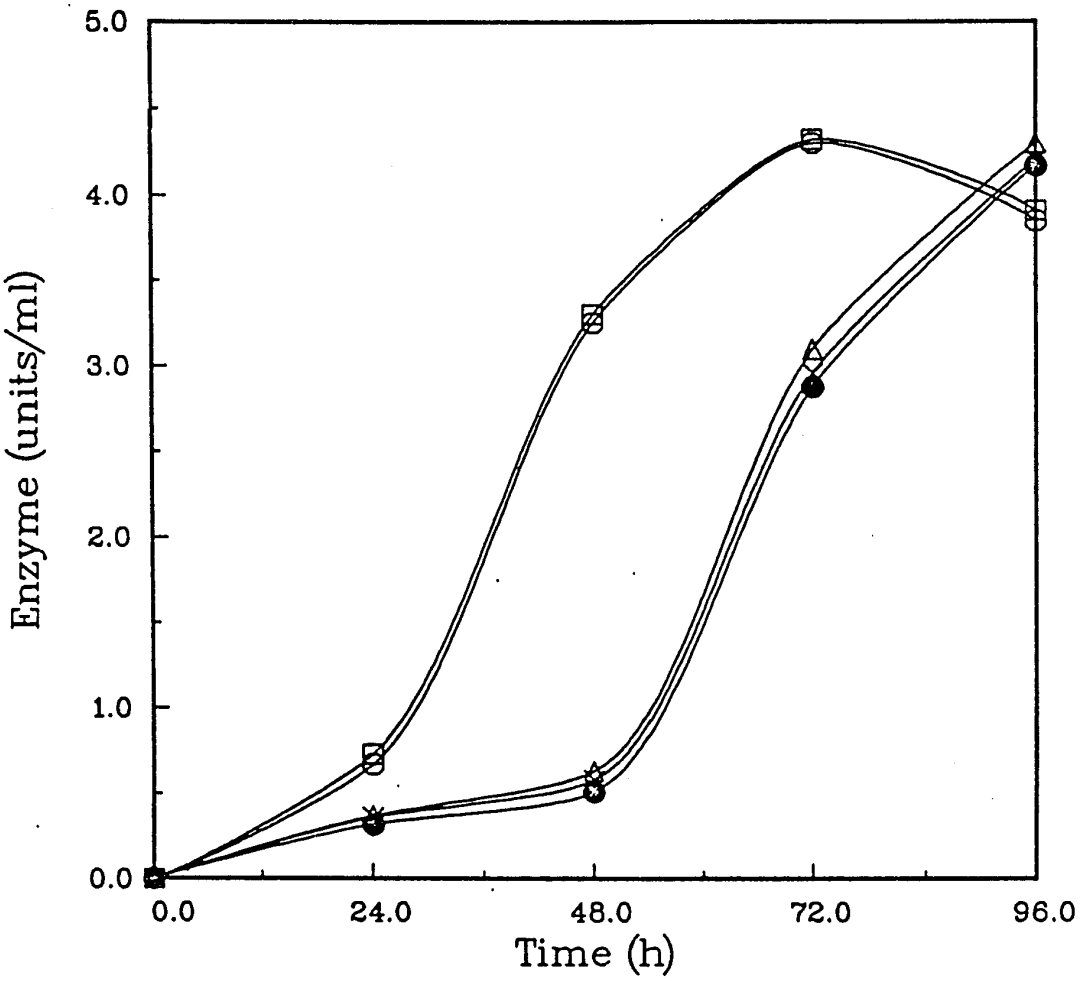

Figure 5.17: Effect of initial pH on enzyme production. (□–distilled water 5.7, ○ –buffer 5.7, △– buffer 5.4, ×–buffer.5.2, •–5.0)

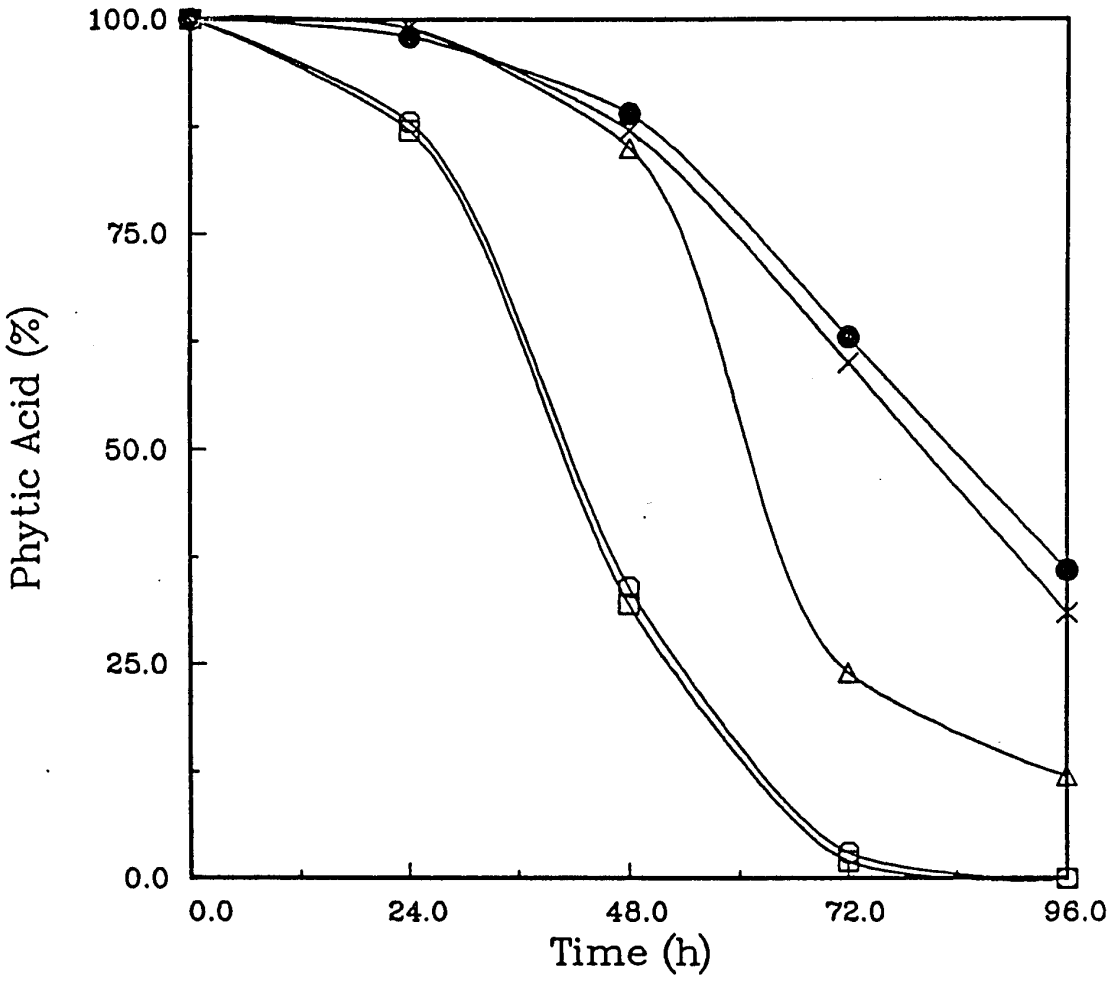

Figure 5.18: Effect of initial pH on phytic acid content reduction. (□–distilled water 5.7, ○–buffer 5.7, △– buffer 5.4, ×–buffer 5.2, •–5.0)

5.4 Nutritional Factors

The effects of supplementing the cultural medium with certain components, namely: Glucose, Tween–80, Triton X–100, sodium oleate, and Phosphate on enzyme production and phytic acid content reduction were studied, and are hereby discussed.

5.4.1 Effect of Added Glucose

Since glucose is one of the very often used available nutrient for the growth of the microorganism and for the production of products, the effect of increasing its concentration in the culture medium was considered. A concentration range of 2–24 g glucose/100 g solid medium was used.

The effect of added glucose on enzyme production is shown in Fig. 5.19. Better enzyme production was observed in systems containing added glucose of up to 6 g; while systems containing 12 g and 24 g of added glucose produced less and lesser amounts of enzyme, respectively. Therefore inhibition was caused by high glucose concentration. A similar trend of substrate inhibition was obtained by Nair, V. (1990) from a submerged culture to the effect that they applied a fed batch technique to enhance better enzyme production by eliminating the effect of high glucose concentrations.

The addition of glucose to the fermentation medium showed increased rates of phytic acid content reduction (Fig. 5.20). After 72 h of incubation the control system still contained 10% of the phytic acid content; the system containing 6 g of added glucose had attained complete phytic acid content reduction; while the systems with 12 g and 24 g of added glucose still had 21% and 48% of the phytic

acid content, respectively.

The effect of added glucose on biomass production in systems supplemented with glucose showed a similar trend as for the phytic acid content reduction and enzyme production. Increase in amounts of supplemented glucose up to 6 g improved biomass production; but further increase in the added glucose of 12 g and 24 g resulted in poorer biomass production (Fig. 5.21).

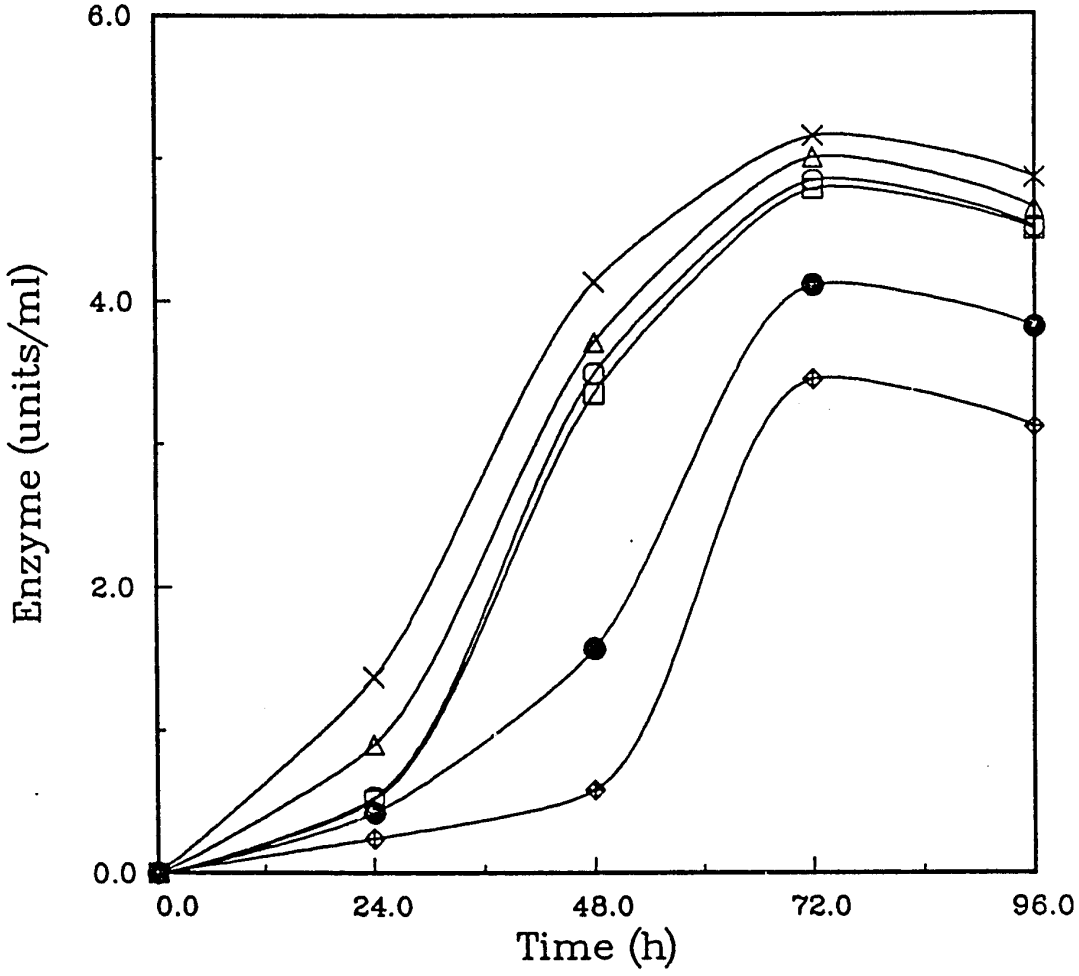

Figure 5.19: Effect of added glucose on enzyme production. Amount of added glucose (g) (□–no glucose, ○–2, △–4, ×–6, ●–12, ◆–24)

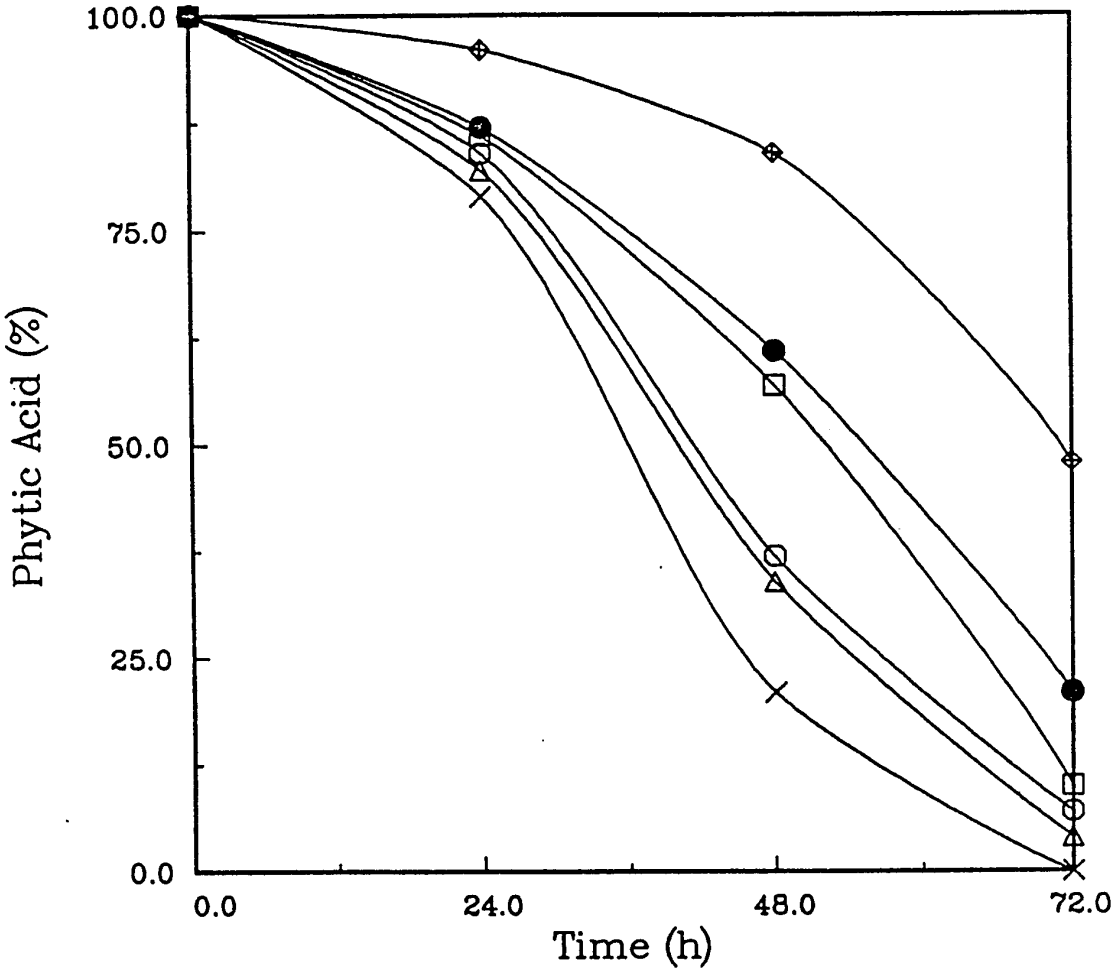

Figure 5.20: Effect of added glucose on phytic acid content reduction. Amount of added glucose (g) (□–no glucose, ○–2, △–4, ×–6, •–12, ⊕–24)

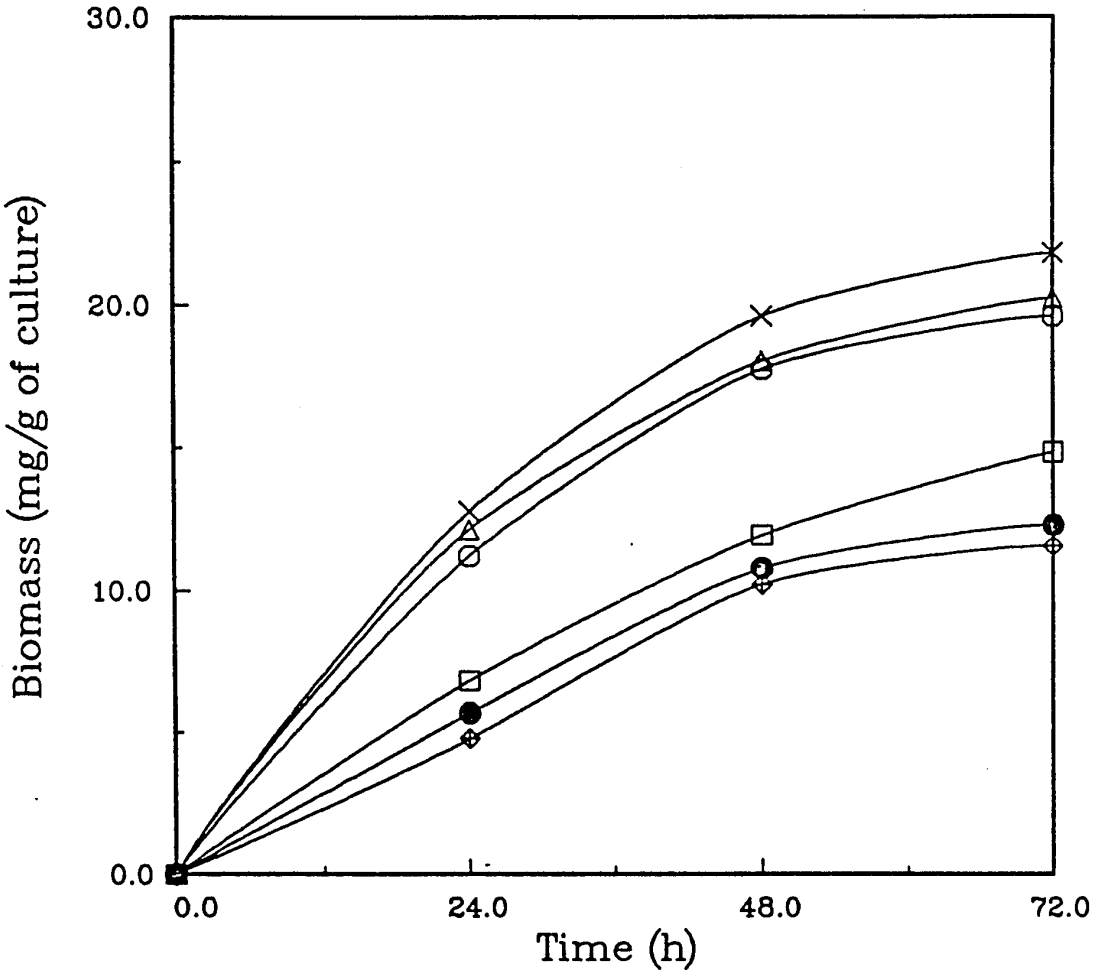

Figure 5.21: Effect of added glucose on biomass production. Amount of added glucose (g) (□–no glucose, ○–2, △–4, ×–6, ●–12, ◈–24)

5.4.2 Effect of Surfactants

Various surfactants have been used in bacterial cultures to assist in cell growth and enzyme production. It has been generally known that surfactants play a role in enzyme production and secretion, but explanation of how the surfactants act to increase the enzyme yield is largely conjectural (Reese and Maguire, 1969).

In this study, sodium oleate, Tween–80 and Triton X–100 were separately added to the solid medium to study their effects on phytase production and phytic acid content reduction. The addition of surfactants at the level of 0.5% to the solid medium was done.

The effect of surfactants on enzyme production is as shown in Fig. 5.22. The system with sodium oleate and Tween–80 produced more enzyme than the control system having no surfactant; with the system containing sodium oleate having the highest enzyme production, and the medium containing Triton X–100 producing less enzyme than the control system. Han and Gallagher 1987), working on liquid cultures also obtained an improved enzyme production in their system containing 0.5% sodium oleate in the culture medium. Reese and Maguire, (1969) also observed that addition of surfactants in culture medium generally increased the enzyme yield but the effects varied from organism to organism and enzyme to enzyme.

Enzyme production at particular times of incubation in a medium supplemented with various amounts of sodium oleate showed that, at the 48 h and 72 h of incubation the system with 0.1% sodium oleate consistently produced the largest amount of enzyme; while the system with 5% sodium oleate also consistently produced lesser amount of enzyme than the control system (Fig. 5.23).

The addition of certain surfactants into the solid culture proved to enhance the

phytic acid content reduction. Fig. 5.24 shows that the addition of sodium oleate resulted in a marked increase in the rate of the phytic acid content reduction. The effect of the addition of Tween–80 in the phytic acid content reduction was not very apparent; while Triton X–100 had a slight negative effect. Phytic acid content reduction at particular times of fermentation in medium supplemented with various amounts of sodium oleate, showed that after 42 h of incubation phytic acid had been completely eliminated in systems separately containing 0.1%, 0.5%, and 1% sodium oleate; while 9% of the phytic acid content was still present in the control system, and 22% in the system containing 5% sodium oleate. After 48 h of incubation no phytic acid was present in the control system but the system with 5% sodium oleate still had 13% of the phytic acid content (Fig. 5.25).

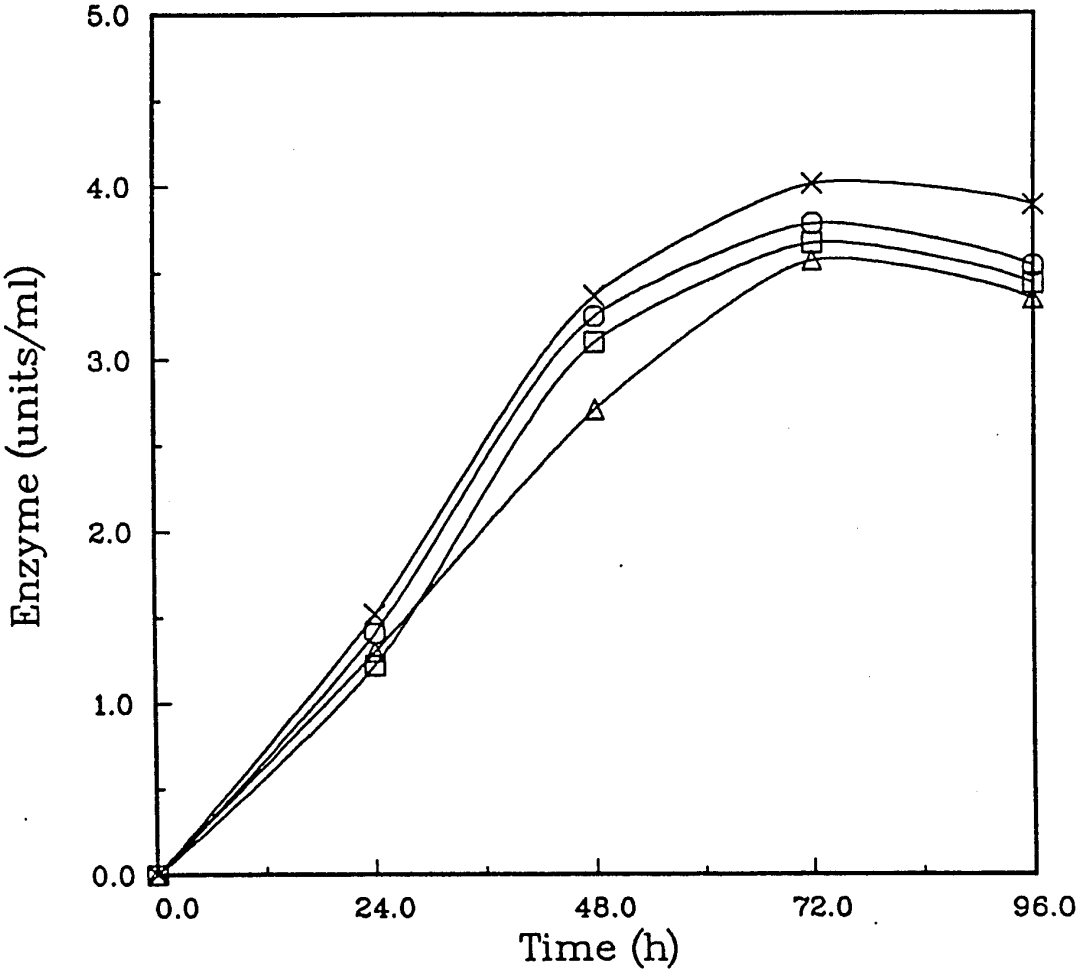

Figure 5.22: Effect of surfactants on enzyme production. (□–no surfactant, ○ –Tween–80, △–Trion X –100, ×– sodium oleate)

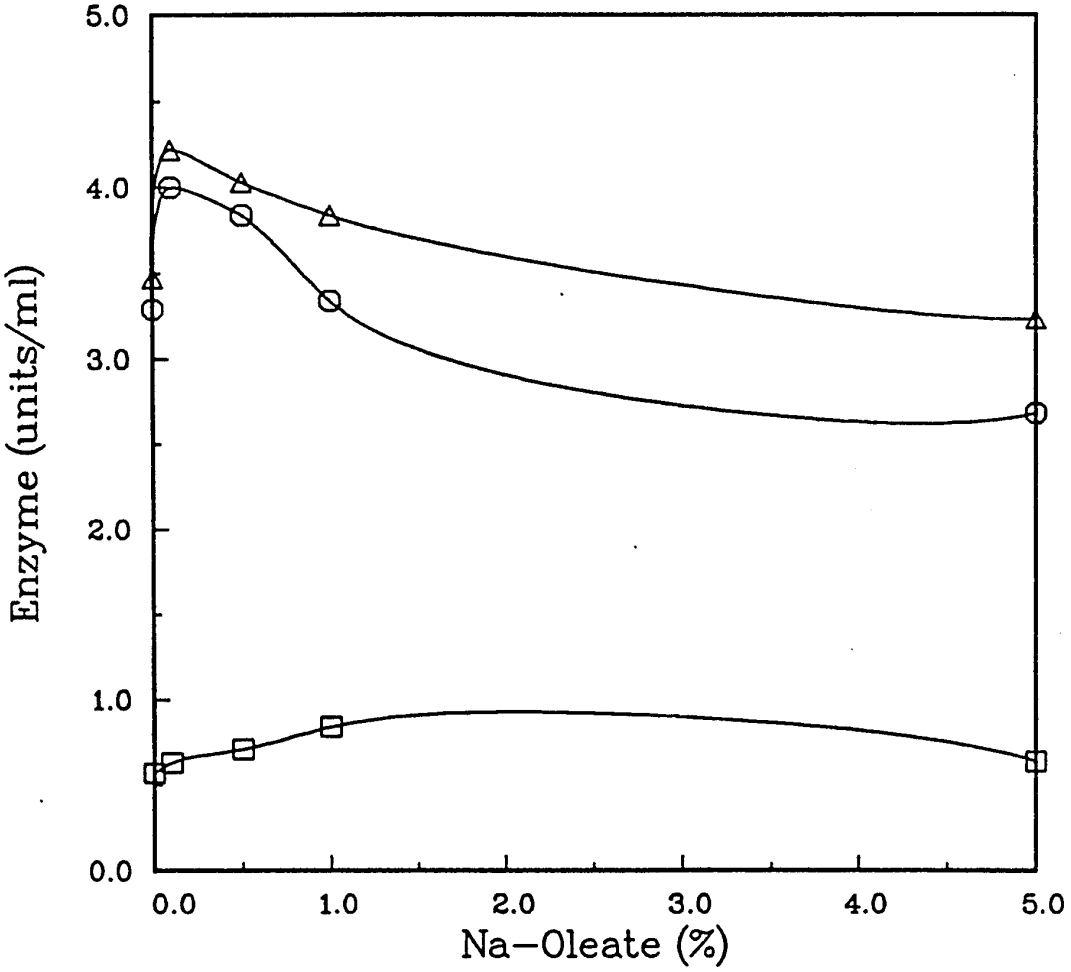

Figure 5.23: Enzyme production at particular times of fermentation in a medium containing various amounts of sodium oleate. (□–24, ○–48, △–72)

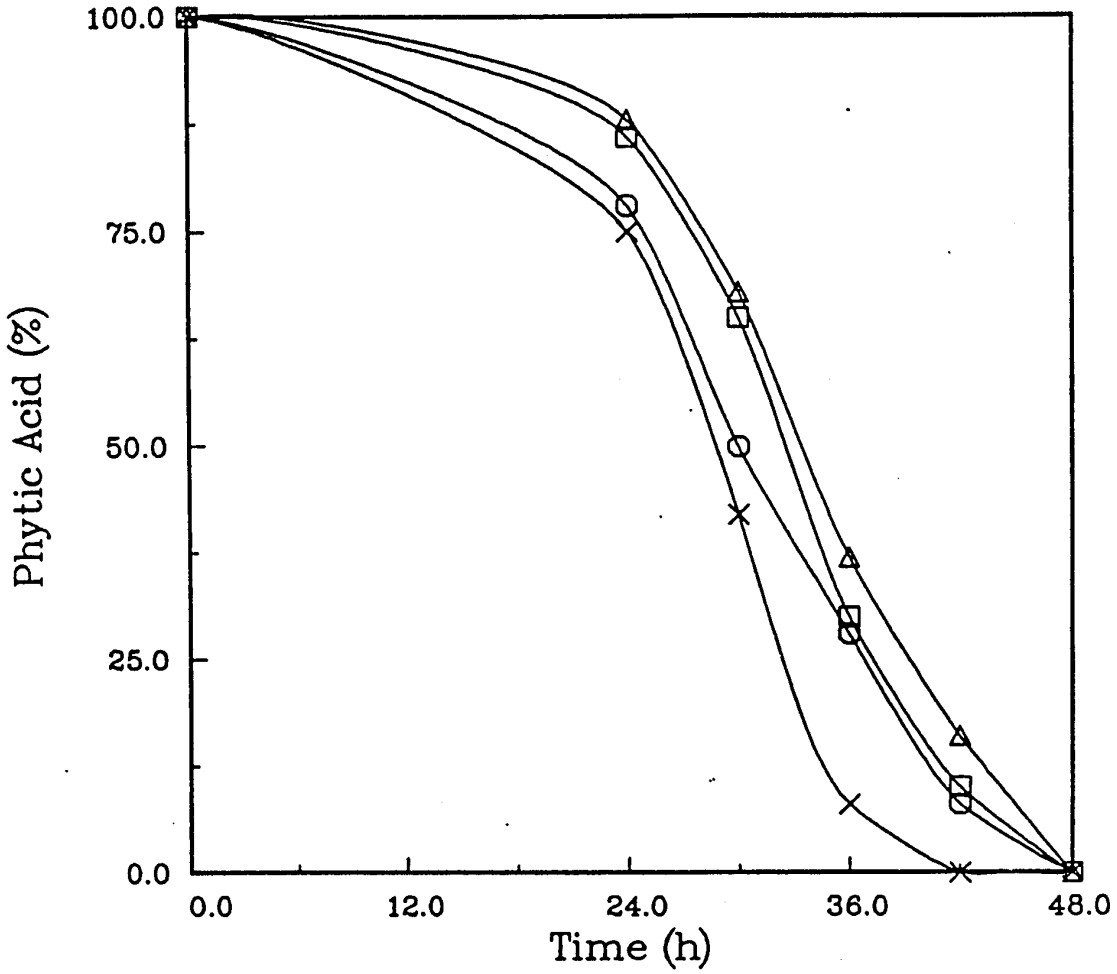

Figure 5.24: Effect of surfactants on phytic acid content reduction. (□–no surfactant, ○–Tween–80, △–Triton X–100, ×–sodium oleate)

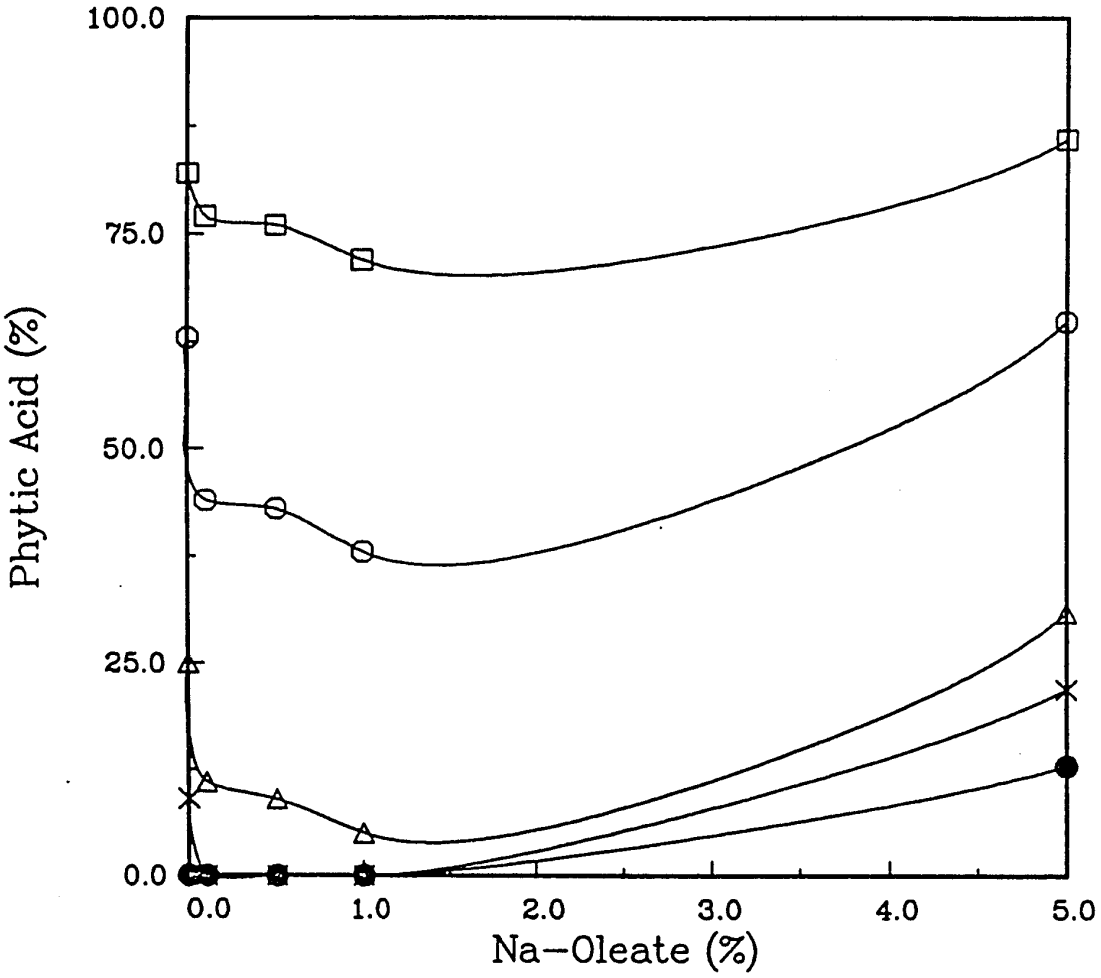

Figure 5.25: Phytic acid content reduction at particular times of fermentation in a medium containing various amounts of sodium oleate. (□–24, ○–30, △–36, ×–42, •–48)

5.4.3 Effect of Added Phosphate

The initial phosphate level in the growth medium is important for the production of phosphatase. In general, a high level of inorganic phosphate (PO_4—Pi) inhibits the synthesis of phosphatase; and the type and amount of phosphatase synthesized is dependent on the concentration of Pi present in the growth medium. Therefore the optimal amount of Pi in the growth medium should be carefully determined (Shieh et al., 1969). Phosphate in the range of 1–50 mg/100 g of solid medium was used in this study; and it was added in the form of Potassium dihydrogen phosphate.

The systems containing 1–5 mg phosphate yielded better enzyme production, with the 1 mg phosphate system producing the greatest amount of enzyme. Apparent inhibition is shown by the systems containing 10–50 mg phosphate, with 50 mg phosphate system producing the least amount of enzyme (Fig. 5.26).

The enzyme production at particular times of fermentation in a medium with various amounts of added phosphate (Fig. 5.27) shows that at 24 h, 48 h, and 72 h of incubation, the system containing 1 mg phosphate had consistently produced more enzyme than the systems with higher phosphate concentrations and the system without any added phosphate. Han et al., 1987 obtained a similar trend for phytase production by *Aspergillus ficuum* on semisolid substrate using soybean meal. They obtained phosphate concentration of 10 mg Pi/100 g substrate in the growth medium to be suitable for improved enzyme production; while higher levels above 10 mg Pi/100 g substrate inhibited phytase production.

The addition of phosphate in the form of potassium dihydrogen phosphate in the solid culture affected the rate of phytic acid content reduction. Fig. 5.28 shows that the addition of phosphate of up to an amount of 50 mg in the medium

increased the phytic acid content reduction rate. This increase in rate might be explained by the better cell growth in the systems with higher concentrations of phosphate. The system with the highest amount of added phosphate (50 mg) had the fastest reduction rate and obtained complete phytic acid content reduction within the incubation period at a time when the control system still contained 18% of the phytic acid.

Phytic acid content reduction at particular times of fermentation in a medium supplemented with various amounts of phosphate is shown in Fig. 5.29. The results show that at 24 h, 36 h, and 48 h of incubation, the systems containing higher concentrations of phosphate consistently gave faster phytic acid content reduction than the control system which had no added phosphate. After 42 h of incubation, the systems containing 5–50 mg phosphate had completely reduced their phytic acid content, while the control system still had 18%, and the system with 1 mg phosphate had 13% phytic acid content. This trend may be explained by the fact that even though lesser amounts of enzymes were produced in the systems with higher phosphate concentrations, these systems showed better cell growth which may have led to better distribution of the produced enzyme, and thus enhanced faster phytic acid content reduction.

The effect of added phosphate on biomass production is shown in Fig. 5.30. Increase in added phosphate of up to 10 mg in the system resulted in increased biomass production; while the addition of 50 mg into the system showed slight but appreciable decrease in the biomass production.

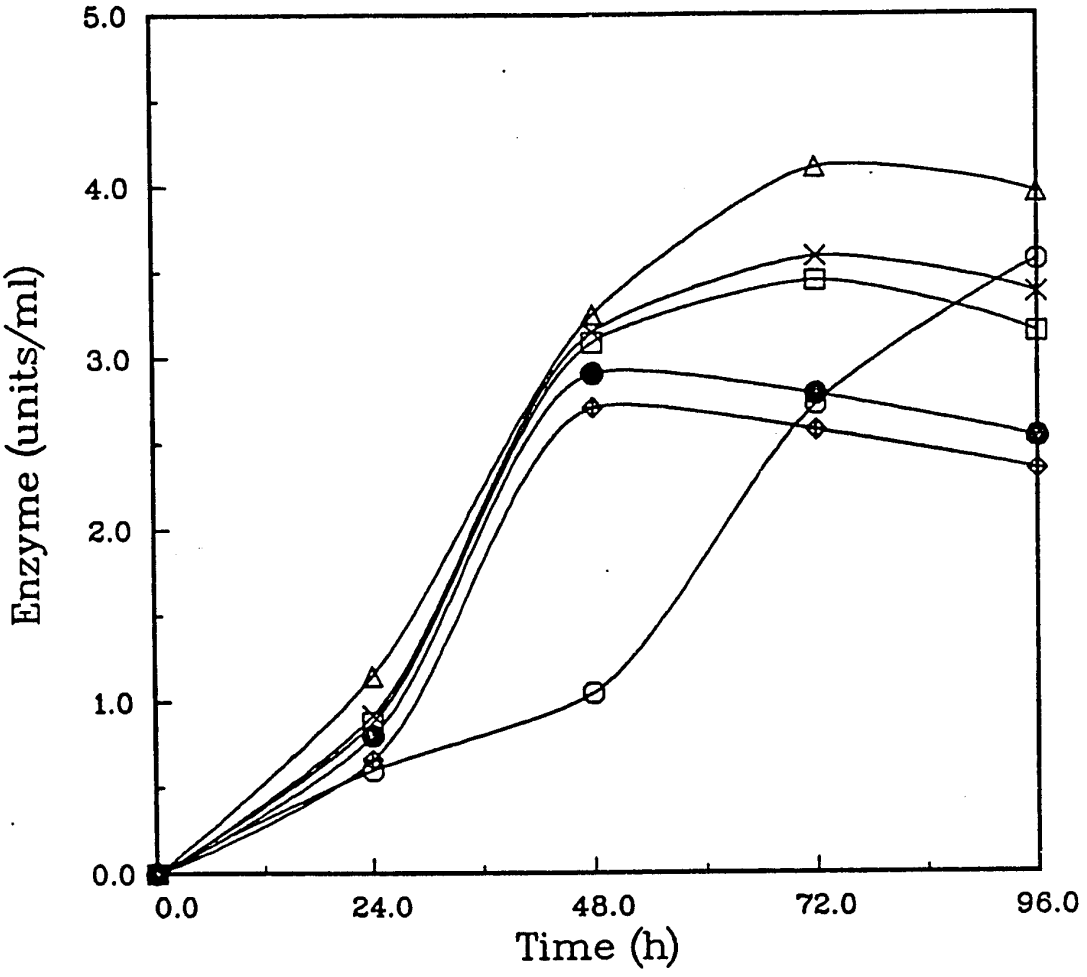

Figure 5.26: Effect of added phosphate on enzyme production in medium prepared with distilled water. Amount of added phosphate (mg) (□–no phosphate, ○–no phosphate (in buffer 4.7), △–1, ×–5, •–10, ◈–50)

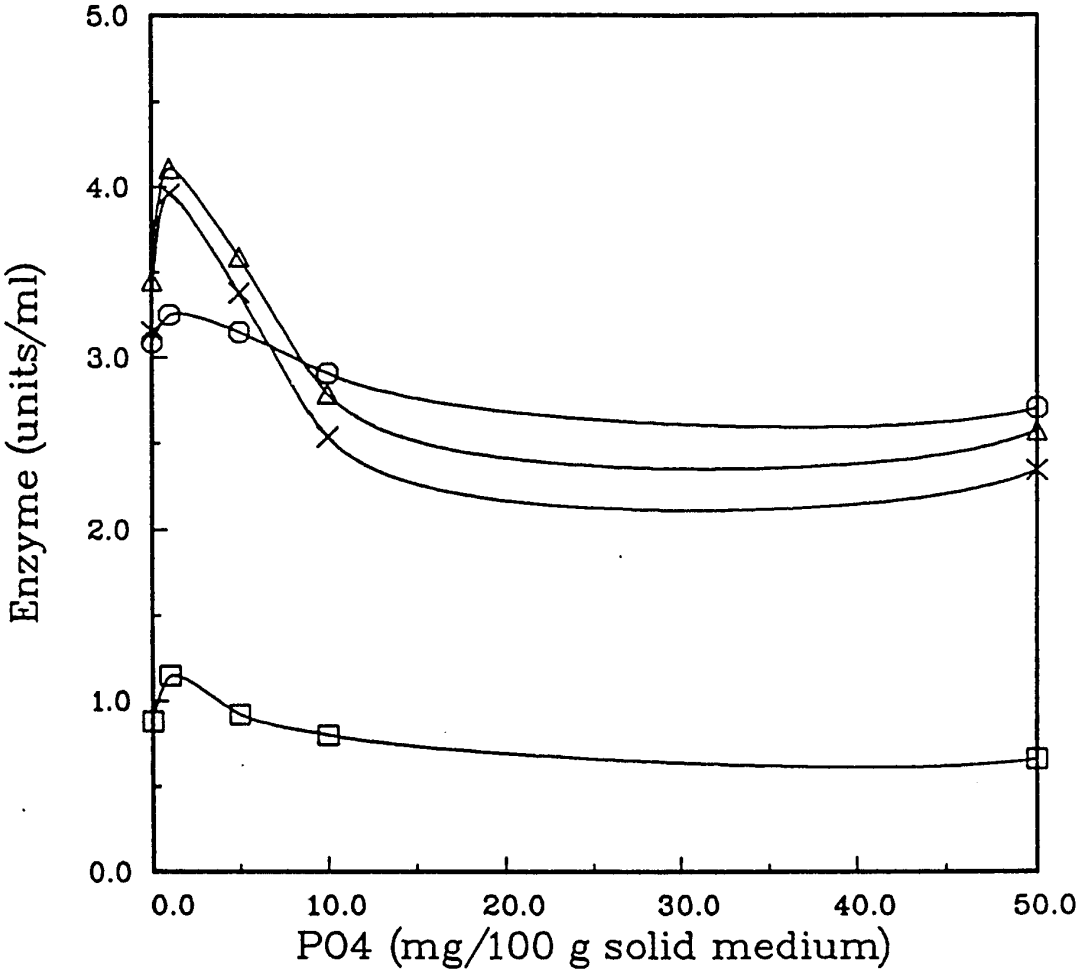

Figure 5.27: Enzyme production at particular times of fermentation in medium supplemented with various amounts of phosphate. Time of fermentation (h) (□–24, ○–48, △–72, ×–96)

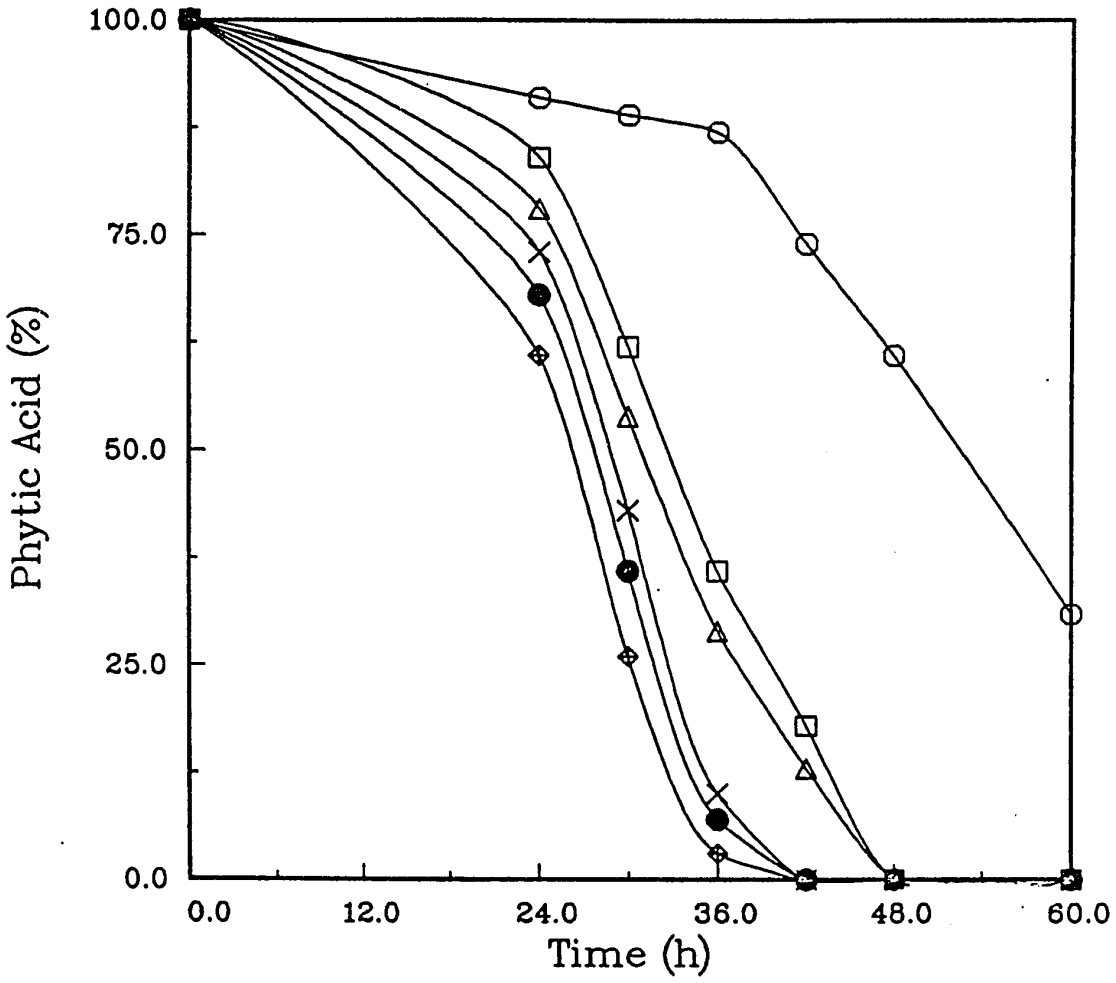

Figure 5.28: Effect of added phosphate on phytic acid content reduction in medium prepared with distilled water. Amount of added phosphate (mg) (□–no phosphate, ○ –no phosphate (in buffer 4.7), △–1, ×–5, •–10, ⊕–50)

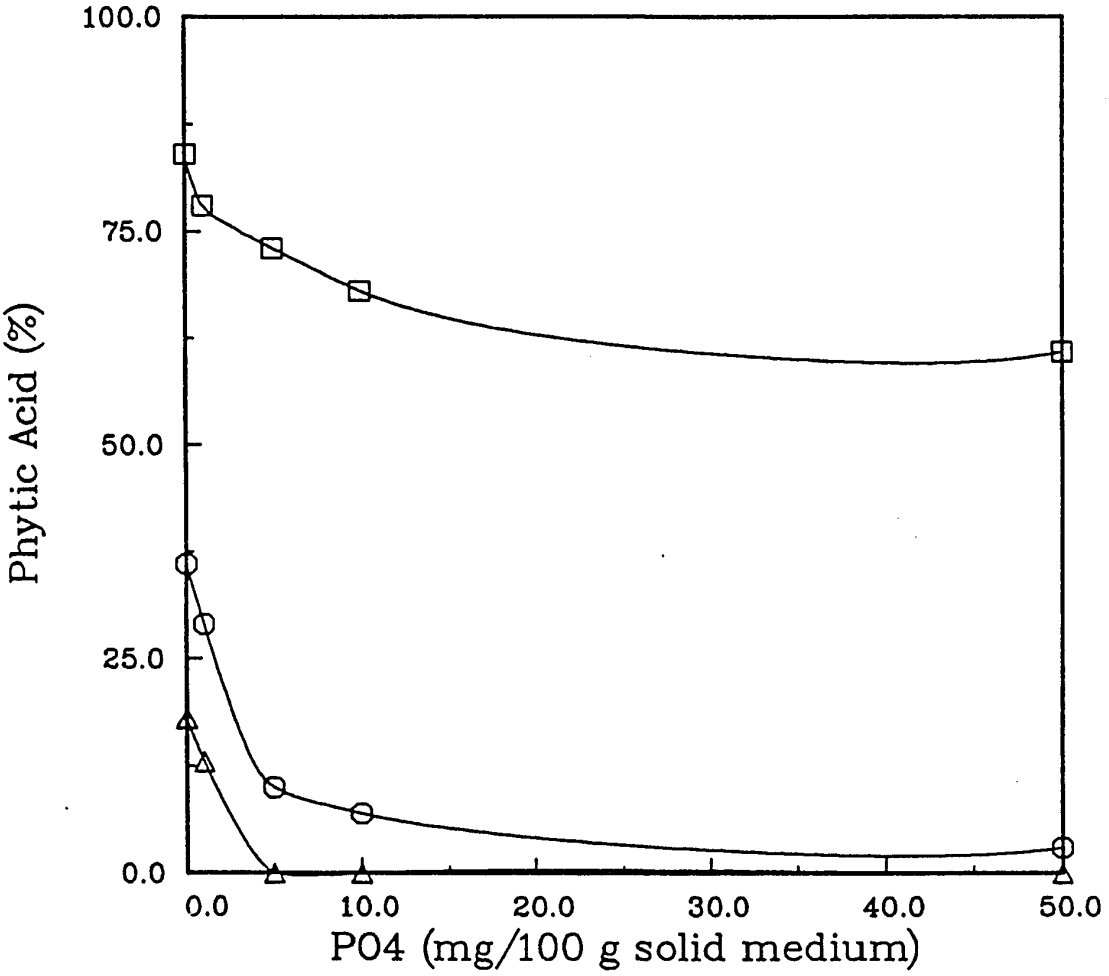

Figure 5.29: Phytic acid content reduction at particular times of fermentation in medium supplemented with various amounts of phosphate. Time of fermentation (h) (□–24, ○ –36, △–42)

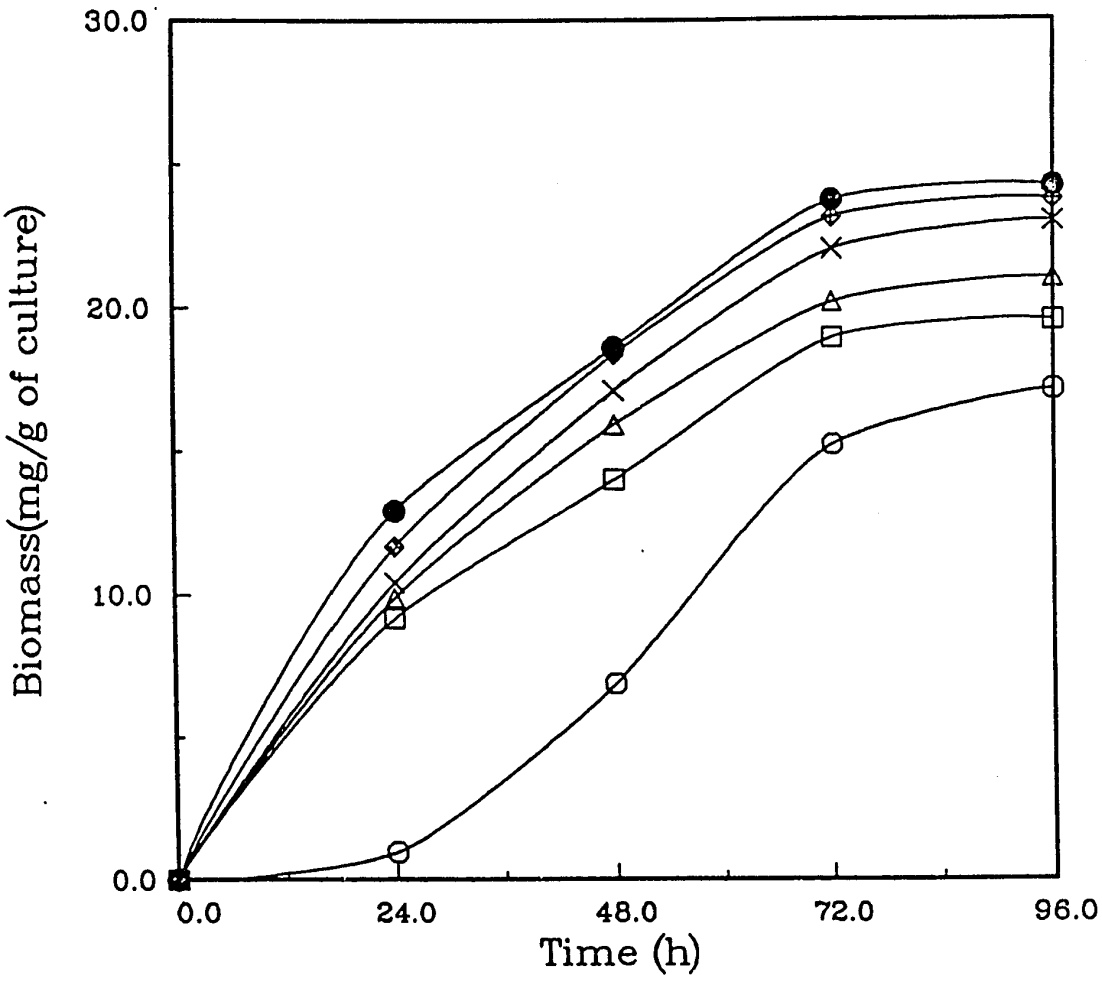

Figure 5.30: Effect of added phosphate on biomass production in medium prepared with distilled water. Amount of added phosphate (mg) (□–no phosphate, ○–no phosphate (in buffer 4.7), △–1, ×–5, •–10, ◈–50)

The effect of added phosphate in buffered systems up to a concentration of 50 mg Pi/100 g of solid medium, resulted in increased enzyme production (Fig.5.31). A lag phase was found to be common to all systems prepared with buffer solutions below 5.7.

The enzyme production at particular times of fermentation in a buffered medium supplemented with phosphate showed that at 24 h, 48 h, 72 h, and 96 h of incubation, the system with 50 mg added phosphate produced more enzymes than the systems with lower phosphate concentrations (Fig. 5.32)

The effect of added phosphate on the phytic acid content reduction in buffered systems was also studied (Fig. 5.33). The addition of phosphate into the systems had a negative effect on the phytic acid content reduction; and the rate of reduction decreased with increasing amounts of the added phosphate. The control system having no added ytic acid content by the end phosphate obtained complete reduction of phytic acid content by the end of the incubation period; while the system containing 50 mg of added phosphate still had 29% of the phytic acid content present in its medium.

Phytic acid content reduction at particular times of fermentation in a buffered medium supplemented with various amounts of phosphate showed the consistent faster reduction in the control system with no added phosphate over the supplemented systems at 24 h, 36 h, 48 h, 72 h, and 96 h of incubation (Fig. 5.34). At 96 h of incubation, the control system had obtained complete reduction of the phytic acid content.

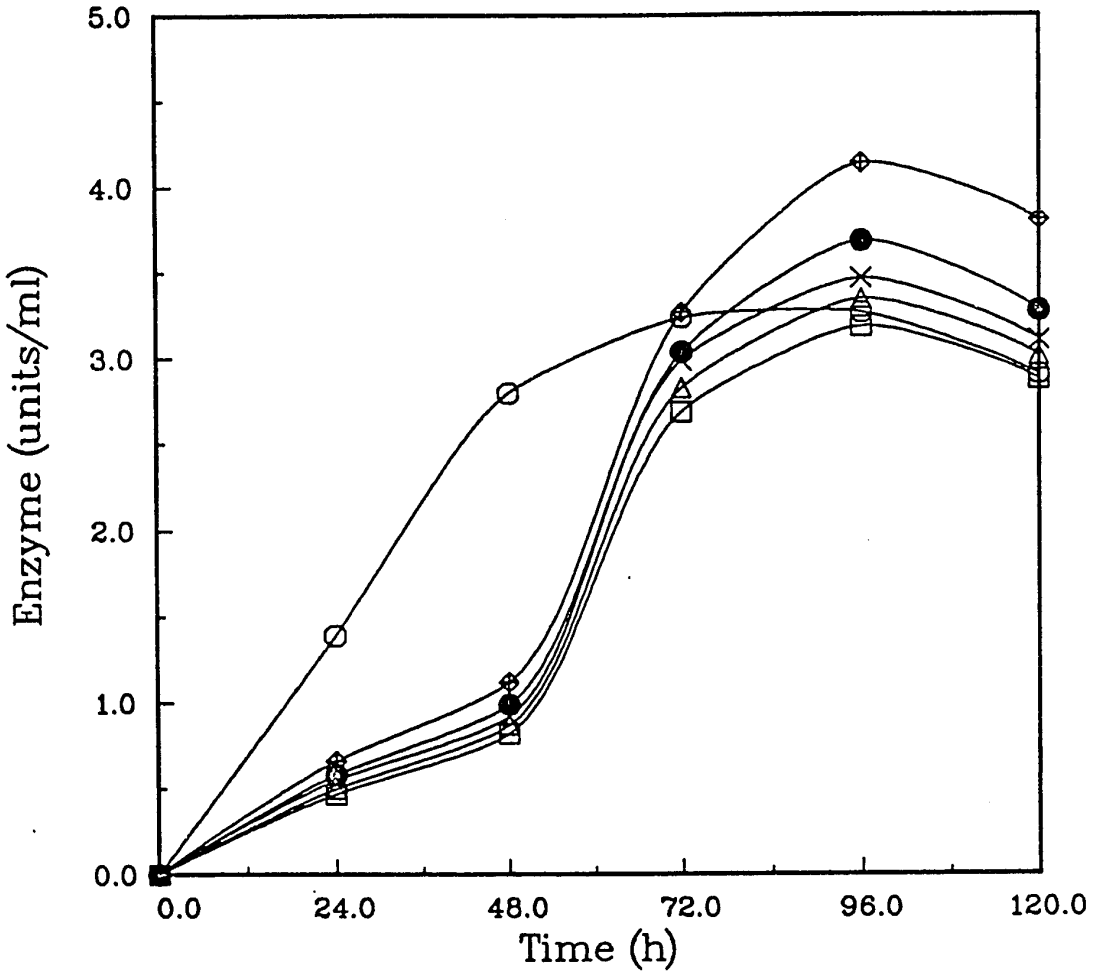

Figure 5.31: Effect of added phosphate on enzyme production in a buffered (pH 4.7) medium. Amount of phosphate (mg) (□–no phosphate, ○– no phosphate (in distilled water), △–1, × –5, •–10, ◈–50)

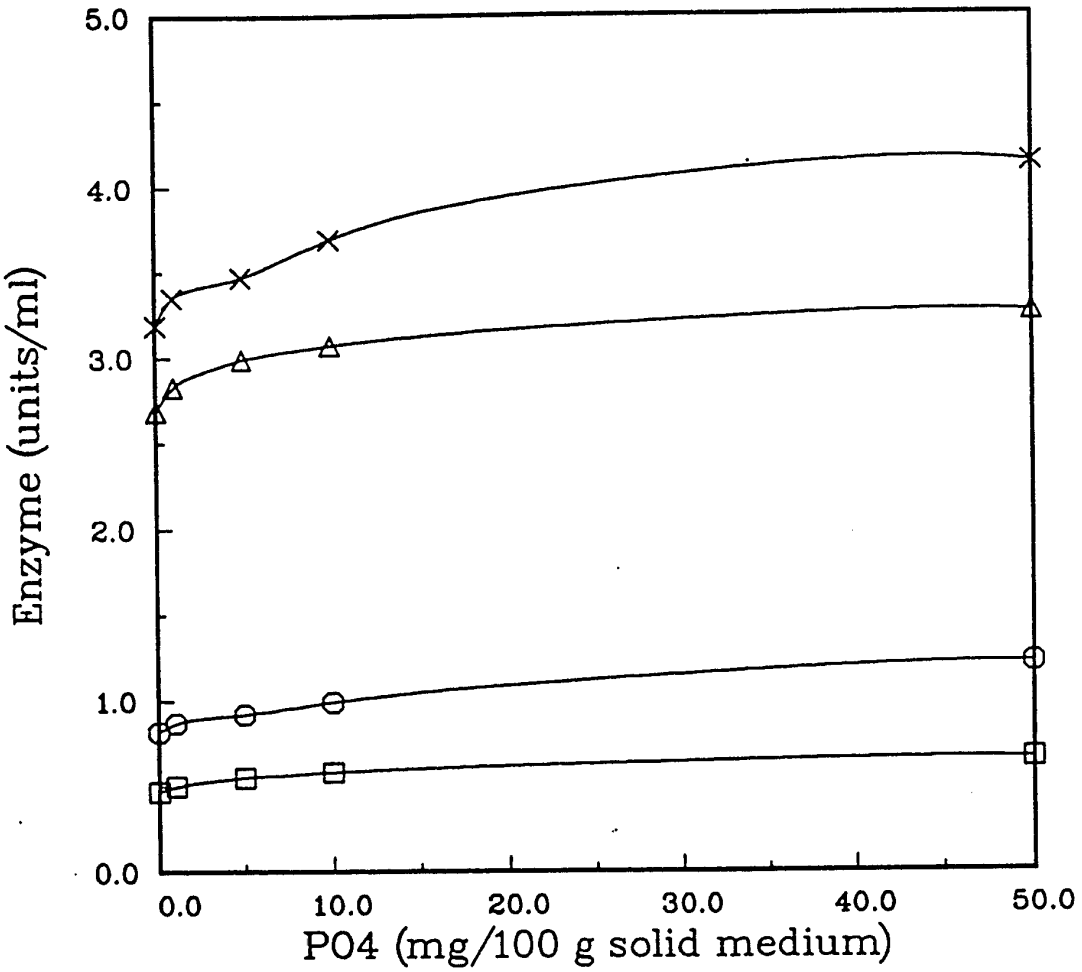

Figure 5.32: Enzyme production at particular times of fermentation in buffered (pH 4.7) medium supplemented with various amounts of phosphate. Time of fermentation (h) (□–24, ○–48, △–72, ×–96)

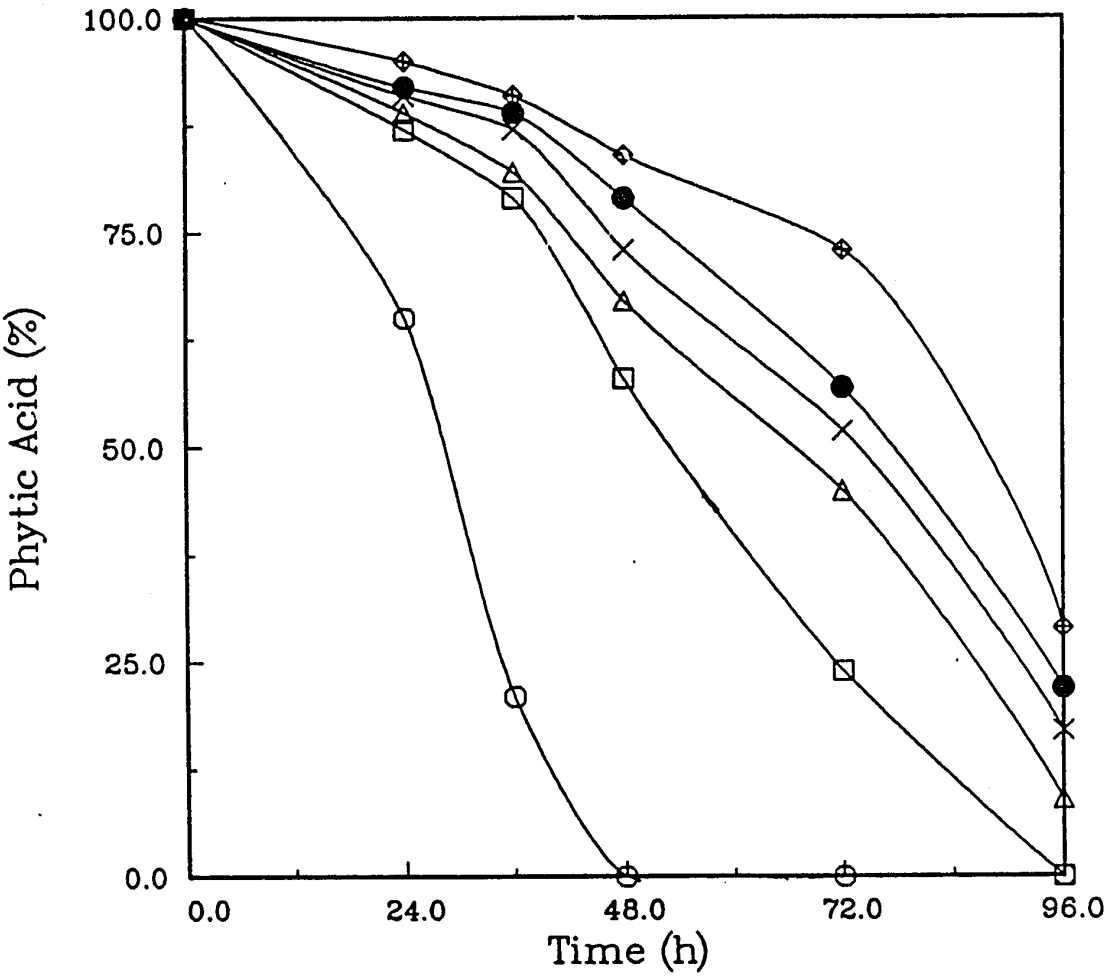

Figure 5.33: Effect of added phosphate on phytic acid content reduction in buffered (pH 4.7) medium. Amount of phosphate (mg) (□–no phosphate, ○ – no phosphate (in distilled water), △–1, × –5, •–10, ◈–50)

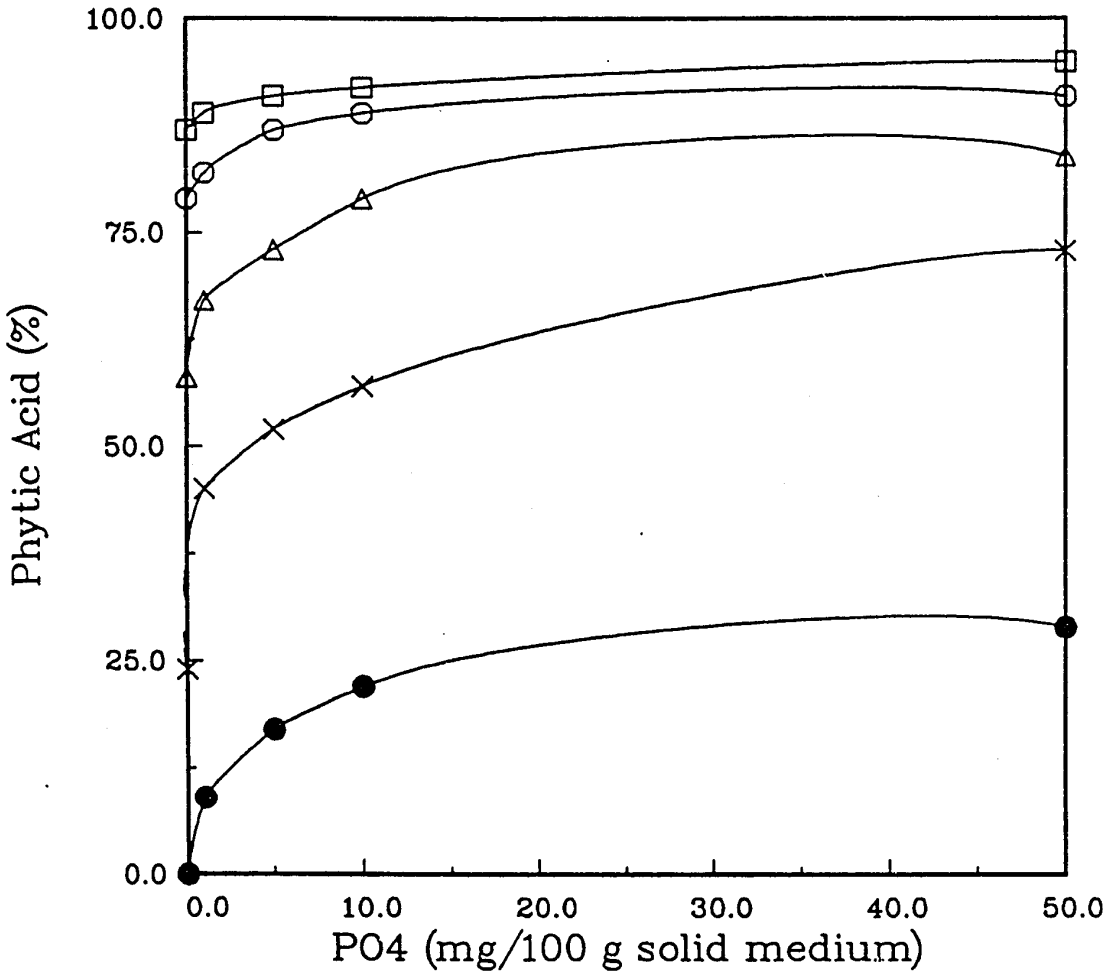

Figure 5.34: Phytic acid content reduction at particular times of fermentation in buffered (pH 4.7) medium supplemented with various amounts of phosphate. Time of fermentation (h) (□–24, ○ –36, △–48, ×–72, •–96)

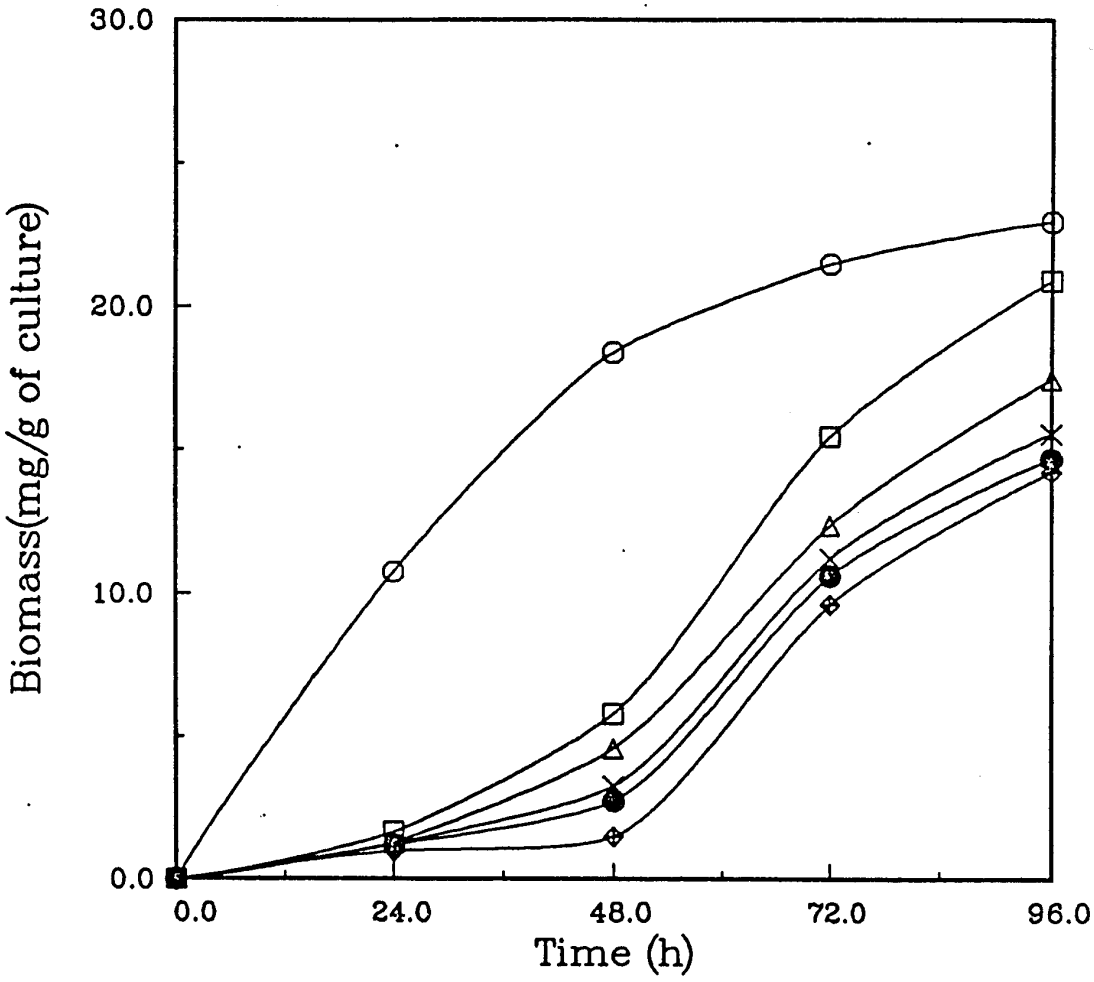

Figure 5.35: Effect of added phosphate on biomass production in a buffered (pH 4.7) medium. Amount of phosphate (mg) (□–no phosphate, ○– no phosphate (in distilled water), △–1, × –5, •–10, ◈–50)

The biomass production in the buffered systems containing added phosphate was also found to decrease with increased phosphate concentration (Fig.5.35). This decrease in cell growth might perhaps explain the reduced rates in phytic acid content reduction with increased phosphate concentration in buffered solutions.

5.4.4 Effect of Combined Oleate and Phosphate

Having obtained positive effects on enzyme production and phytic acid content reduction by the separate addition of phosphate and sodium oleate, the effects of combining these two components in a single solid medium was considered.

The effect of combined sodium oleate and phosphate on enzyme production is shown in Fig. 5.36. The systems which contained either 1 mg of phosphate or 0.1% sodium oleate produced more enzymes than the control system having no additive; while systems which contained both portions of the 1 mg phosphate and 0.1% sodium oleate combined produced lesser amounts of enzyme than the control system.

This same synergistic effect of combined sodium oleate and phosphate is seen on phytic acid content reduction as shown in Fig. 5.37. The systems which separately contained 1 mg phosphate and 0.1% sodium oleate showed increased rate in phytic acid content reduction compared to the control system within the first 36 h of incubation; but these two systems and control system all obtained complete phytic acid content reduction after 48 h of incubation. The system which contained both portions of 1 mg phosphate and 0.1% sodium oleate combined in it still had 3% phytic acid left in the medium after 48 h of incubation.

The same effect caused by the combined portions of phosphate and sodium oleate

was also observed in the biomass production (Fig. 5.38) with a similar trend as for phytic acid content reduction and enzyme production. Better biomass production was obtained in the two systems which contained either 1 mg phosphate or 0.1% sodium oleate, than in the control; while the system containing both portions of the additives resulted in a poorer biomass production than in the control system.

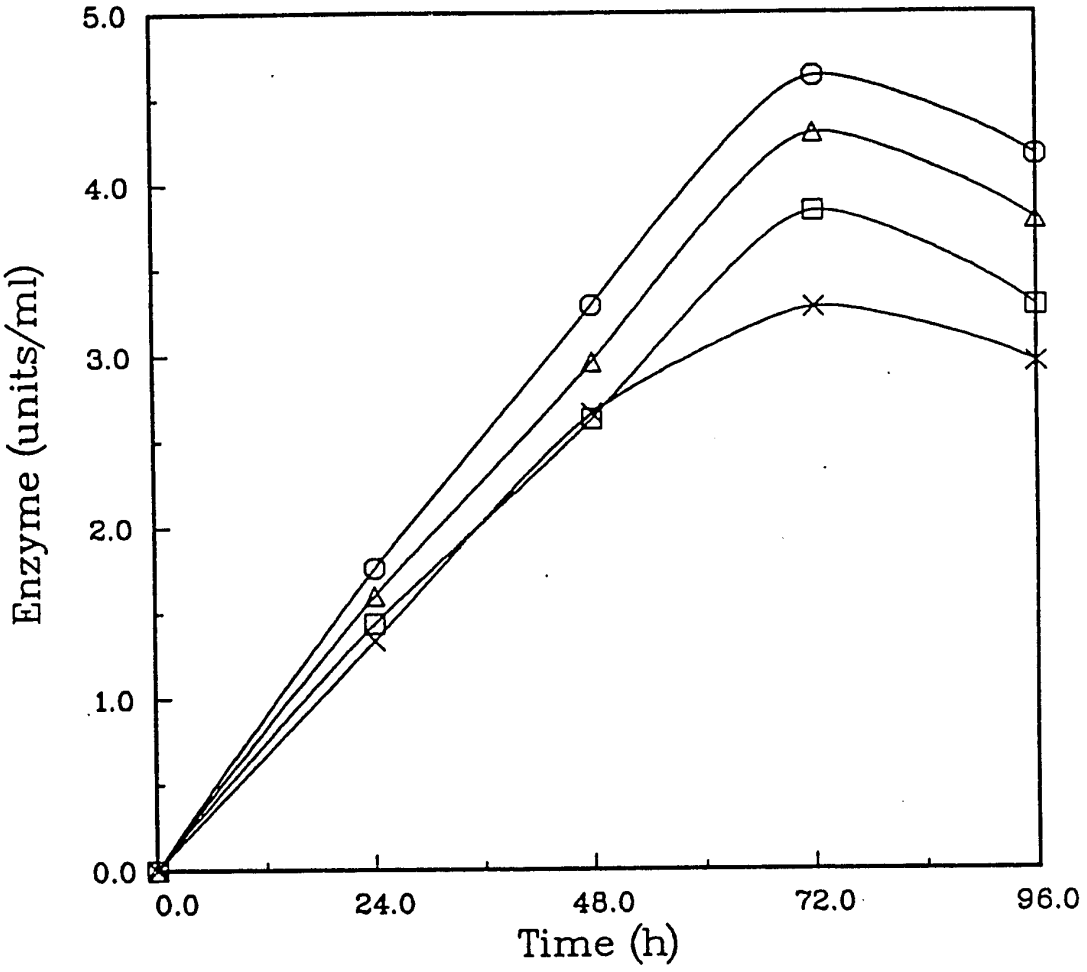

Figure 5.36: Synergistic effect of combined sodium oleate and phosphate on enzyme production. (□–no additive, ○ –1 mg phosphate, △ –0.1% sodium oleate, ×–1 mg phosphate + 0.1% sodium oleate)

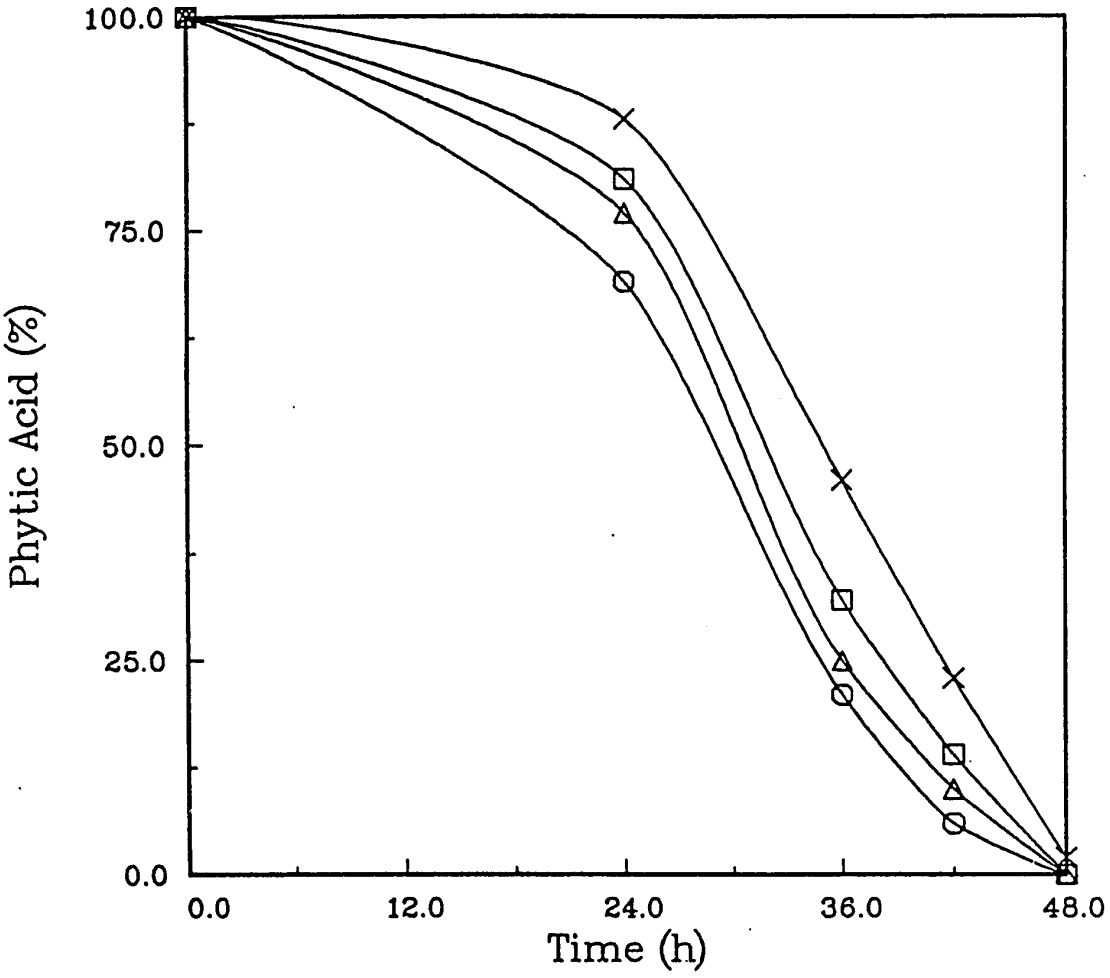

Figure 5.37: Synergistic effect of combined sodium oleate and phosphate on phytic acid content reduction. (□–no additive, ○–1 mg phosphate, △ –0.1% sodium oleate, ×–1 mg phosphate + 0.1% sodium oleate)

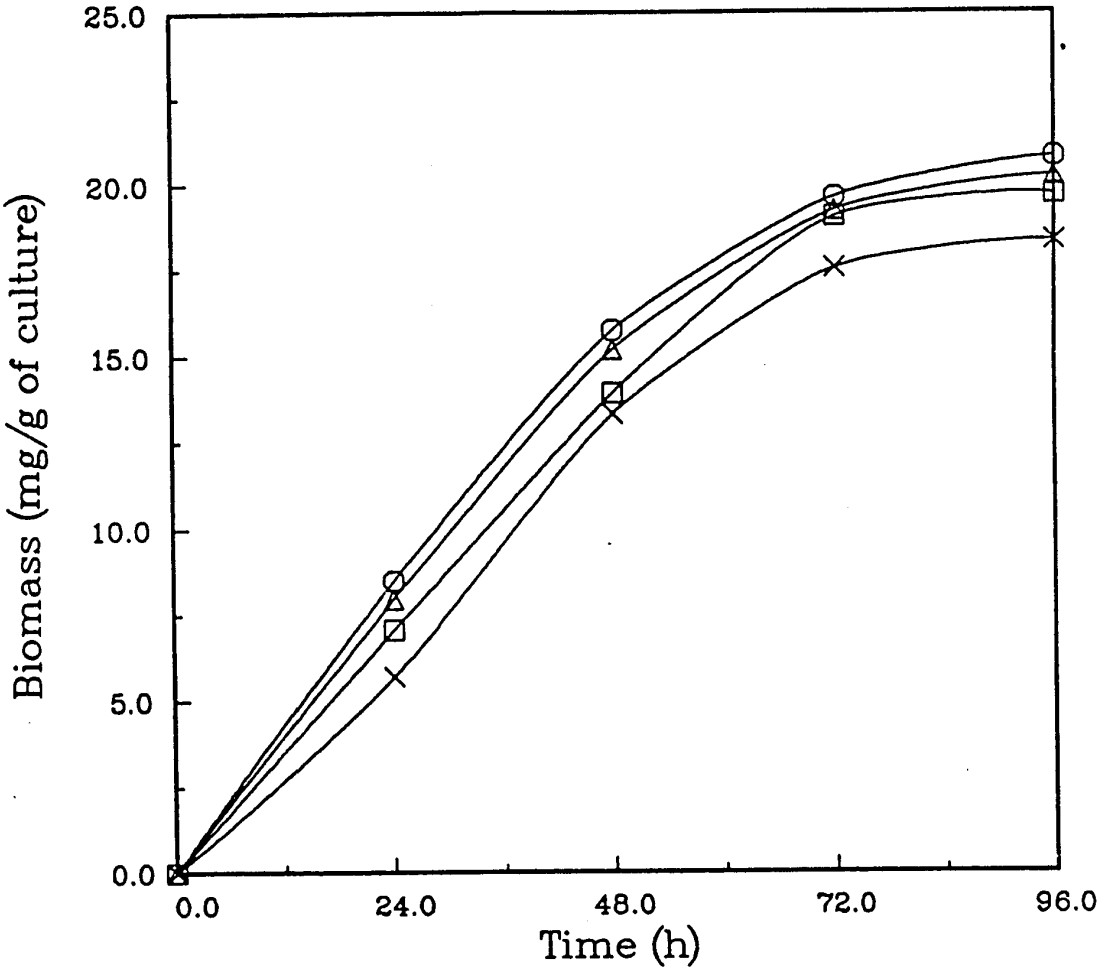

Figure 5.38: Synergistic effect of combined sodium oleate and phosphate on biomass production. (□–no additive, ○–1 mg phosphate, △ –0.1% sodium oleate, ×–1 mg phosphate + 0.1% sodium oleate)

5.5 Changes in Protein and Carbohydrate Contents, Enzyme and Biomass production

General changes in protein and available carbohydrate contents, enzyme and biomass production were followed in a fermentation process (Fig. 5.39). When *A. ficuum* was grown in a solid medium, no lag phases were observed for enzyme and biomass production for medium prepared with distilled water. Hence growth accelerated quickly until a biomass concentration of 17.5 mg dry wt/g culture was attained; then growth slowed gradually. This trend of biomass production is supported by an increase in protein content up to the 72 h of fermentation. Available carbohydrate utilization corresponds to the cell growth, being rapid first, then slowing. The enzyme production in the medium increased rapidly during early growth, reaching a maximum at 72 h, then slowly decreasing.

The trend of results for biomass and enzyme production, and carbohydrate utilization obtained in this work are similar to those of Mitchell et al., (1991). They grew *Rhizopus oligosporus* in membrane filter culture as a model solid-state fermentation. They obtained increase in enzyme activity up to about 26 h of incubation; and thereafter enzyme decay commenced. Biomass production was still found to be increasing after 46 h of incubation, while the starch being used up by the microorganism had been reduced to 75% by the 46 h of incubation.

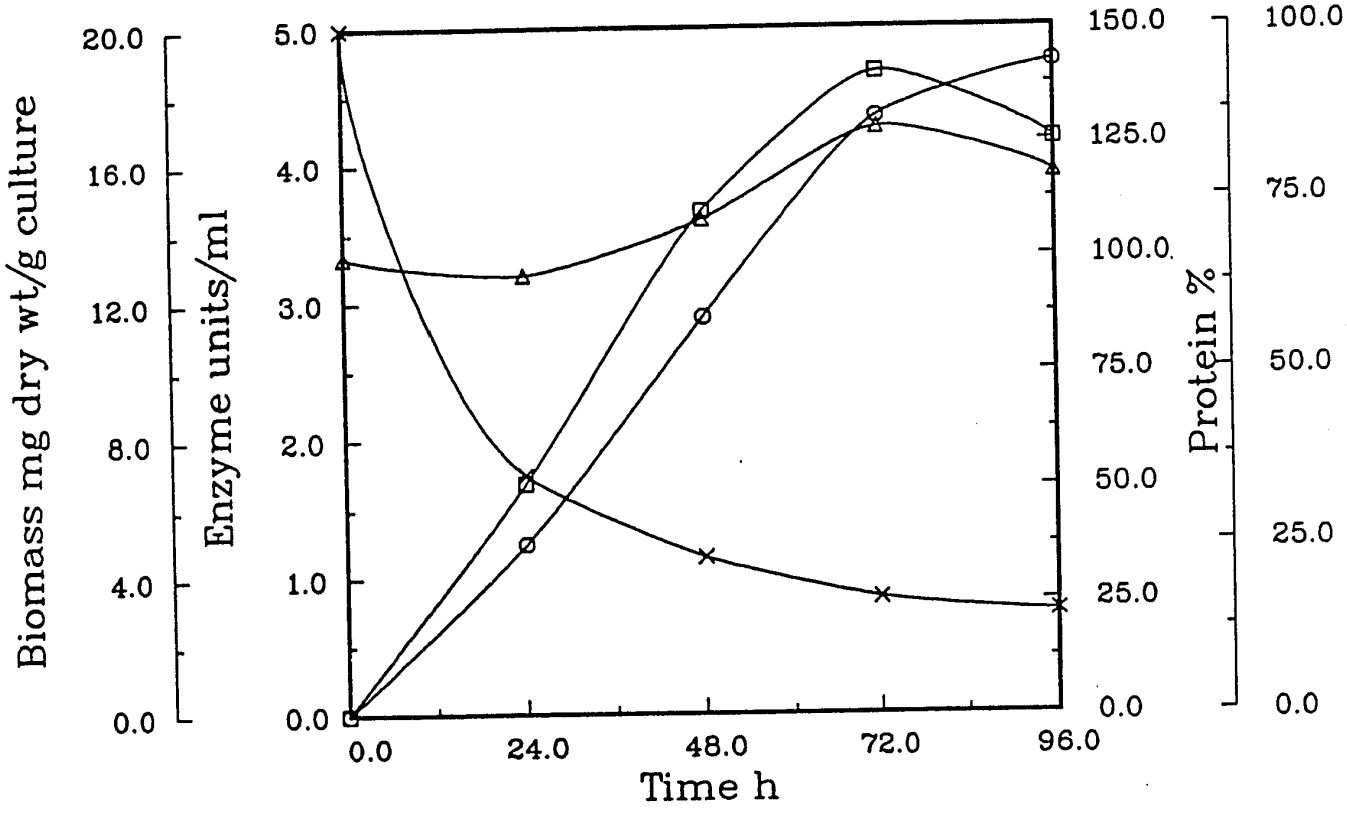

Figure 5.39: Enzyme, biomass and protein production; carbohydrate utilization in an SSF. (□–enzyme, ○–biomass, △–protein, ×–available carbohydrate)

To determine what ratio of culture:extractant could be used for a single extraction of enzyme from the culture, a 1:5 and 1:15 ratios were considered. A solid medium was prepared using 220 mg biomass as inoculum, and the culture was grown for 96 h. A test was carried out on a series of extractions. The results in Table 5.2 shows that the first extraction of enzyme, using 1:5 ratio gave 70% extraction. The second extraction on the same culture, still using 1:5 ratio gave 19%; and a third extraction on the same culture, with 1:5 ratio, gave 11%. Using an extraction ratio of 1:15, and assuming the same total enzyme units for 1:5 ratio for the three consecutive extractions, gave about 80% extraction. Based on these results, extraction of enzyme from culture was done once for each sample, using 1:5 culture extractant ratio.

Table 5.2: Extraction of Enzyme from meal culture

	Extraction of Enzyme			
Ratio of	First	Second	Third	First
Culture:Extractant	1:5	1:5	1:5	1:15
Extract collected (ml)	250	250	250	750
Enzyme (units/ml)	2.62	0.72	0.42	0.99
Total Enzyme (units)	655	180	105	749
% of Grand total	70	19	11	80

Chapter 6

Conclusions and Recommendations

6.1 Conclusions

A. ficuum NRRL 3135 was used for the production of phytase and reduction of the phytic acid content in canola meal by a solid state fermentation technique. The results show that *A. ficuum* was capable of completely eliminating the phytic acid in the meal. The production of phytase and the rate of the phytic acid content reduction were both found to be influenced by a number of physical, chemical and nutritional factors.

Phytase production and phytic acid content reduction were both found to increase with increasing biomass in inoculum of up to 170 mg.

Increasing the age of inoculum up to 7 days was found to increase enzyme production as well as rate of phytic acid content reduction.

The optimum moisture content of the solid medium used in this study was found to be 64%.

Increase in time of homogenization proved to enhance improved enzyme production and phytic acid content reduction. From the figure on enzyme production, 36 h of incubation is the suggested length of time required for damaged cell reconstruction. Phytase production and phytic acid content reduction were both found to increase with increasing initial pH of medium from 5.0 to 5.7; the systems prepared with distilled water giving the best results.

The addition of 1–5 mg Pi/100 g solid medium was found to increase enzyme production and phytic acid content reduction in systems prepared with distilled water; while increasing concentrations up to 50 mg Pi/100 g solid medium favoured improved enzyme production but produced a negative effect in the phytic acid content reduction.

The addition of sodium oleate to the culture medium improved enzyme production and phytic acid content reduction.

Using combined portions of sodium oleate and phosphate resulted in synergistic effects on enzyme production and phytic acid content reduction.

The study of some of the characteristics of the enzyme produced in the solid culture showed the K_m and V_{max} values to be 0.39 mM and 5.7 units/h^{-1}, respectively, when sodium phytate was used as substrate.

In the solid culture the biomass increased up to the 144 h of incubation, though the amount of biomass produced varied with the age and amount of inoculum used for the inoculation.

In all the buffered systems (pH below 5.7), there was a lag phase in the cell growth and enzyme production as opposed to systems prepared with distilled water, whose cell growth and enzyme production experienced no lag phase. For the first 48 h of

incubation, there was no apparent cell growth in all buffered systems (pH below 5.7); while the systems prepared with distilled water and with buffer of pH 5.7 had shown considerable cell growth.

The results of appreciable production of cell biomass and increased protein content improved the nutritional quality of canola meal as animal feed.

6.2 Recommendations

The most difficult problem encountered in this work was how to obtain representative samples from the solid medium for the various analyses, without hindering the cell growth by continuous breaking and destruction of mycelia in the mixing procedure. The cell growth was found to be best on the surface of the mass of substrate; so was the phytic acid content reduction and enzyme production. Because of the thickness of the mass of substrate (no less than 5 cm), adequate mixing was necessary if representative media samples were to be taken for analyses; and also for introducing the microorganism to fresh nutrient sources. This continuous mixing greatly reduced rate of cell growth, and hence biomass production. A solid state system with substrate layer of minimum thickness is recommended if biomass production is of outstanding importance; as this would minimize or completely eliminate mixing of medium.

The inoculation of each solid medium was done with freshly grown and homogenized liquid culture. It is worth trying an old solid culture (grown for about 3 days) as inoculum for a fresh solid medium. This would safe materials and time spent on growing a liquid culture for each solid medium.

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Appendix A

Standard Curves

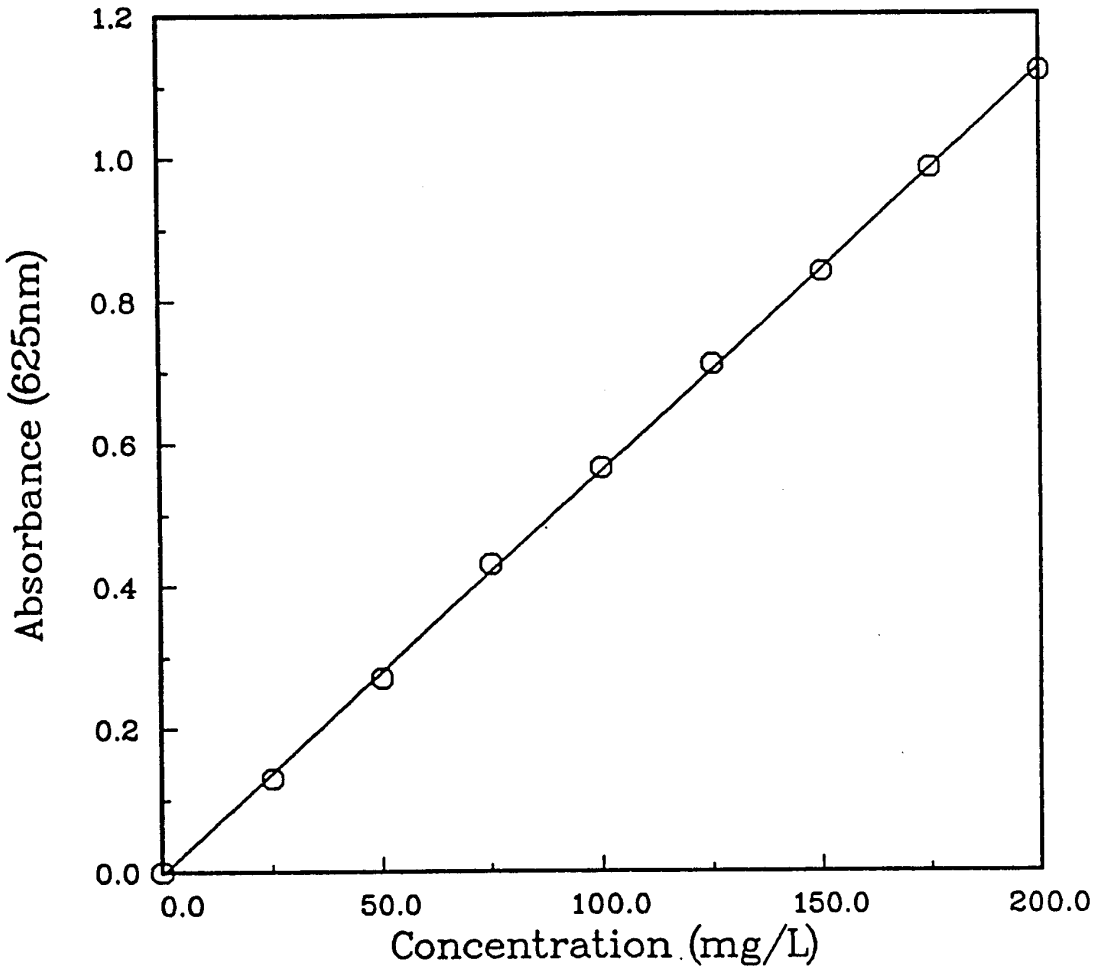

Figure A.1: Standard curve for measurement of total available carbohydrates (AOAC, 1975), using sucrose as the standard.

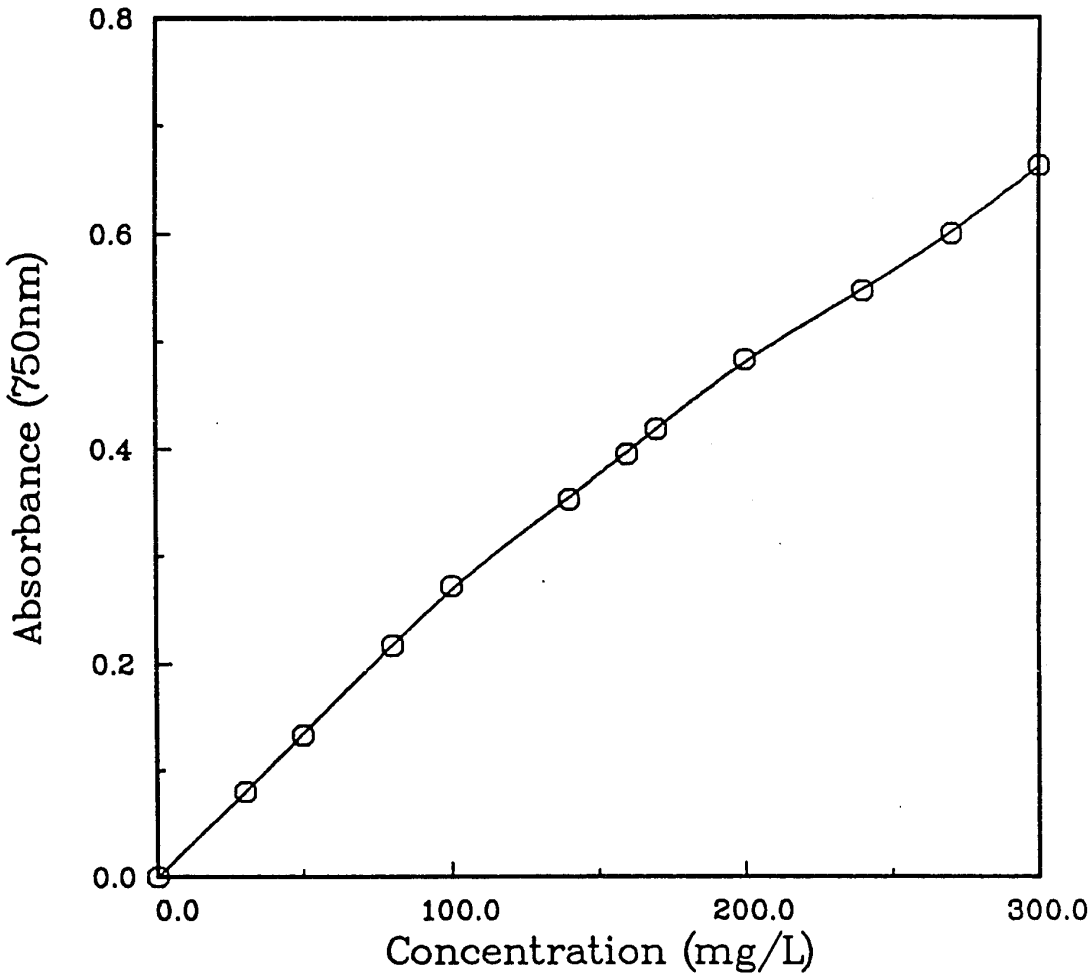

Figure A.2: Standard curve for measurement of protein by the method described by Lowry et al., (1975)

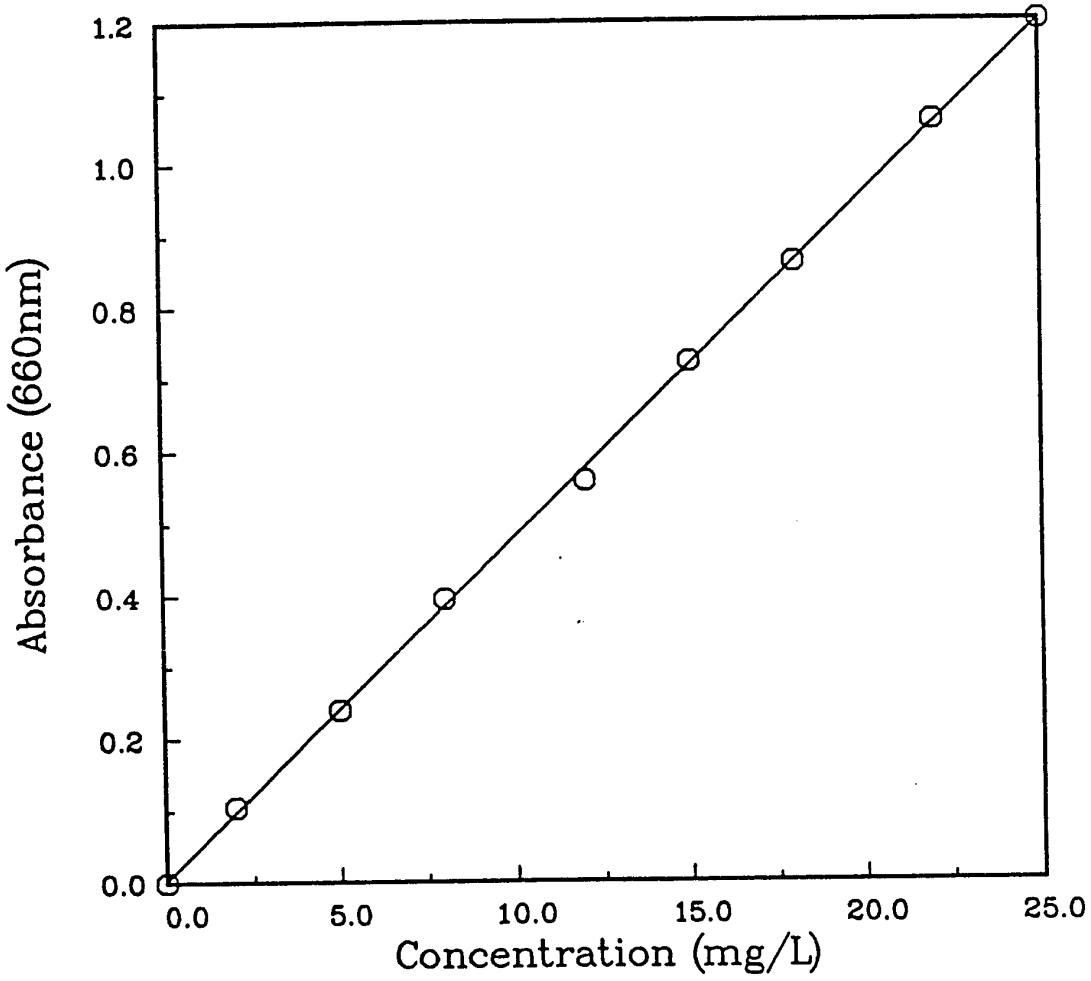

Figure A.3: Standard curve for measurement of inorganic phosphorus by the method described by Harland and Harland, (1980)

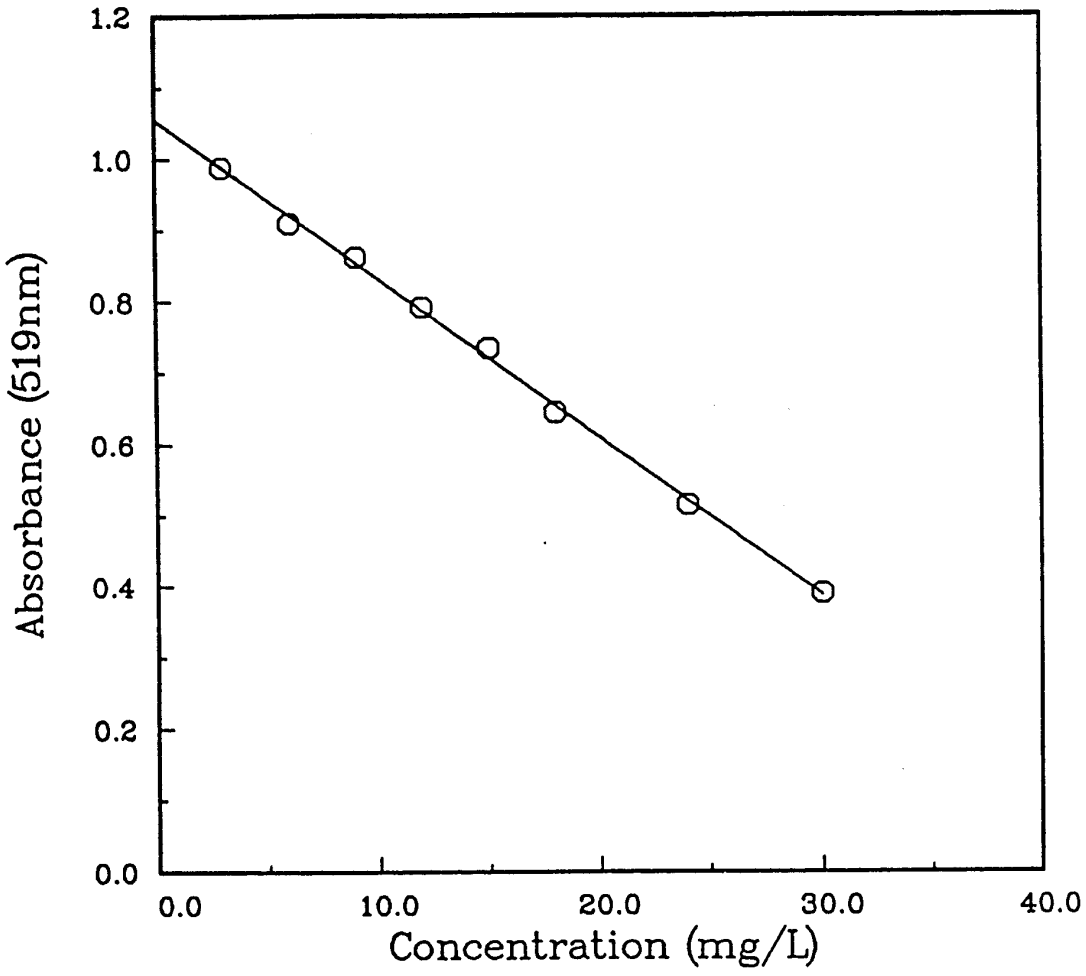

Figure A.4: Standard curve for direct measurement of phytic acid concentration by the method described by Haug and Lantzch, (1983)

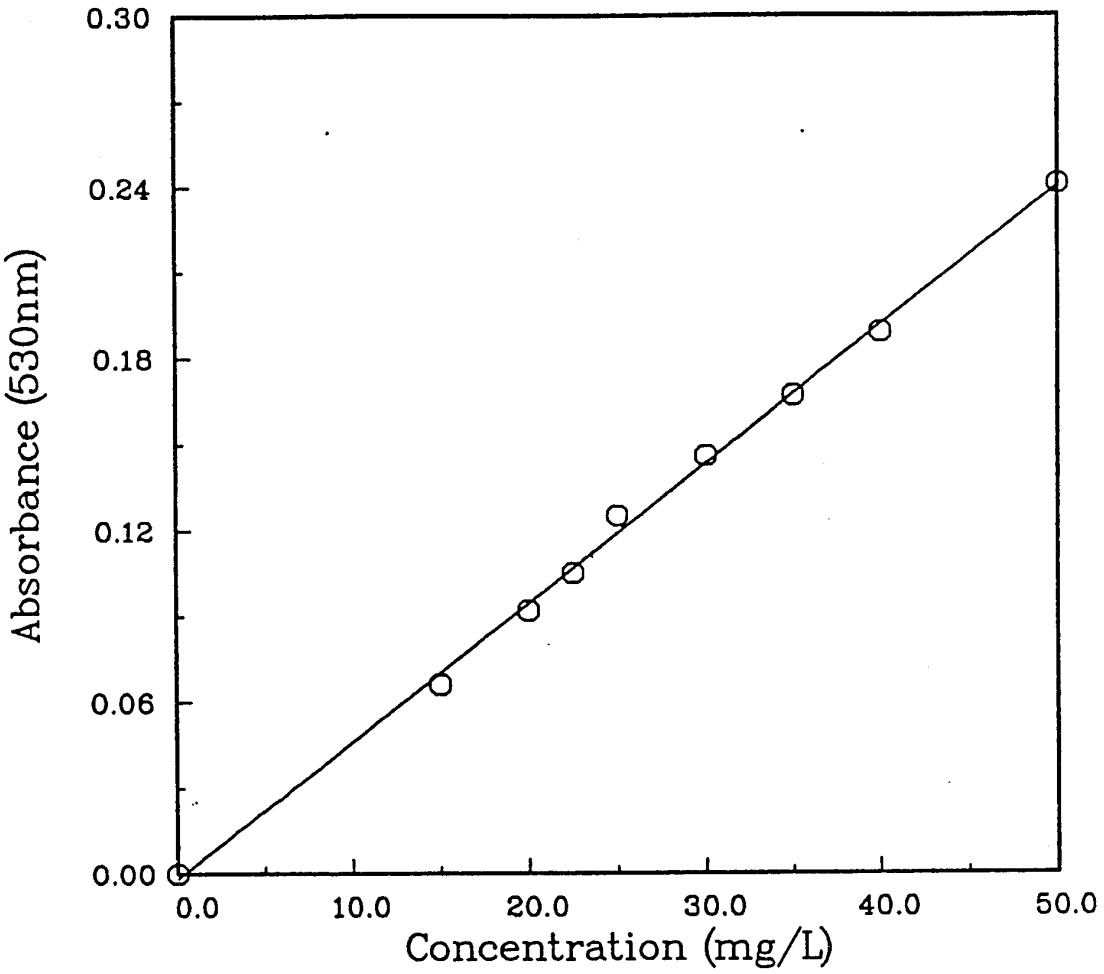

Figure A.5: Standard curve for measurement of glucosamine by the method described by Blix (1948)

Appendix B

Plots of biomass and glucosamine

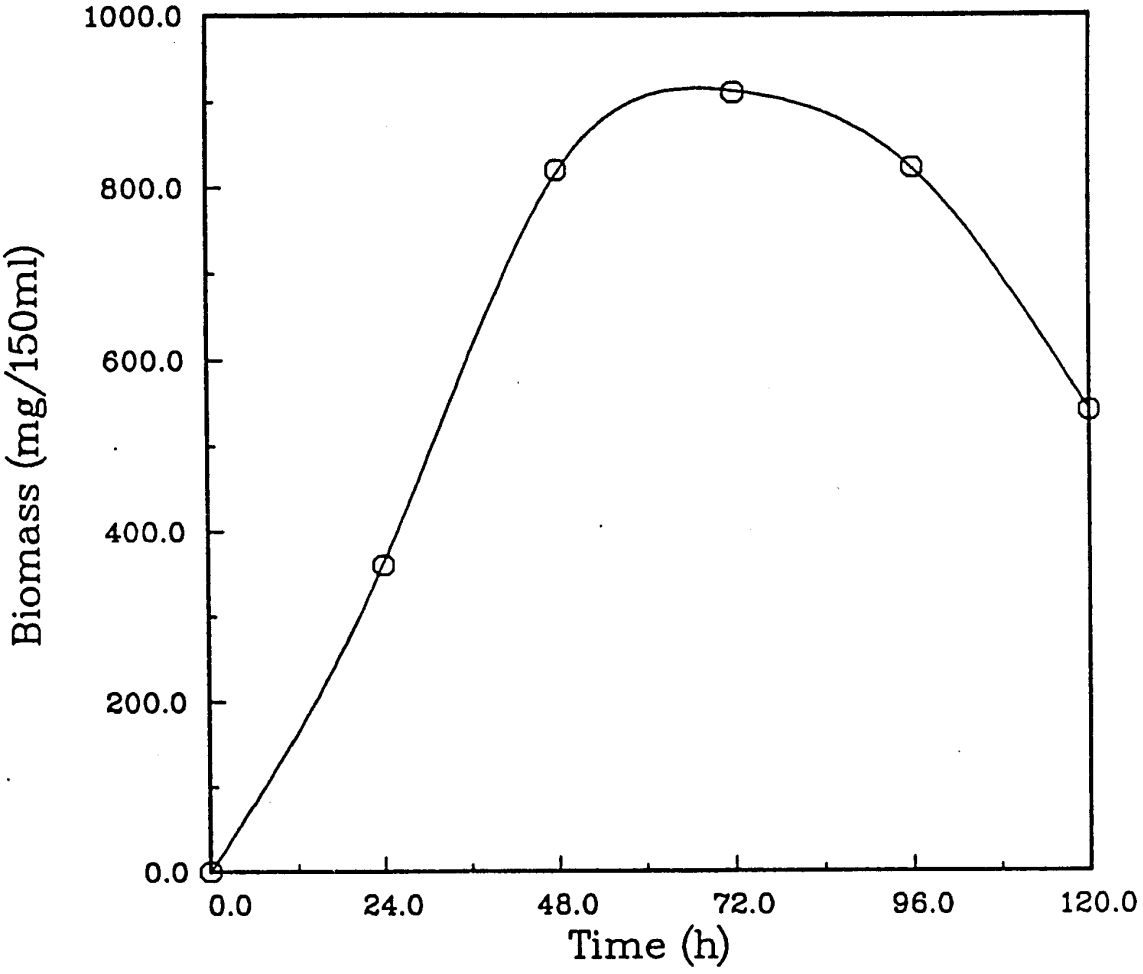

Figure B.1: Changes in dry biomass in a liquid culture

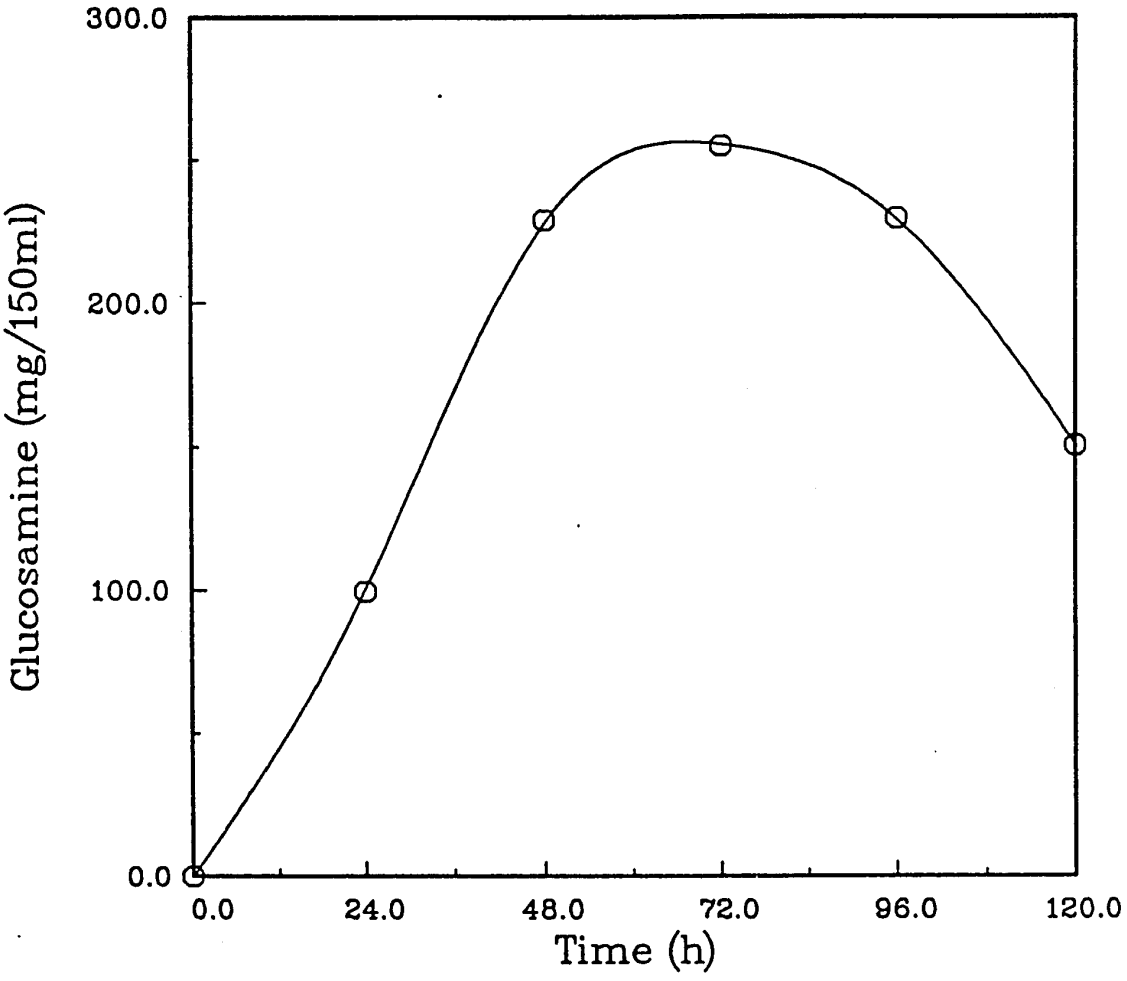

Figure B.2: Changes in glucosamine content (based on dry biomass) in a liquid culture

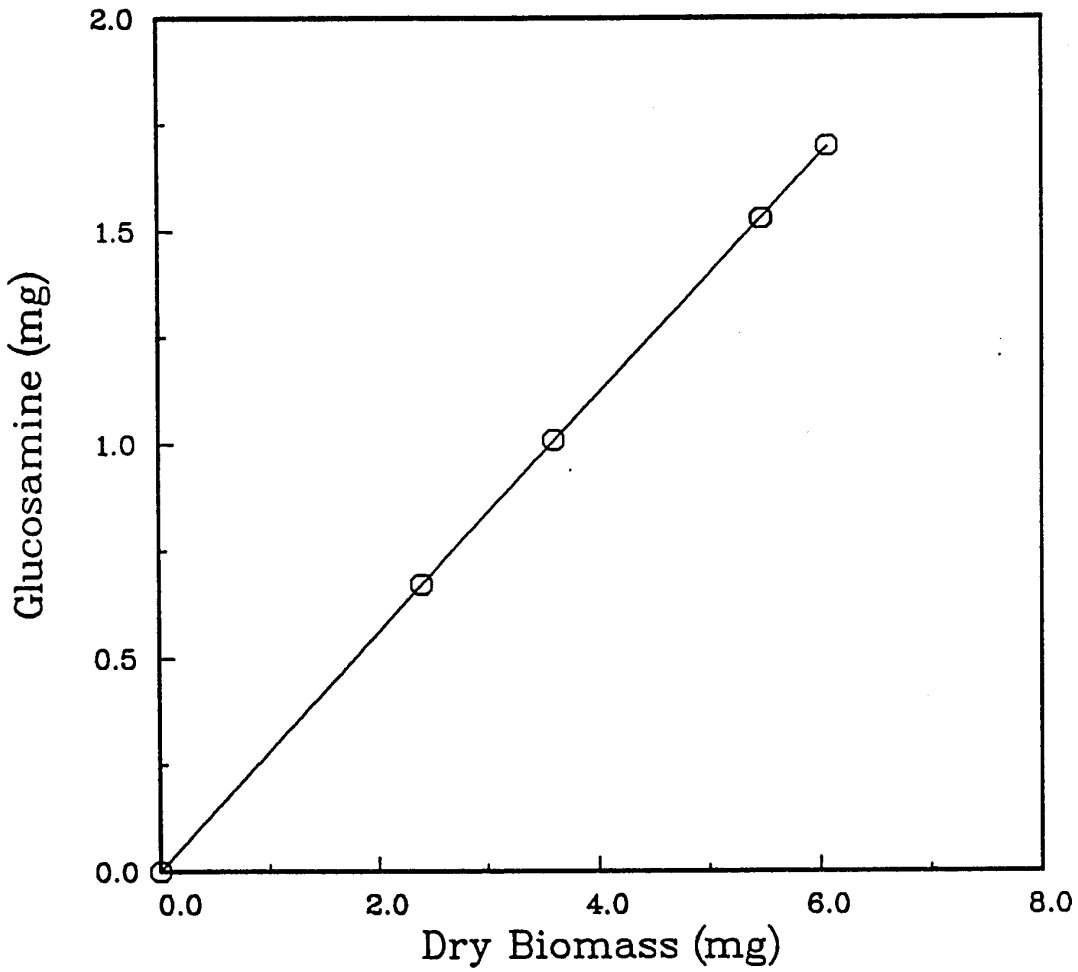

Figure B.3: Changes in dry weight biomass and glucosamine in a liquid culture

Appendix C

Tables

Table C.1: Effect of Incubation time on enzyme activity at 60°C and pH 4.8

Time (min)	Activity (units)
0	0
10	1.2
30	4.2
45	5.1
60	5.2

Table C.2: Effect of enzyme concentration on its activity at 60°C and pH 4.8

Enzyme (ml)	Activity (units)
0	0
0.1	0.42
0.5	8.00
1.0	14.98
1.5	19.00
2.0	20.50
2.5	22.10
3.0	22.80
3.5	23.40

Table C.3: Effect of Phytic acid concentration on enzyme activity at 60°C and pH 4.8

Phytic acid conc. (mM)	Activity (units)
0	0
0.5	3.00
0.9	4.00
1.5	4.60
1.95	4.76
2.45	4.88
3.25	5.10

Table C.4: Determining K_m and V_{max} values

$1/\text{conc. (mM)}^{-1}$	$1/\text{Activity (units/h)}^{-1}$
2.0	0.310
1.1	0.250
0.67	0.215
0.51	0.210
0.42	0.207
0.31	0.196

Table C.5: Effect of Inoculum concentration on phytic acid content

Time (h)	Inoculum (mg)			
	130	130 (in buffer 4.7)	150	170
	Phytic acid content (%)			
0	100	100	100	100
24	98	99	97	95
30	89	97	82	74
48	23	91	20	15
54	11	83	7	0
62	0	47	0	0
72	0	13	0	0
84	0	0	0	0

Table C.6: Effect of Inoculum concentration on enzyme production

Time (h)	Inoculum (mg)			
	130	130 (in buffer 4.7)	150	170
	Enzyme (units/ml)			
0	0	0	0	0
24	0.55	0.21	0.57	0.64
30	0.76	0.37	0.78	0.91
48	2.40	1.08	2.54	2.85
54	2.98	1.40	3.13	3.25
72	3.18	2.27	3.30	3.44
84	3.09	2.97	3.14	3.16
96	2.87	3.20	2.96	2.99

Table C.7: Effect of inoculum age on phytic acid content

Time (h)	Inoculum age (days)					
	2	3	4	5	6	7
	Phytic acid content (%)					
0	100	100	100	100	100	100
24	98	97	85	77	54	38
48	74	67	31	20	8	0
72	58	43	2	0	0	0
96	37	29	0	0	0	0
120	18	5	0	0	0	0

Table C.8: Effect of inoculum age on enzyme production

Time (h)	Inoculum age (days)					
	2	3	4	5	6	7
	Enzyme (units/ml)					
0	0.	0.	0.	0.	0.	0.
24	0.49	0.54	1.05	0.87	0.62	0.33
48	1.01	1.22	3.39	3.73	4.68	4.97
72	1.97	2.34	4.36	5.05	5.21	5.54
96	2.75	3.41	3.89	4.81	4.98	5.31
120	3.36	4.32	3.60	4.52	4.72	5.09

Table C.9: Effect of homogenization time on phytic acid content

Time (h)	Homogenization time (seconds)				
	10	30	60	120	240
	Phytic acid content (%)				
0	100	100	100	100	100
24	86	85	80	79	71
30	63	61	52	48	40
48	20	14	10	7	0
54	11	4	0	0	0
72	0	0	0.	0	0

Table C.10: Effect of homogenization time on enzyme production

Time (h)	Homogenization time (seconds)				
	10	30	60	120	240
	Enzyme (units/ml)				
0	0.	0.	0.	0.	0.
24	1.69	1.43	1.25	1.16	1.02
48	1.97	2.22	2.37	2.68	2.92
72	2.25	3.01	3.30	3.65	3.81
96	2.15	2.84	3.10	3.57	3.67

Table C.11: Phytic acid content reduction at particular periods of fermentation in medium containing inoculum homogenized for various times

Homogenization time (seconds)	Fermentation time (h)			
	24	30	48	54
	Phytic acid content (%)			
10	86	63	20	11
30	85	61	14	4
60	80	52	10	0
120	79	48	0	0
240	71	40	0	0

Table C.12: Enzyme production at particular periods of fermentation in medium containing inoculum homogenized for various times

Homogenization time (seconds)	Fermentation time (h)			
	24	48	72	96
	Enzyme (units/ml)			
10	1.69	1.97	2.25	2.15
30	1.43	2.22	3.01	2.84
60	1.25	2.37	3.30	3.10
120	1.16	2.68	3.65	3.57
240	1.02	2.92	3.81	3.67

Table C.13: Effect of initial moisture content on phytic acid content

Time (h)	Moisture Content (%)				
	21	31	39	45	50
	Phytic acid content (%)				
0	100	100	100	100	100
24	101	97	96	94	91
48	100	94	88	79	66
72	100	83	76	58	47
96	100	79	59	46	35
120	100	75	48	30	20

Table C.14: Effect of initial moisture content on enzyme production

Time (h)	Moisture Content (%)				
	21	31	39	45	50
	Enzyme (units/ml)				
0	0.	0.	0.	0.	0.
24	0.	0.36	0.57	0.82	0.95
48	0.	1.18	1.80	2.63	3.17
72	0.	1.49	2.36	3.20	4.03
96	0.	1.33	2.12	3.09	3.89
120	0.	1.20	2.03	2.92	3.72

Table C.15: Effect of higher initial moisture content on phytic acid content

Time (h)	Moisture Content (%)					
	31	39	50	60	64	68
	Phytic acid content (%)					
0	100	100	100	100	100	100
24	94	92	89	82	77	79
48	85	80	68	36	28	31
72	74	66	48	24	18	22
96	51	43	29	5	0	2
120	46	32	12	0	0	0

Table C.16: Effect of higher initial moisture content on enzyme production

Time (h)	Moisture Content(%)					
	31	39	50	60	64	68
	Enzyme (units/ml)					
0	0.	0.	0.	0.	0.	0.
24	0.32	0.64	1.08	1.54	1.69	1.49
48	1.43	2.38	3.12	3.41	3.66	2.46
72	2.21	3.02	4.22	4.58	4.67	3.24
96	1.96	2.63	3.64	4.27	4.18	2.67

Table C.17: Effect of initial pH on phytic acid content

Time (h)	buffer solutions (pH)				
	distilled water	5.7	5.4	5.2	5.0
	Phytic acid content (%)				
0	100	100	100	100	100
24	87	88	99	99	98
48	32	34	85	87	89
72	2	3	24	60	63
96	0	0	12	31	36

Table C.18: Effect of initial pH on enzyme production

Time (h)	buffer solutions (pH)				
	distilled water	5.7	5.4	5.2	5.0
	Enzyme (Units/ml)				
0	0.	0.	0.	0.	0.
24	0.73	0.67	0.37	0.36	0.32
48	3.30	3.25	0.63	0.58	0.51
72	4.32	4.30	3.10	2.96	2.88
96	3.91	3.85	4.30	4.20	4.17

Table C.19: Effect of added glucose concentration on phytic acid content

Time (h)	Glucose (g)					
	0	2	4	6	12	24
	Phytic acid content (%)					
0	100	100	100	100	100	100
24	86	84	82	79	87	96
48	57	37	34	21	61	84
72	10	7	4	0	21	48

Table C.20: Effect of added glucose concentration on enzyme production

Time (h)	Glucose (g)					
	0	2	4	6	12	24
	Enzyme (units/ml)					
0	0.	0.	0.	0.	0.	0.
24	0.51	0.53	0.90	1.37	0.42	0.24
48	3.35	3.49	3.81	4.13	1.57	0.58
72	4.78	4.84	4.45	4.60	4.11	3.45
96	4.26	4.28	4.30	4.32	3.82	3.12

Table C.21: Effect of added glucose concentration on biomass production

Time (h)	Glucose (g)					
	0	2	4	6	12	24
	Biomass (mg dry wt/g culture)					
0	0.	0.	0.	0.	0.	0.
24	6.82	11.21	12.14	12.78	5.67	4.78
48	11.94	17.74	18.03	19.60	10.78	10.21
72	14.86	19.60	20.24	21.81	12.31	11.56

Table C.22: Effect of surfactants on phytic acid content

Time (h)	Surfactants			
	no surfactant	Tween–80	Triton X–100	Na–O
	Phytic acid content (%)			
0	100	100	100	100
24	86	78	88	75
30	65	50	68	42
36	30	28	37	8
42	10	8	16	0
48	0	0	0	0

Table C.23: Effect of surfactants on enzyme production

Time (h)	Surfactants			
	no surfactant	Tween–80	Triton X–100	Na–O
	Enzyme (units/ml)			
0	0.	0.	0.	0.
24	1.22	1.41	1.30	1.52
48	3.10	3.25	2.71	3.37
72	3.67	3.78	3.57	4.01
96	3.44	3.54	3.35	3.89

Table C.24: Effect of sodium oleate concentration on phytic acid content

Time (h)	Sodium oleate (%)					
	0	0 (in buffer 4.7)	0.1	0.5	1	5
	Phytic acid content (%)					
0	100	100	100	100	100	100
24	82	88	77	76	72	86
30	63	74	44	40	38	65
36	25	61	11	9	5	31
42	9	45	0	0	0	22
48	0	38	0	0	0	13
60	0	13	0	0	0	2
72	0	0	0	0	0	0

Table C.25: Effect of sodium oleate concentration on enzyme production

Time (h)	Sodium oleate (%)					
	0	0 (in buffer 4.7)	0.1	0.5	1	5
	Enzyme (units/ml)					
0	0.	0.	0.	0.	0.	0.
24	0.57	0.31	0.63	0.71	0.84	0.64
48	3.29	1.15	4.00	3.84	3.34	2.69
72	3.47	3.13	4.22	4.03	3.84	3.24
96	3.21	2.88	4.01	3.79	3.62	3.03

Table C.26: Effect of Phosphate concentration on phytic acid content (in distilled water)

Time (h)	Phosphate (mg)					
	0	0 (in buffer 4.7)	1	5	10	50
	Phytic acid content (%)					
0	100	100	100	100	100	100
24	84	91	78	73	68	61
30	62	89	54	43	36	26
36	36	87	29	10	7	31
42	18	74	13	0	0	0
48	0	61	0	0	0	0
60	0	31	0	0	0	0

Table C.27: Effect of Phosphate concentration on enzyme production (in distilled water)

Time (h)	Phosphate (mg)					
	0	0 (in buffer 4.7)	1	5	10	50
	Enzyme (units/ml)					
0	0.	0.	0.	0.	0.	0.
24	0.88	0.60	1.15	0.92	0.80	0.66
48	3.09	1.05	3.25	3.15	2.91	2.71
72	3.45	2.73	4.11	3.59	2.79	2.58
96	3.15	3.57	3.96	3.38	2.54	2.35

Table C.28: Effect of Phosphate concentration on biomass production (in distilled water)

Time (h)	Phosphate (mg)					
	0	0 (in buffer 4.7)	1	5	10	50
	Biomass (mg dry wt/g culture)					
0	0.	0.	0.	0.	0.	0.
24	9.16	0.96	9.88	10.41	11.66	12.91
48	13.99	6.86	15.91	17.09	18.89	18.34
72	18.95	15.23	20.20	22.02	23.71	23.12
96	19.56	17.16	21.03	22.99	24.21	23.74

Table C.29: Phytic acid content reduction at particular periods of fermentation using different Phosphate concentrations in medium prepared with distilled water

Phosphate conc. (mg/50 g culture)	Fermentation time (h)		
	24	36	42
	Phytic acid content (%)		
0	84	36	18
1	78	29	13
5	73	10	0
10	68	7	0
50	61	3	0

Table C.30: Enzyme production at particular periods of fermentation using different Phosphate centrations in medium prepared with distilled water

Phosphate conc. (mg/50 g culture)	Fermentation time (h)			
	24	48	72	96
	Enzyme (units/ml)			
0	0.88	3.09	3.45	3.15
1	1.15	3.25	4.11	3.96
5	0.92	3.15	3.59	3.38
10	0.80	2.91	2.79	2.54
50	0.66	2.71	2.58	2.35

Table C.31: Effect of Phosphate concentration on phytic acid content (in buffer 4.7)

Time (h)	Phosphate conc. (mg)					
	0	0 (in distilled water)	1	5	10	50
	Phytic acid content (%)					
0	100	100	100	100	100	100
24	87	65	89	91	92	95
36	79	21	82	87	89	91
48	58	0	67	73	79	84
72	24	0	45	52	57	73
96	0	0	9	17	22	29

Table C.32: Effect of Phosphate centration on enzyme production (in buffer 4.7)

Time (h)	Phosphate conc. (mg)					
	0	0 (in distilled water)	1	5	10	50
	Enzyme (units/ml)					
0	0.	0.	0.	0.	0.	0.
24	0.47	1.39	0.50	0.55	0.58	0.66
48	0.82	2.80	0.87	0.92	0.99	1.22
72	2.69	3.24	2.83	2.99	3.07	3.27
96	3.19	3.08	3.35	3.47	3.69	4.14
120	2.88	2.91	3.02	3.11	3.28	3.81

Table C.33: Effect of Phosphate concentration on biomass production (in buffer 4.7)

Time (h)	Phosphate conc. (mg)					
	0	0 (in distilled water)	1	5	10	50
	Biomass (mg dry wt/g culture)					
0	0.	0.	0.	0.	0.	0.
24	1.64	10.75	1.29	1.24	1.18	0.97
48	5.79	18.39	4.57	3.25	2.72	1.47
72	15.47	21.50	12.36	11.22	10.61	9.61
96	20.93	22.97	17.47	15.57	14.68	14.22

Table C.34: Phytic acid content reduction at particular periods of fermentation using different Phosphate concentrations in buffered medium

Phosphate conc. (mg) (mg/50 g cultre)	Fermentation time (h)				
	24	36	48	72	96
	Phytic acid content (%)				
0	87	79	58	24	0
1	89	82	67	45	9
5	91	87	73	52	17
10	92	89	79	57	22
50	95	91	84	73	29

Table C.35: Enzyme production at particular periods of fermentation using different Phosphate concentrations in buffered medium

Phosphate conc. (mg/50 g culture)	Fermentation time (h)			
	24	48	72	96
	Enzyme (units/ml)			
0	0.47	0.82	2.69	3.19
1	0.50	0.87	2.83	3.35
5	0.55	0.92	2.99	3.47
10	0.58	0.99	3.07	3.69
50	0.66	1.22	3.27	4.14

Table C.36: Effect of phosphate, sodium oleate, phosphate + sodium oleate on phytic acid content

Time (h)	Additives			
	no additive	1 mg PO_4	0.1% Na–O	1 mg PO_4 + 0.1% Na–O
	Phytic acid content (%)			
0	100	100	100	100
24	81	69	77	88
36	32	21	25	46
42	14	6	10	23
48	0	0	0	2

Table C.37: Effect of phosphate, sodium oleate, phosphate + sodium oleate on enzyme production

Time (h)	Additives			
	no additive	1 mg PO_4	0.1% Na–O	1 mg PO_4 + 0.1% Na–O
	Enzyme (units/ml)			
0	0.	0.	0	0
24	1.44	1.76	1.60	1.34
48	2.63	3.29	2.96	2.66
72	3.84	4.63	4.30	3.28
96	3.29	4.17	3.79	2.96

Table C.38: Effect of phosphate, sodium oleate, phosphate + sodium oleate on biomass production

Time (h)	Additives			
	no additive	1 mg PO_4	0.1% Na-O	1 mg PO_4 + 0.1% Na-O
	Biomass (mg dry wt/g culture)			
0	0.	0.	0	0
24	7.07	8.56	7.96	5.71
48	13.93	15.75	5.19	13.32
72	19.07	19.64	19.25	17.57
96	19.71	20.79	20.24	18.36

Table C.39: Changes in Phytic acid, Available carbohydrates and protein in an SSF process

Time (h)	Meal components		
	Phytic acid	Available carbohydrate	Protein
	Content (%)		
0	100	100	100
24	78	38	96
48	30	30	108
72	17	25	128
96	0	23	118

Table C.40: Changes in biomass and enzyme activity in an SSF process

Time (h)	Biomass (mg dry wt/g culture)	Enzyme (units/ml)
0	0.	0.
24	5.0	1.69
48	11.56	3.66
72	17.38	4.67
96	18.97	4.18

Table C.41: Following dry weight biomass in a liquid culture

Time (h)	Biomass (mg/150 ml)
0	0
24	360
48	820
72	910
96	822
120	540

Table C.42: Following glucosamine content in dry biomass in a liquid culture

Time (h)	Glucosamine (mg/150 ml)
0	0
24	100.8
48	229.6
72	254.8
96	230.0
120	151.2

Table C.43: Following dry weight biomass and glucosamine content in a liquid culture

Dry wt. biomass (mg)	Glucosamine (mg)
0	0
2.40	0.67
3.60	1.01
5.47	1.53
5.48	1.53
6.07	1.70