National Library of Canada
Acquisitions and Bibliographic Services Branch
395 Wellington Street
Ottawa, Ontario
K1A 0N4

Bibliothèque nationale du Canada
Direction des acquisitions et des services bibliographiques
395, rue Wellington
Ottawa (Ontario)
K1A 0N4

NOTICE

The quality of this microform is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

Reproduction in full or in part of this microform is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30, and subsequent amendments.

AVIS

La qualité de cette microforme dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

La reproduction, même partielle, de cette microforme est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30, et ses amendements subséquents.

Canada

Production of Phytase and Reduction of Phytic Acid Content in Canola Meal by Solid State Fermentation Using *Aspergillus carbonarius*

by

Sameer Al-Asheh

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
Chemical Engineering

Department of Chemical Engineering
University of Ottawa

Ottawa, Canada, 1993

Sameer Al-Asheh, Ottawa, Canada, 1993

National Library of Canada

Acquisitions and Bibliographic Services Branch

395 Wellington Street
Ottawa, Ontario
K1A 0N4

Bibliothèque nationale du Canada

Direction des acquisitions et des services bibliographiques

395, rue Wellington
Ottawa (Ontario)
K1A 0N4

The author has granted an irrevocable non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of his/her thesis by any means and in any form or format, making this thesis available to interested persons.

L'auteur a accordé une licence irrévocable et non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de sa thèse de quelque manière et sous quelque forme que ce soit pour mettre des exemplaires de cette thèse à la disposition des personnes intéressées.

The author retains ownership of the copyright in his/her thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without his/her permission.

L'auteur conserve la propriété du droit d'auteur qui protège sa thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

ISBN 0-315-89616-7

Canada

UNIVERSITÉ D'OTTAWA
UNIVERSITY OF OTTAWA

ABSTRACT

Solid state fermentation (SSF) with canola meal as substrate was carried out to study the production of phytase and the reduction of the phytic acid content in the meal using *Aspergillus carbonarius* NRC 401124.

Some characteristics of the phytase were studied. K_m and v_{max} values of 0.345 mM and and 0.8071 units were determined when sodium phytate was used as the substrate for this enzyme. The enzyme showed an optimum pH and temperature of 4.7 and 53°C respectively. It was demonstrated that an activation of the enzyme occured when it was preincubated at higher temperatures for a period of time. The energy of activation and the entropy and enthalpy changes were evaluated to be 7800 cal/mole, 74 cal/(mole.K) and 24000 cal/mole respectively for this enzymatic reaction. Effects of the extractant:solid state culture ratio and the time of extraction on the single step extraction of phytase were evaluated. Models were proposed for some of the studied parameters.

It was found that the production of phytase was growth associated and that the maximum activity was attained after 72 h of incubation during SSF process.

Apparent increases of about 25% and 10% of protein content of canola meal were noticed after 48 h and 72 h of the process respectively. A 25% reduction in the total carbohydrates concentration was reached at the end of fermentation.

The rate of the reduction of phytic acid content of the meal depended on the physical parameters of the SSF. The optimum moisture content was found to be 53%. When the effect of homogenization was studied, it was found that the most suitable time of the homogenization was 120 s for the growth of the biomass, production of the enzyme and thus also for the reduction of the phytic acid content in the meal. The biomass growth, phytase production and phytic acid content reduction increased with an increase in the inoculum size. Although both sterilized and unsterilized meal inoculated with the microorganism can be used for the reduction of the phytic acid content better results were obtained with the former.

The effect of particle size of the meal was considered. The optimum particle size for this process was found to be 1.4 mm, and negative results were noticed

with particle sizes higher than 1.4 mm.

The effect of added glucose on the process was considered. It was found that the increase in glucose amount up to and including 6 g per system in the initial medium resulted in an increase in the rate of the biomass growth, enzyme concentration and the rate of phytic acid content reduction in canola meal. Inhibition of the rates of the above processes was noticed when the initial glucose amounts were above those mentioned. The canola meal systems with more than 6 g of glucose had longer growth phases than those with lower glucose amounts, and this resulted in their higher maximum enzyme activities.

The effect of added phosphate in the process was considered. The addition of 1 mg of phosphate per system remarkably increased the biomass and enzyme productions and phytic acid content reduction.

When the effect of surfactant was studied, it was found that sodium oleate increased the biomass and enzyme productions and the rate of phytic acid content reduction, while Triton X-100 gave a negative effect. A positive effect was obtained for systems containing combined portions of oleate and phosphate.

Some results from liquid medium were obtained and are presented in this work.

A logistic law was used to model the biomass production. Models which relate the enzyme production and the phytic acid content reduction with the biomass concentration during a solid-state fermentation (SSF) process are given in this work and they fit the experimental data produced in this work reasonably well.

ACKNOWLEDGEMENT

I wish to express my gratitude to Dr. Z. Duvnjak for his continuous encouragement and invaluable suggestions throughout the research. I would like also to express my sincere thanks to the Jordan University of Science and Technology (J.U.S.T.) for its scholarship program.

I would like to thank Mr. K. Lacki and S. Boudriga for their support throughout this project.

It is with heart felt gratitute that I commend my father and my soul mother; my beloved wife Nessren and my brother Taiseer for their unyielding support, encouragement, and love throughout this period of study.

Contents

List of Tables

List of Figures

Nomenclature

C_g	glucose concentration, g per system
C_p	phosphate concentration, mg per system
E	energy of activation, cal/mole
K_1, K_2	Equilibrium constants, mM
K_d	Equilibrium constant
K_m	Michaelis Menton constant, mM
$K_{t_{inc}}$	constant in Eq. 3.42, min
K_t	constant in Eq. 3.43, min
K_V	constant in Eq. 3.44, ml/g
P	phytic acid concentration, %
P_0	initial phytic acid content, %
Pi	orthophosphate released, mg/ml
Q	extracted enzyme, units/g
Q_{max}	maximum extracted enzyme, units/g
R	ideal gas constant, cal/(mole.K)
T	temperature, °C
T_{max}	maximum temperature acheived, °C
V_E	volume of extractant, ml/g SSC
X	biomass, g/g SSC
X_m	maximum biomass achieved, g/g SSC
X_0	initial biomass, g/g SSC
a	empirical parameter in Eq. 3.11, units/g
b	empirical parameter in Eq. 3.11, h^{-1}
e_a	fraction of the enzyme in the active form
e_i	fraction of the enzyme in the inactive form
e_0	total fraction of the enzyme
k_d	empirical constant in Eq. 3.12, day^{-1}
k_p	rate constant in Eqs. 3.17 and 3.19, h^{-1}
k_v	rate constant in Eq. 3.10, units/g.h
h^+	hydrogen concentration, M
s	substrate concentration, mM
t	fermentation time, h
t_{max}	time for maximum enzyme activity, h
t_E	extraction time, min
t_{inc}	incubation time, min

v	enzyme activity, units/g
v_{max}	maximum enzyme activity acheived, units/g
v_0	initial enzyme activity, units/g
v_{opt}	optimum enzyme activity in Eq. 3.40, units/g
ΔG_d	free energy, cal/mole
ΔH_d	enthalpy of deactivation, cal/mole
ΔS_d	entropy of deactivation, cal/(mole.K)
ΔS^*	entropy of deactivation, cal/(mole.K)
μ_m	maximum specific growth rate of biomass , h^{-1}

Abbreviations

SSC	solid state culture.
E	enzyme.
E_a	activated enzyme.
E_i	inactivated enzyme.
SSF	solid state fermentation.

Chapter 1

Introduction

Canada is the second largest rapeseed producer and the largest rapeseed exporter in the world. Rapeseed was brought into Canada in 1942 when it was used only as a lubricant due to its adhesive property to wet metal surfaces. In the 1970's, Canadian scientists developed new rapeseed varieties which are relatively low in glucosinolates and erucic acid and are called "Canola seeds". Since then, the market value of canola has increased considerably. Canada produced more than 4.3 $\times 10^6$ metric tons of canola seeds (which are a variety of rapeseed) in the year 1991/1992 (Statistics Canada, 1993).

Canola seed is very rich in oil (40-45%) and protein (20-25%). The extracted oil from canola seeds is characterized by a low level of cholesterol and saturated fatty acid (Harris, 1988). It is used commercially for the production of margarine, shortening and salad oil. The production of canola oil in Canada in the year 1991/1992 was 0.742 $\times 10^6$ metric tons, 8% of the total canola/rapeseed oil production in the world.

Canola meal is a by-product of canola oil production. The production of

canola meal in Canada in the same year was 1.097 $\times 10^6$ metric tons (Statistics Canada, 1993). It is widely used in Canada in rations for poultry, pigs and cattle because of its high nutritional value and its availability at a competitive price with other protein supplements. The protein content of canola meal depends up on the cultivar from which the meal is produced. On average, canola meal made from a mixture of low glucosinolate cultivars can be expected to contain 37 to 38% protein (Clandinin, 1986). The crude fiber content of canola meal is higher than that of soybean meal (11-15%), and the fiber in canola meal has been shown to have beneficial values in rations for ruminants (Clandinin, 1986).

The level of available phosphorus in canola meal is higher than that in soybean meal. Phytic acid (myo-inositol hexaphosphoric acid) is the main storage form of phosphorus in many seeds and cereal; up to 80% phosphorus was found to be in the form of phytic acid (Lolas and Markakis, 1977).

Harland and Harland (1980) reported that no phytate is present in bananas, celery, citrus fruits, lettuce, mushrooms, onion and prunes. Very low amounts of phytate were found in apple, carrots, broccoli, and green beens. Moderate amounts of phytate exist in potatoes, figs, artichokes and strawberries. The greatest amounts of phytate have been reported in legumes, cereals and nuts. Canola meal contains 4-6% phytic acid. Phytic acid binds to multivalent cations such as Zn^{2+}, Ca^{2+} and Fe^{3+} and so reduces their bioavailability. In spite of the lower availability of minerals in canola meal versus that in soybean, canola meal is a better source of available calcium, iron, manganese, phosphorus and selenium than

soybean (Clandinin, 1986). It has also been reported that phytic acid inhibits enzymes such as α- amylase (Sharma et al., 1978), trypsin, trysinase and pepsin (Graf, 1986). Taking the above into consideration the phytic acid content in canola meal should be decreased to enhance the value of this commodity.

It has also been found that phytase (EC 3.1.3.8), which is a phosphomonoesterase, hydrolyses phytic acid and a number of other phosphoesters (Courtois and Joseph, 1947; Courtois and Perez, 1948). Some phytase activity has been reported in many plants as in wheat (Peers, 1953) and mung beans (Mandal et al., 1972), which can hydrolyze the phytic acid in them. But there are also plants such as canola that do not contain phytase (Stone et al., 1984); in such cases, the phytic acid content can be reduced either by chemical methods, but a partial loss of nutritional contituents such as proteins and minerals has been noticed when these techniques were applied (Gillberg and Tornell, 1976; Ford et al., 1978; Alli and Houde,1987), or using externally added phytases. Solid state fermentation or submerged processes can be used for the production of microbial phytase.

Solid state fermentation (SSF) may have great potential; it is attractive because of high solid matter content in the medium and, therefore, reduces the costs of drying of the products (Mitchell et al., 1991). Although it looks like a simple production method, the growth of microorganisms on solid substrates is poorly understood (Moo-Young et al., 1979); difficulties also arise because of the heterogeneity and complexity of the media in the SSF process and the problems related to the measurement of fermentation parameters, the most critical of which is the

biomass (Mitchell et al., 1990).

SSF has been used to study the growth of microorganisms, such as *Aspergillus niger* on casava meal (Raimbault and Alazarad, 1980), to enrich raw materials with proteins. It has also been applied to investigate the production of a number of enzymes including lipolytic ones (Rivera-Munoz et al., 1991) by filamentous fungi.

SSF and submerged processes have also been used for the production of phytase (Han and Gallagher, 1986; Nair and Duvnjak, 1990) by *Aspergillus ficuum*. It has also been reported that many organisms can produce this enzyme; among these, *Aspergillus carbonarius* has also been reported to be able to produce a considerable amount of extracellular phytase in a liquid medium (Shieh and Ware, 1968).

The objectives of this work are;

- to study the production of phytase in canola meal by solid state fermentation using *Aspergillus carbonarius* NRC 401124.
- to study the characteristics of phytase.
- to study the reduction of phytic acid content in canola meal by solid state fermentation using *Aspergillus carbonarius* NRC 401124.
- to develop a mathematical model which relates the fermentation variables with the time of fermentation.

Chapter 2

Literature Review

2.1 Canola Oil

The oil from canola seeds is much lower in harmful materials such as erucic acid and glucosinolate than other rapeseed oils. Its production has increased by over 300% in the last decade (Vaisey-Genser and Harris, 1987). Zuk et al., (1981) concluded that canola oil can be substituted for diesel fuel without major changes in engine performance and there was only low levels of particulates, NO_x and aldehydes in exhaust emissions when canola oil was used. The problem arises in cold weather because of the higher viscosity of canola oil; however, this problem can be avoided by the addition of alcohol to the oil in different proportions to reduce the viscosity and makes the mixture more feasible. Unfortunately this process failed because of its higher cost with respect to the crude oil.

Commercial hexane specially refined for use in the vegetable oil industry can be used for the extraction of canola oil from canola seeds.

2.2 Canola Meal

Canola meal is very rich in protein. It is a by-product of canola oil production. It contains 37-38% proteins, 28-32% carbohydrates, 11-15% crude fibre, 4-6% phytic acid and a number of minerals such as calcium, iron, manganese, phosphorus, selenium and zinc (Clandinin, 1986). Canola meal is used for livestock and poultry because it is a good source of vitamins, containing appreciable quantities of biotin, folic acid, niacin, riboflavin and thiamine (Clandinin, 1986). It can also be used as an organic fertilizer (Clandinin, 1986).

Glucosinolates, phytates and phenolic compounds are the main harmful materials in canola oil. Attempts have been made to reduce these constituents (Clandinin, 1986).

2.3 Phytic Acid

2.3.1 Structure

Phytic acid (myo-inositol hexaphosphoric acid), designated as IP6, a naturally occuring plant acid, exhibits six acidic phosphate groups bound to a myo-inositol ring. Its structure has not been clearly demonstrated.

Brown et al. (1961) showed that phytic acid exists in two different structures proposed by Anderson and Neuberg. Neuberg has proposed a structure,

$C_6H_{24}O_{27}P_6$, with 18 acid hydrogens bound to the inositol phosphate nucleus. Anderson proposed a structure, $C_6H_{18}O_{24}P_6$, with 12 acid hydrogens bound to the inositol phosphate nucleus; this structure was found in plant seeds. These structures are shown in Fig. 2.1.

2.3.2 Occurence

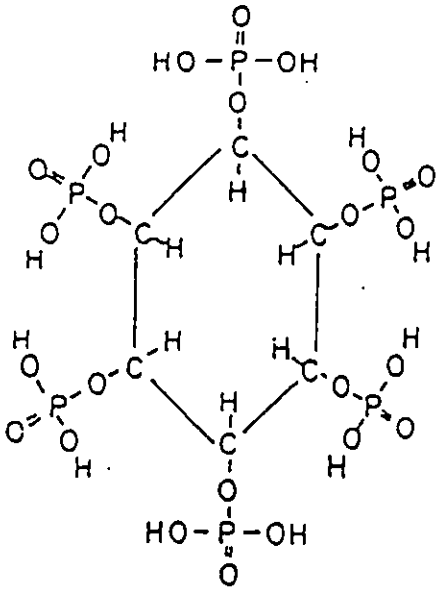
A great variety of seeds contain important quantities of phytic acid and its salts (Harland and Morris, 1985). Most cereal grains and legume seeds have been reported to contain phytic acid approximately 0.5-5.0% by weight as their main phosphorus source.

The levels of phytic acid in 18 varieties of barley were found to range from 0.97 to 1.08% dry weight (Lolas and Makakis, 1975). Phytic acid is also involved with minerals bioavailability in humans (Bieth et al., 1989). A considerable amount of phytic acid is present in pearl millet (Chauhan et al., 1986; Mahjan and Chauhan, 1987). Han and Wilfred (1988) reported that soybean meal and cottonseed meal contain about 2.2 and 4.4% (w/w) phytate, respectively.

2.3.3 Function

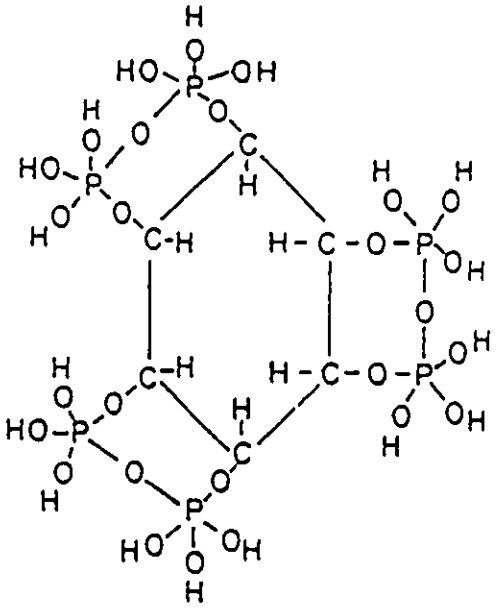
Phytic acid is found mainly in plant seeds, where it functions as a reserve material for phosphorus. Phytic acid has strong negative charge over most of the pH scale, suggesting a tremendous potential for complexing positively charged molecules such as cations or proteins with the formation of complexes that may be

(a)



ANDERSON

(b)



NEUBERG

Figure 2.1: Structures of phytic acid; (a)-Anderson structure, (b)- Nerberg structure

insoluble or otherwise unavailable under physiological conditions (Cheryan, 1980). This leads to decrease in the bioavailability of many essential minerals, especially zinc (Erdman, 1979).

At low pH, for example, $-NH_3^+$ groups on protein will bind to the phosphate groups of phytic acid (Fig. 2.2 a). Multivalent metals ions such as Ca^{2+}, Fe^{3+} and Zn^{2+} may also interact with phytic acid in a similar fashion (Reddy et al., 1989).

At intermediate pH range (4-9), both phytic acid and proteins have a net negative charge, but complexation occurs through multivalent cation-mediated interaction (Fig. 2.2 b).

At high pH the interaction between protein and phytic acid is diminished; however, the nature of interaction is not clearly understood (Reddy et al., 1989)

2.3.4 Applications

The ability of phytic acid to chelate metallic cations gives rise to a variety of food, industrial and biological applications. These include its use as a preservative and antioxidant in food (Graf et al., 1987); or as an anti-corrosion agent in various coating materials (Graf, 1983). In the biological area, technetium-99m complexed by phytate produces a clinically useful radiopharmaceutical (Graf and Eaton, 1985). In the particular case of hemoglobin, phytic acid is known to alter the hemoglobin-oxygen affinity Phytic acid can be used to remove metals from liquid products such as wines and other beverages.

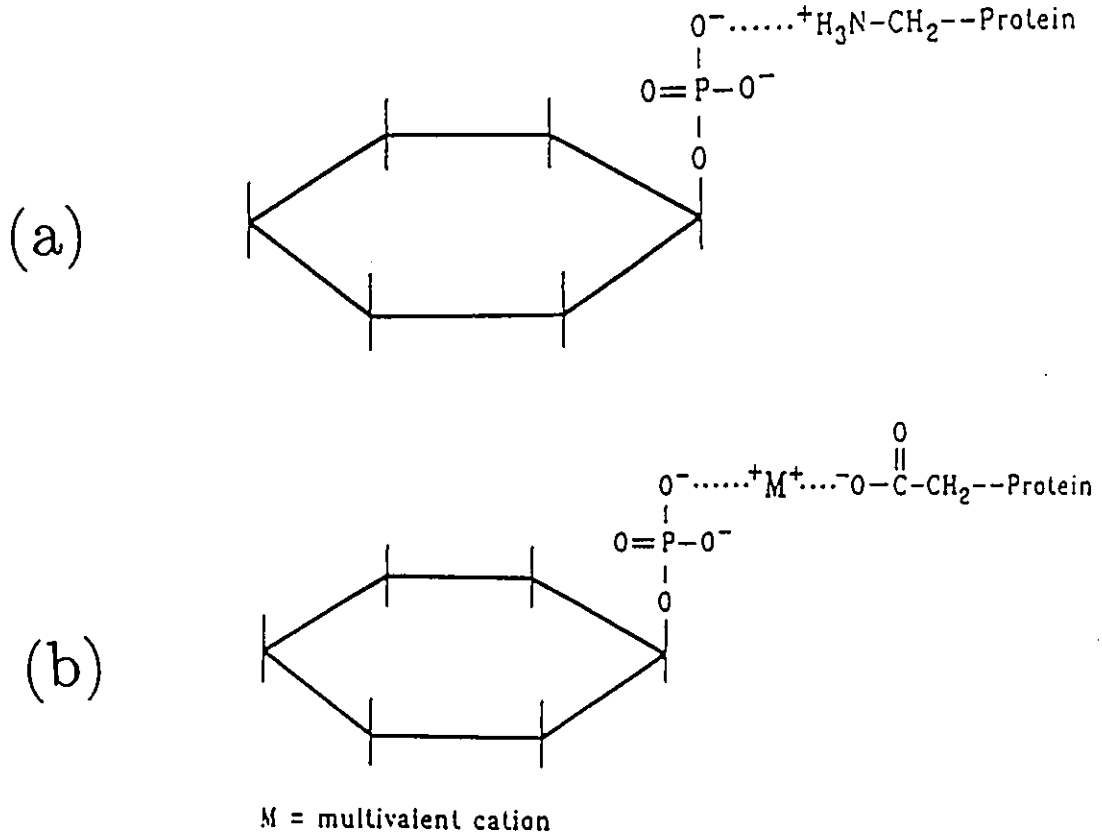


Figure 2.2: Complexation of phytic acid with protein; (a)-low pH, (b)- intermediate pH

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

2.3.5 Phytate Removal

Several techniques have been reported for the removal of phytate from their origins. Different methods were reported for partial elimination of phytate from soya products, such as precipitating it as barium salt, dialyzing it against sodium chloride solution, and treating it with a strong anionic exchange resin.

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

Ford et al. (1978) studied the removal of phytic acid from soybeans by a lipid-protein concentrate process. They concluded that by changing both the molar concentration of the calcium chloride solution used and the pH of the initial acid slurry, various amounts of phytic acid and mineral elements were recovered in the acid precipitated crude. Their results also showed that the removal of 10-90% of phytic acid was associated with zinc recovery from 10-90% and calcium concentrations equalling twice that of the original starting flour.

Hartman (1979) proposed the precipitation of phytate from soya protein by adjusting the solution at 28°C to pH 11.6. The precipitated phytates are then removed by centrifugation or by filtration. In the last step, the extract is treated

by filtration , resulting in a reduction of the phytate content from 2.6% to 0.1%.

Han and Wilfred (1988) concluded that a majority (more than 50%) of phytic acid in soybean and cottonseed was easily removed by washing with water or 1 N HCl. Temperature (25-50°C) and pH (3.7-5.6) of the extracting media, extraction time (1-24 h), and particle size of the seed meals had little effect on the amount of phytate extracted.

Removal of phytic acid of brown and white varieties of Bengal grams has been studied (Khan et al., 1988). This process envolved soaking, boiling, roasting and frying. Loss of phytic acid in presoaked grams increased with resting time.

Apparently, phytates are fairly stable to heat. In the case of soy isolate, autoclaving for 4 h at 115 °C is required to destroy most of the phytic acid (Rackis, 1974). The disadvantage of this technique arises because of amino acid destruction. 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%. 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 it increased the rate of enzymatic hydrolysis in the heated sample.

Phytic acid of chickpea seed was reduced to a range value of 16-60% and 16-64% by roasting and autoclaving respectively depending upon the cultivar (Hussain et al., 1989). In the cotyledon, the phytic acid was reduced to a range value of 32-68% and 18-68% during roasting and autoclaving respectively (Hussain et

al., 1989).

The enzyme phytase exists in seeds and in many microorganisms. Soaking the seeds reduces the phytic acid content through enzymatic destruction. During bread making, a cosiderable part of phytic acid is destroyed by enzymatic activity of the yeast (Laskin, 1982). Han and Wilfred (1988) concluded that the application of exogenous phytase or activation of endogenous phytase can hydrolyzed water-insoluble phytate in the seed meal. He also stated that the coapplication of phytase with cellulase produced a synergistic effect in hydrolyzing phytate in soybean meal.

The enzymes from microorganisms have been used very effectively and successfully in the reduction of phytic acid content in products. For example, the solid state fermentation technique was used by Nair and Duvnjak (1990) to produce phytase from *Aspergillus ficuum* NRRL 3135 on canola meal. The produced enzyme catalyzed degradation of the phytic acid which was present in the meal, completely eliminating it, thus rendering the commodity more suitable for animal feed.

2.4 Solid State Fermentation

Many advances have been made in the successful propagation of fungi for commercial purposes. One of these is the solid state fermentation (SSF) process, the process known for hundreds of years.

SSF process has been defined by different researchers. Mitchell et al. (1990) stated that the solid state fermentation is the utilization for microbial growth of water insoluble materials in the absence or near absence of free water. According to Durand and Gereau (1988) the definition of SSF shows that microorganisms are intimately bound to the solid matrix, which involves difficulties for biomass measurements. Generally, solid state fermentations involve cultivation of mycelial organisms on media comprising three phases:

1. a solid phase which is generally a vegetable substrate containing the microorganism nutrients;

2. a liquid phase bound to the solid matrix where different mass transfer occurs;

3. a gaseous phase.

Ralph (1976) considered two principal types of microbial behaviour under SSF process;

(*i*) that in which a solid material serves as the main source of nutrients for the organisms which intimately associate themselves with the surface and interstices of the material;

(*ii*) that in which a nutritionally inert solid support provides some advantage to the organism in respect to access to nutrients.

Food fermentations such as cheese making and oriental fungus processed foods, preservation of fish and animal products, and production of vinegar and gallic acid are types of solid state fermentation systems that have existed for

many hundreds or thousands of years (Laskin, 1982).

Most SSF processes were used for the production of enzymes (Hesseltine, 1972; Han and Anderson, 1975). The famous example is the *koji* process for making enzymes needed in soybean fermentation. The process is based on using wooden trays in which cereals, such as rice and wheat, were allowed to mold after mixing with *koji* molds as inoculum. The problem with the tray method is the difficulty in controlling the operating conditions. Although rotary drums have been used to replace trays, low enzymes production has been reported due to the damage of mycelia by the rotation of the drum.

Various workers have used solid state fermentation techniques for mass production of spores needed in the transformation of organic compounds such as steroids, antibiotics, fatty acids and carbohydrates (Laskin, 1982). Butler (1975) has given comprehensive accounts of myocotoxicoses; he reported that the toxic substances, usually of fungal origin, may be produced on a wide range of solid substrates such as leaves, stems, grains and seeds. Aflatoxins, the most publicized of the myocotoxins, are produced by certain strains of *Aspergillus flavus* and *Aspergillus parasiticus* (Laskin, 1982; Detry et al., 1972). It has been shown that aflatoxins may be produced by solid state fermentations on a wide range of foodstuffs such as rice, wheat, and oats and also on forages (Laskin, 1982).

Conversion of agricultural wastes such as crop residues by fermentation to more useful products has recieved much attention in recent years. Han and Anderson (1975) fermented rice straw and cane sugar bagasse with *Cellulomonas* and

Alcaligenes species. They were able to increase the digestibility of cellulose from 29% to 73%.

Moo-Young et al. (1979) have developed a fermentation process for the bioconversion of agricultural wastes into proteinaceous food products based on mass microbial cultivation of *Chaetomium cellulolyticum* in a solid state fermentation-system.

Raimbault et al. (1977) and Senez (1978) have reported a simple and inexpensive process of solid state fermentation for protein enrichment of starchy wastes materials from cassava, banana potatoes and other sources. Raimbault and Alazard (1980) and Raimbault et al. (1985) also investigated the growth of *A. niger* in a SSF process for the protein enrichment of starchy materials such as cassava. They studied the growth kinetics, changes in pH, protein and carbohydrates, and found that the optimum conditions for the growth of *A niger* were 50-55% moisture, 35°C, and 2 $\times 10^7$ spores initially added per gram of substrate.

Composting may be considered as a two-phase SSF process. During the first phase, available sugars are fermented by diverse organisms leading to the production of heat which in the second phase is favourable for thermophilic microbes to attack less readily available sugar sources such as polysaccharides (Hesseltine, 1972).

The solid state fermentation has also great use in the production of other valuable materials besides proteins and enzymes, and also in the reduction and/or elimination of some undesired constituents in substrates.

2.4.1 Advantages and Disadvantages of SSF

There are many advantages of solid state fermentation processes over the conventional submerged stirred tank system on both laboratory and large scale. Some of the advantages for work involving fungi are as follows:

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

2. The space required by the fermentation equipment is small relative to yield of product because less water is used and substrate is concentrated.

3. Since spores are used directly in the fermentation, neither seed tanks nor performed inoculum is necessary.

4. The conditions under which the fungus grows are more like the conditions in its natural habitat.

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

6. The desired product may be readily extracted from the vessel by addition of solvent directly.

7. Low level of contamination, due to reduced moisture content as opposed to submerged culture.

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

These advantages, in addition to some of the general characteristics of solid state fermentation, strongly support the application of SSF technique in industrial production of biochemicals and other products.

Some of the disadvantages are:

1. The type of organisms are limited to those which can grow at reduced moisture levels, namely fungi, some yeasts, and some bacteria including streptomycetes.

2. Heat becomes a problem when large quantities of moist substrates are used.

3. Large amount of inoculum is required.

4. Chances of contamination are high if water is to be added during the course of fermentation.

5. Using monitoring devices to determine moisture, pH., etc., becomes a problem. Also controlling pH, etc., is difficult.

6. Sometimes pretreatment of the substrate may be required.

7. Power requirement may be high for continuous agitation of solid material.

2.4.2 Phytic Acid Reduction by SSF

Hydrolysis of phytic acid in canola meal prior to its utilization by animals would increase the availability of inorganic phosphorus and other minerals in the animal diet. One of the most effective ways of phytic acid hydrolysis is solid state fermentation.

Fardiaz and Markakis (1981) studied the reduction of phytic acid content in oncom using *R. oligosporus*. They reduced phytic acid content from 1.36% to 0.05% after 72 h fermentation, while oncom prepared with the Indonesian strain of *Neurospora* contained higher amount of phytic acid content (0.7%).

During bread making, a considerable part of phytic acid is destroyed by enzymatic activity of the yeast. Using soy-fortified flour (10% soy flour and 90% wheat flour), the amount of phytic acid decreased by 46% to 88% depending on the amount of yeast used (Ranhotra et al., 1974). Without adding any yeast, approximately 46% reduction in phytic acid was observed, which was due to phytase activity in wheat. When they added 9 g of yeast per lb of bread, 88% of phytic acid was hydrolyzed; there was no further reduction in phytic acid even after the addition of 15 g of yeast. They found that the phytase activity in wheat and yeast is quite high. Increased hydrolysis of phytate was accompanied by an increase in the level of inorganic phosphorus which may have inhibited further hydrolysis.

Han and Wilfred (1988) investigated the removal of phytic acid content from soybean and cottonseed enzymatically. He obtained a synergistic effect in the hy-

drolysis of phytate by co-application of phytase and cellulase; with the application of phytase, 37% of phytate in soybean meal was hydrolyzed during 3 h incubation at 37°C, but in the sample treated with a combination of phytase and cellulase the highest level of hydrolysis (44%) of phytate was obtained. Treatment with other enzymes, however, had little effect on the ability of phytate to be hydrolyzed in soybean meal. They also reported that incubating cottonseed in the presence of water in the temperature range 30-60°C significantly reduced phytate content in the seed meal.

Chang (1977) studied the removal of phytate from whole dry beans by enzymatic hydrolysis. He found that incubation of presoaked beans in water at 60°C for 10 h lowered their phytate content by 90%. Incubation in pH 5.5 buffer at 50°C for 24 h also lowered it by 90%. When leaching was eliminated by incubating in water-saturated air at 60°C overnight, 33% of the phytate was destroyed by hydrolysis. Similar treatments were effective with mung beans, lima beans, and wheat.

Sudarmadji and Makakis (1977) fermented soybeans into tempeh by *Rhizopus oligosporus* NRRL 2710. The phytic acid content of soybeans was reduced by about 33% as a result of this fermentation, while an equivalent amount of phosphate was released in the tempeh. They also reduced phytic acid by 14% by boiling the seeds for 30 minutes. They concluded that the reduction of phytic acid was due to phytase elaborated by the mould during fermentation. The optimum pH of *Rhizopus oligosporus* phytase activity was reported to be 5.6 and K_m value

was 0.28 mM using sodium phytate (10^{-3} M) as the substrate.

Harland and Harland (1980) performed experiments with three breads (rye, wheat, and whole white) to investigate the reduction of phytic acid content by doubling the yeast in each recipe and extending the fermentation time. They obtained 65% reduction of phytic acid content in rye bread and 33% in both white and whole wheat breads. It was observed that an increase in yeasts cells and fermentation time increased the phytic acid reduction. The major reduction of phytic acid in all three breads occured during the first two hours of rising period.

Stone et al. (1984) investigated the autolysis of phytic acid in canola meal, wheat bran and fish silage blends. They reported that there was no autolysis of phytic acid in acidified canola meal even after 21 days, which confirmed the absence of phytase in canola meal. They found that by adding 10% wheat bran, 100% hydrolysis of phytic acid was possible in 35 days. Addition of 50% wheat bran hydrolyzed 100% of phytic acid in 28 days.

Nair and Duvnjak (1990) investigated the reduction of phytic acid content in canola meal. It has bean demonstrated that *Rhizopus oligosporus* NRRL 2990, *Aspergillus niger* NRC 5765, *Aspergillus carbonarius* NRC 401121, *Aspergillus ficuum* NRRL 3135 and a wild strain of *Saccharomyces cerevisiae* can be used for this purpose. *Aspergillus ficuum* was found as a good producer of phytase, and complete reduction of phytic acid content in canola meal was obtained using this microorganism.

2.5 Phytase

Phytase is a phosphomonoesterase which dephosphorylates 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, in a stepwise mannar (Irving and Cosgrove, 1972).

IUPAC-IUB recognise 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-pentakisphosphate and Pi (Cosgrove, 1980).

Phytase is widely distributed in plant and animal tissues, in many species of fungi and in certain bacteria (Cosgrove, 1966). It is found in wheat germ (Ullah, 1988), in mung bean cotyledons (Mandal et al., 1972), in Pseudomonas (Shieh and Ware, 1968), and in Bacillus subtilis (Howson and Davis, 1983). Courtois and his collabolators have carried out extensive work on the phytase occurence, specificity and mechanism of reaction (Peers, 1953; Courtois and Joseph, 1947).

2.5.1 The Function and Production of Phytase

Phytase is known as the first enzyme which liberates inorganic phosphate from organic phosphorus (Peers, 1953). Many workers (Peers, 1953) have shown that the proportion of inorganic phosphate to phytate increases progressively during germination with wheat, oats, barley maize and rice as a result of the increase

in phytase activity during germination.

Peers (1953) extracted phytase from wheat flour using distilled water. The ratio of flour to water was 1:6. The crude enzyme was partially purified by ammonium sulfate saturation. He had determined the phytase activity of a number of different wheats and he found an increase of 6.5 fold in the phytase activity when a soft wheat germinates.

Microorganisms from various culture collections were tested for their phytase-producing ability (Shieh and Ware, 1968). A number of the *Aspergillus niger* strains produced extracellular phytase which dephosphorylated calcium phytate in acidic solution. Shieh and Ware concluded that of the 30 mold culture filtrates that attacked calcium phytate, 28 were from molds of the genus *Aspergillus*. The most active group was *Aspergillus niger*. Many other species of *Aspergillus* produced considerable amounts of intracellular phytase activity but negligible amount of phytase activity in the culture filtrate. For *A. ficuum* NRRL 3135 a special interest was shown because it did not sporulate in most media and produced the highest phytase activity.

Gibbins and Norris (1963) extracted phytase from dwarf beans (*Phaseolus vulgaris*). The cotyledons were homogenized with 5 volumes of 0.01 M malate buffer (pH 6.4) in a blender. After centrifuging for 30 minutes, the supernatant was adjusted to pH 4.8 with acetic acid and partially purified by ammonium sulfate saturation. The fraction precipitating between 30% and 50% saturation contained the most activity. Further purification was done by diethyl amino ethyl (DEAE)

cellulose chromotography and ultrafiltration. The crude enzyme had an activity of 0.025 units and the purified enzyme had a maximum activity of 0.1 units.

Irving and Cosgrove (1972) demonstrated chromatographically that the fungus *Aspergillus ficuum* NRRL 3135 can produce two types of enzymes; an extracellular nonspecific orthophosphoric monoester phosphohydrolase (EC 3.1.3.2) with an optimum pH of 2.0, as well as an extracellular myo-inositol hexaphosphate phosphohydrolase (EC 3.1.3.8; phytase) with pH optima of 2.0 and 5.5. Both of these enzymes are also known to hydrolyze myo-inositol hexaphosphate.

Han and Gallagher (1987) studied the effect of nutritional and cultural conditions on cell growth and phosphatase production by *Aspergillus ficuum* in a submerged culture. They also found that the organism produced a non-specific phosphomonoesterase rather than a phytin specific phosphatase. The enzyme hydrolyzed a variety of phosphates and produced orthophosphates. The organism slowly released the phosphatase, and the enzyme activity in the growth medium increased continually during one month growth period. For a high level of phosphatase production, low levels (1-5 mg) of initial phosphorus were necessary and polyphosphates were the desired form rather than monophosphate. They also found that the addition of surfactants, such as polyoxyethylene ethers and sodium oleate, to fungal culture medium markedly increased the level of phosphate production.

Chang (1967) extracted phytase from germinating corn seeds, and studied the properties of the enzyme. Germination of corn seeds was conducted in dark-

ness at 25-27°C. The germinated plants were harvested at intervals of 24, 48, 72 and 96 hours. The endosperm and scutellar tissues were removed from the plant and homogenized in a solution containing methanol, chloroform and water (12:5:3 v/v). The materials were then dried and ground to a fine powder. The phytase activity was measured for its ability to release inorganic phosphorus from phytate. The activity was reported to be 0.03 units/mg of dried material.

Phytase was isolated from mung beans by Mandal et al. (1972). After 72 hours of germination, the cotyledons were homogenized with 0.05 M Tris-HCl buffer (pH 7.0) for 10 min. with a pestle and mortar. The homogenate was filtered and centrifuged for 25 min. at 10000 × g. The supernatant was designated as the crude enzyme which was then subjected to further purification. The specific activity increased from 0.001 units/mg of total protein for the crude enzyme to 0.08 units/mg for the purified. The phytase (EC 3.1.3.8) of Navy beans (*Phaseolus vulgaris*) was extracted with 2% $CaCl_2$ and purified by ammonium sulfate fractination and DEAE-cellulose chromotography. The ratio of extractant to beans was 10:1. The filtrate was centrifuged and used as crude enzyme. The crude enzyme was also purified further. The purified phytase showed broad specificity, and the fraction between 35% and 70% of saturation with ammonium sulfate had the highest activity.

Han and Gallagher (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 minimum 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%. *A. ficuum* grew well on raw (unheated) substrate containing a minimal amount of water and produced as much phytase as on heated substrate.

2.5.2 The Characteristics of Phytase

The phytase of wheat studied by Peers (1953) showed an optimum pH and temperature of 5.15 and 55°C, respectively. Peers also observed that the enzyme activity decreased by 88% when the enzyme was heated for 6 minutes at 100°C, while only very little activity was lost after the heat treatment at 62.8°C for the same period of time. The Michaelis-Menten constant (K_m) and the maximum reaction velocity (v_{max}) for the purified enzyme were found to be 0.33 $\times 10^{-3}$ M and 127 μg.P/h/ml, respectively. A linear relationship was found between the enzyme

concentration and its activity up to 50 mg.whole meal/ml and 400 units purified enzyme/ml. Peers studied the influence of a number of inorganic and organic salts on phytase action, and found that heavy metal salts completely inhibit the enzyme probably by removing the phytate from the solution.

Ullah (1988) studied the characteristics of purified phytase of *A. ficuum* 3135 using sodium phytate as substrate. He observed that phytate concentration in excess of 125 μM inhibited enzyme activity at both pH 5.0 and 2.5, the inhibition was more prominent at pH 2.5 than at 5.0. At a substrate concentration of 4 mM the phytase activity was totaly inhibited at pH 2.5; but at pH 5.0 only 13% less catalytic activity was observed. He found that *A. ficuum* phytase performed optimum substrate concentration at 1.0-2.0 mM at pH 5.0; but under identical condition 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. He also concluded that the enzyme had varied sensitivity toward cations. While Ca^{2+} and Fe^{2+} produced no effect on the catalytic rate of the enzyme, Cu^{+}, Cu^{2+}, Zn^{2+} and Fe^{3+} were found to be inhibitory. Mn^{2+} was observed to enhance enzyme activity by 33% at 50 μM.

Studying the characteristics and kinetics of partially purified samples of acid phosphatase (EC 3.1.3.2) and of the phytase (EC 3.1.3.8) from *A. ficuum*, Irving and Cosgrove (1974) found the optimum pH 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 the optima at pH 2.5 and 5.3, with the suggestion of a third at pH 2.8. The

K_m value of the acid phosphatase was 0.127 mM with sodium myo-inositol hexakisphosphate as substrate at pH 2.2; while K_m value of the phytase at pH 2.5 was 0.0244 mM and at pH 5.3 it was 0.0129 mM. Neither enzyme was inhibited by 1 mM oxalate, 1 mM citrate, 1 mM EDTA or 1 mM (+)-tartarate. The activity of the acid phosphatase was reduced to 17% and that of phytase at pH 2.5 to 69% by 1 mM flouride. The phytase was not inhibited at pH 5.3 by any of the anions tested.

The phytase of Navy beans (*Phaseolus vulgans*) studied by Lolas and Markakis (1977) showed an optimum pH of 5.3 and K_m of 0.018 mM with phytic acid as the substrate. The optimum temperature was 50°C. The activation energy of the enzymatic hydrolysis of phytic acid was 11,500 cal/mole. The enzyme was inhibited by high concentrations of phytic acid. The activity was increased by about 35% in the presence of 1 mM of Co^{2+}. In studying the stability of the enzyme, it has been shown that heating up to 50°C for 10 min. did not depress the enzyme activity, but at 65°C the activity decreased to about 45% of the control value and at 80°C the enzyme was almost completely inactivated.

The optimum pH and K_m value of the phytase extracted from dwarf beans (Gibbins and Norris, 1963) were found to be 5.2 and 0.15 mM, respectively. The enzyme was inhibited by higher substrate concentrations (4 mM sodium phytate). Calcium and magnesium ions were found to have stimulatory effect, while iodoacetamide and floride ions had an inhibitory effect on the phytase activity.

Mandal et al. (1972) studied the characteristics of phytase isolated from

mung bean cotoyledons. The enzyme was stable at 0°C, had a pH optimum at 7.5 and optimum temperature of 57°C. Inhibition by phytate has been found to be competitive, the K_i and K_m values with phytate were reported to be 0.4-0.43 mM and 0.65 mM, respectively, and v_{max} was found to be 0.13 units.

Sadarmadji and Markakis (1977) studied the phytase of *Rhizopus oligosporus* NRRL 2710 of soybean. They reported that the optimum concentration of phytate as substrate was in the neighbourhood of 1 mM. The optimum pH for this enzymatic hydrolysis was 5.6 in the presence of either acetate or citrate buffer. A Michaelis-Menten constant of 0.28 mM was calculated for the phytase-phytate reaction.

Chang (1967) investigated the characteristics of phytase in corn seeds. Optimum incubation temperature of the enzyme was 50°C, optimum pH was 5.6, and the Michaelis-Menten constant (K_m) was 0.09 mM. Heating up to 50°C for 10 min. did not depress the enzyme activity. However, at 60°C, the activity was depressed to about 40% of the control value and at 80°C, 90°C and 100°C was practically completely inhibited. Responses of phytase to various concentrations of enzyme were found to be linear up to 98 units of enzyme tested. The enzyme was activated by calcium chloride, and was inhibited by sodium flouride.

Ghareub (1990) studied the characteristics of phytase produced from *Aspergillus carneus* in a culture filtrate. The maximum activity of the crude enzyme occurred at 35°C and pH 5.6. The pure enzyme was found to be stable between pH 5.5-6.2; about 68% of enzyme activity was lost by heating at 45°C for 60 min..

The pure phytase retained its activity over a long period when stored at 4°C.

Nair and Duvnjak (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, respectively. Optimum pH and temperature values were found to be 5.0 and 60°C, respectively. Preincubation of the enzyme at 60°C for 30 min. did not cause any significant decrease in the enzyme activity; while 70% of the enzyme activity was lost after 3 h of preincubation; 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, 15% loss of activity was observed.

2.6 *Aspergillus carbonarius*

Shieh and Ware (1968) considered *A. carbonarius* NRRL 368 and *A. carbonarius* PCC 104 among those *Aspergillus* varieties and strains which can produce extracellular acid phytase. The activity reported for the enzyme produced from both organisms, NRRL 368 and PCC 104, are 1.9 and 1.5 units/ml, respectively.

Markakis and Diamantoglous (1990) studied the enzymatic activity of the isolated fungi from leaves. They reported that *Alternaria alternate*, *Aspergillus carbonarius* and *Pencillium glabrum* were the most common fungi isolated during the survey. They also reported that *Aspergillus carbonarius* presented the higher tannase activity.

Mixed cultures of *Aspergillus carbonarius* and *Pencillium glabrum* were used

to study the carb tannin reduction and the enrichment of carob pod with fungal protein in carb slurries (Markakis and Diamantoglous, 1990).

Chapter 3

Mathematical Modelling

This chapter discusses the developement of semi-mechanistic mathematical models used to describe the biomass production, the enzyme formation and phytic acid utilization during SSF process. Some models for the characteristics of the enzyme also will be discussed.

3.1 Modelling of a SSF System

The kinetics and mathematical modelling of microbial growth in SSF have received little attention due to the difficulties associated with these processes. Some of these difficulties include:

1. The systems are geometrically complex due to non-homogeneous 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 agricultural by-products 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. A critical problem is the penetration of fungal hyphae into 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.

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

3.1.1 Growth Kinetics of the Biomass

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 appropriate term *exponential phase*, is generally used. Naturally in a closed vessel the cells can not 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.

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 or biomass concentration. Thus:

$$\frac{dX}{dt} = f(X) \tag{3.1}$$

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

$$f(X) = \mu_m X \tag{3.2}$$

where μ_m is the maximum rate of growth of the biomass, and X is the biomass concentration.

Malthus' prediction of the death phase, resulting from unrestrained population growth, has not yet been realized, and transition to a stationary population is generally observed for microbial growth.

Although there are a number of models for growth in SSF, the only real previous attempt to model fungal growth kinetics in SSF is the logistic model of Okazaki et al. (1980) when they studied the growth of *koji* mold on the surface culture of steamed rice grains. It is an improvement of Malthus' model. This model covered the mycelial growth in the logarithmic and stationary phases, and, therefore, it was used in this work to test its applicability in representing the relationship between time of fermentation and the biomass growth, for a given conditions. Okazaki and collaborators introduced the following logistic equation:

$$\frac{dX}{dt} = \mu_m X(1 - \frac{X}{X_m}) \tag{3.3}$$

where X is the biomass concentration, X_m is the maximum biomass concentration that can be attained, μ_m is the maximum specific growth rate, and t is the time

of fermentation.

Providing the following initial condition:

$$t = 0 \qquad X = X_0 \tag{3.4}$$

where X_0 is the initial experimental biomass concentration. Eq. 3.3 on integration gives:

$$X = \frac{X_m}{1 + \beta e^{-\mu_m t}} \tag{3.5}$$

where:

$$\beta = \frac{X_m}{X_0} - 1 \tag{3.6}$$

This expression enables one to calculate the biomass concentration (X) for a particular condition at any time of fermentation if X_m and μ_m are known. They can be obtained by fitting the experimental data of biomass during fermentation with the logistic law expression. For example in the study of the effect of glucose concentration on the SSF process a set of data was obtained for each glucose concentration. The above expression was fitted to each set of data and so the X_m and μ_m values were obtained for each glucose concentration.

3.1.2 Modelling Enzyme Activity During SSF

In solid state fermentation process the enzyme activity increases during a certain period of time then it starts decrease. Separate models were given for the increasing and decreasing parts of the enzyme activity curve.

Increasing phase of enzyme production

For the increasing part of the enzyme production curve two models were given; one is based on the logistic law and the other is an empirical one.

a. *Using the logistic law*

It is possible to assume that the enzyme production during the enzyme increasing phase is proportional to the biomass concentration; this was also suggested by Okazaki et al. (1980). Taking this assumption into consideration the following equation can be written:

$$\frac{dv}{dt} = k_v X \tag{3.7}$$

where v is the enzyme activity and k_v is a rate constant.

Substituting Eq. 3.5 into 3.7 gives:

$$\frac{dv}{dt} = \frac{k_v X_m}{1 + \beta e^{-\mu_m t}} \tag{3.8}$$

The following initial condition is used:

$$t = 0 \qquad v = v_0 \tag{3.9}$$

where v_0 is the initial enzyme activity (after inoculation). Eq. 3.8 on integration gives the following expression which relates the enzyme concentration with the time of the fermentation process and the logistic law parameters:

$$v = v_0 + (\frac{k_v X_m}{\mu_m}) ln(\frac{\beta + e^{\mu_m t}}{\beta + 1}) \tag{3.10}$$

b. *Empirical model*

In addition to the above model, the phytase formation for the increasing

phase can also be given by the following empirical expression:

$$v = v_0 + atexp(-bt) \tag{3.11}$$

where v_0 is the initial enzyme activity after inoculation, and a and b are empirical constants.

Decay phase of enzyme formation

After the enzyme attained the maximum value, a decrease in its activity had been noticed. Relatively few studies have been made on the decay phase of the enzyme production, perhaps because many industrial batch processes are terminated before this phase begins. The decay of the enzyme formation shown in this work can be represented by the following empirical exponential correlation:

$$v = v_{max}exp(-k_d(t - t_{max})) \tag{3.12}$$

where v is the enzyme activity at the time t, v_{max} is the maximum enzyme activity attained at the time t_{max} and k_d is an empirical constant.

3.1.3 Modelling of Phytic Acid Concentration During SSF

Bearing in mind that the enzyme production is related to the biomass growth, it is also logical to relate the phytic acid content in canola meal with the biomass production. The rate of phytic acid hydrolysis is assumed to be first order with phytic acid concentration, P, and second order with biomass, X. Taking that into

consideration, the following equation can be written:

$$\frac{dP}{dt} = -k_p P X^2 \tag{3.13}$$

were P is the phytic acid concentration and k_p is a rate constant.

Substituting X in Eq. 3.13 with its value from the logistic law gives the following expression:

$$\frac{dP}{dt} = -k_p P \frac{X_m^2}{(1+\beta e^{-\mu_m t})^2} \tag{3.14}$$

and after separating the variables:

$$\frac{dP}{P} = -k_p X_m^2 \frac{dt}{(1+\beta e^{-\mu_m t})^2} \tag{3.15}$$

Providing the following initial condition:

$$t = 0 \qquad P = P_0 \tag{3.16}$$

where P_0 is the initial phytic acid concentration (after inoculation). On integration Eq. 3.15 gives the relationship between the fractional phytic acid concentration and the time of fermentation under given conditions which are taken into consideration, as indicated, by X_m and μ_m values:

$$(\frac{P}{P_0}) = exp(\frac{k_p X_m^2}{\mu_m}(ln(\frac{e^{-\mu_m t}(1+\beta)}{1+\beta e^{-\mu_m t}}) + \frac{1}{1+\beta e^{-\mu_m t}} - \frac{1}{1+\beta})) \tag{3.17}$$

In some cases, phytic acid was assumed to be third order with biomass, X, and then Eq. 3.13 and 3.17 become:

$$\frac{dP}{dt} = -k_p P X^3 \tag{3.18}$$

$$\left(\frac{P}{P_0}\right) = exp\left(\frac{k_p X_m^3}{\mu_m}\left(ln\left(\frac{\beta e^{-\mu_m t}}{1+\beta e^{-\mu_m t}}\right) + \frac{1}{2(1+\beta e^{-\mu_m t})^2} + \frac{1}{1+\beta e^{-\mu_m t}}\right.\right.$$
$$\left.\left. - ln\left(\frac{\beta}{1+\beta}\right) - \frac{1}{2(1+\beta)^2} - \frac{1}{1+\beta}\right)\right) \quad (3.19)$$

3.2 Models for the Characteristics of the Enzyme

3.2.1 Phytase Activity Versus Substrate Concentration

Most enzymatic reactions followed the Michaelis-Menten kinetic equation, which can be written, at a given temperature and pH, as:

$$v = \frac{v_{max} s}{K_m + s} \quad (3.20)$$

where v_{max} is the maximum phytase activity achievable, s is the substrate concentration and K_m is the Michaelis-Menten's constant.

This model was applied to represent the relationship between s and v and to calculate the K_m and v_{max} values for the phytase of *A. carbonarius* that was studied in this work.

3.2.2 Phytase Activity Versus Temperature of Incubation

It is well known that the temperature range of activity of most enzymes is quite limited. The activity of the enzymes increases with an increase in temperature and attains a maximum; if temperature continues increasing denaturation follows.

It can be assumed that the enzyme's inactive (E_i) and active (E_a) forms exist

in equilibrium:

$$E_a \rightleftharpoons E_i \tag{3.21}$$

The relationship between the enzyme activity and temperature at equilibrium was developed previously (Baily and Ollis, 1986), and will be applied in this work to relate phytase activity with temperature of incubation and to calculate some thermodynamic properties of this enzyme. The derivation of this relationship can be summarized as follows:

the equilibrium constant for the above reaction is:

$$\frac{e_i}{e_a} = K_d = exp(\frac{-\Delta G_d}{RT}) = exp(\frac{-\Delta H_d}{RT})\ exp(\frac{\Delta S_d}{R}) \tag{3.22}$$

where ΔG_d, ΔH_d and ΔS_d denote the free energy, enthalpy and entropy of deactivation, respectively, T is the temperature of incubation, R is the ideal gas constant, e_a and e_i are the fractions of the enzyme in the active and inactive form respectively, and K_d is the equilibium constant.

Since the total amount of enzyme is present either in active or inactive forms, it is possible to write:

$$e_a + e_i = e_0 \tag{3.23}$$

where e_0 is the total enzyme present at equilibrium.

Using Eq. 3.22 and Eq. 3.23, it is possible to obtain the following expression:

$$e_a = \frac{e_o}{1 + K_d} \tag{3.24}$$

the enzyme activity at large substrate concentration can be written as:

$$v_{max} = e_a k(T) \tag{3.25}$$

where:

$$k(T) = \alpha(\frac{k_B T}{h})e^{\Delta S^*/R}\ e^{-E/RT} \tag{3.26}$$

k_B and h are Boltzmann's and Plank's constants, respectively, α is a proportionality constant, E is the activation of energy and ΔS^* is the entropy change at the standard condition.

Combining Eqs. 3.22-3.26 to obtain:

$$v = \frac{\beta T e^{-E/RT}}{1 + e^{\Delta S_d/R} e^{-\Delta H_d/RT}} \tag{3.27}$$

where:

$$\beta = \alpha(\frac{k_B}{h})e^{\Delta S^*/R}(e_0) \tag{3.28}$$

A plot of $1/T$ versus $ln(v)$ could give an initial estimate for the constants of Eq. 3.27 as follows: the slope at large $1/T$ values is $-E/RT$, while the slope of the other straight line obtained at higher temperatures is approximately equal to $(\Delta H_d - E)/R$.

ΔS_d could be estimated after noting that, at the temperature T_{max} where $ln(v)$ is maximized:

$$K_d(T_{max}) = \frac{E + RT_{max}}{\Delta H_d - E - RT_{max}} \tag{3.29}$$

Providing that at equilibrium:

$$K_d = \frac{e_i}{e_a} = e^{\Delta S_d/R} e^{-\Delta H_d/RT} \tag{3.30}$$

which results in:

$$\Delta S_d = Rln(K_d e^{\Delta H_d/RT_{max}}) \tag{3.31}$$

After these preliminary calculations, some iterative readjustment may be necessary to refine these initial estimates to obtain a good fit of Eq. 3.27 to the experimental data and to use this equation to predict the v value for each temperature. By applying this procedure, it is assumed that the other parameters such as Michaelis-Menten constant remains constant with temperature.

3.2.3 Phytase Activity Versus pH

Depending upon the pH, the active enzyme form may be a large or small fraction of the total enzyme present. Most catalytic active enzymes pass through a maximum (at the optimum pH) as pH increases and are then deactivated by further increase in pH (Baily and Ollis, 1986). The following model represents the active site ionization state:

$$E \rightleftharpoons E^{-} \rightleftharpoons E^{2-} \tag{3.32}$$

where E^{-} denotes the active enzyme form, while E and E^{2-} are inactive forms obtained by protonation and deprotonation of the active site of E, respectively. K_1 and K_2 are equilibrium constants for the indicated reactions; these can be written at equilibrium as:

$$\frac{h^{+}e^{-}}{e} = K_1 \qquad \frac{h^{+}e^{2-}}{e^{-}} = K_2 \tag{3.33}$$

Where h^{+} is the hydrogen concentration, e and e^{2-} are the fractions of enzymes in the inactive forms E and E^{2-} respectively and e^{-} is the fraction of enzyme in the active form E^{-}.

The total enzyme concentration (e_0) is:

$$e_0 = e + e^- + e^{2-} \tag{3.34}$$

By combining Eq. 3.33 and 3.34, the fraction of the active enzyme, y^-, is:

$$\frac{e^-}{e_0} = y^- = \frac{1}{1 + \frac{h^+}{K_1} + \frac{K_2}{h^+}} \tag{3.35}$$

The relationship providing the influence of pH on the maximum reaction velocity v_{max} has already been reported (Baily and Ollis, 1986). This relationship can be obtained by replacing the total enzyme concentration e_0 with the total active form concentration $e_0 y^-$:

$$v_{max} = k e_0 y^- = \frac{k e_0}{1 + \frac{h^+}{K_1} + \frac{K_2}{h^+}} \tag{3.36}$$

In order to use this relationship to calculate the enzyme activity at a given pH, the following steps have been developed in this work (internal communication: Karol Lacki): providing that Michaelis-Menten's equation can be written as:

$$v = \frac{v_{max}}{1 + \frac{K_m}{s}} \tag{3.37}$$

and substituting Eq. 3.36 in Eq. 3.37 gives:

$$v = \left(\frac{1}{1 + \frac{h^+}{K_1} + \frac{K_2}{h^+}}\right)\left(\frac{k e_0}{1 + \frac{K_m}{s}}\right) \tag{3.38}$$

Differentiating Eq. 3.38 with respect to h^+ with the assumption that K_m is independent of pH at constant temperature and setting this differential form to zero gives the following relationship between the optimum value of the hydrogen

concentration and the equilibrium constants K_1 and K_2:

$$h_{opt} = \sqrt{K_1 K_2} \tag{3.39}$$

This corresponds to:

$$v_{opt} = \left(\frac{1}{1 + \frac{\sqrt{K_1 K_2}}{K_1} + \frac{K_2}{\sqrt{K_1 K_2}}}\right)\left(\frac{k}{1 + \frac{K_m}{s}}\right) \tag{3.40}$$

Dividing Eq. 3.38 by Eq. 3.40 results in the following expression which can be used to predict the phytase activity for any pH value:

$$v = v_{opt} \frac{1 + 2\sqrt{K_2/K_1}}{1 + \frac{h^+}{K_1} + \frac{K_2}{h^+}} \tag{3.41}$$

where v_{opt} is the activity at the optimum pH, and $h^+ = 10^{-pH}$. The equilibrium constants K_1 and K_2 could be obtained from pH versus v data.

3.2.4 Time of Incubation Versus Orthophosphate Liberated

When the enzyme was incubated for different periods of time it was noticed that the following Michaelis-Menten type of equation could be used to represent the relationship between the time of incubation and the enzymatic activity expressed as the amount of orthophosphate, Pi, released from sodium phytate:

$$Pi = \frac{Pi_{max} t_{inc}}{K_{t_{inc}} + t_{inc}} \tag{3.42}$$

where t_{inc} is the time of incubation, and $K_{t_{inc}}$ is the time of incubation at which the orthophosphate released is a half of its maximum amount, Pi_{max}, which is liberated when $t_{inc} \gg K_{t_{inc}}$ (roughly speaking, it is the division between the lower

time of incubation, where Pi is linearly dependent on t_{inc}, and the higher range where Pi becomes independent of t_{inc}).

3.2.5 Phytase Activity Versus Meal to Extractant Ratio

In the production of an enzyme by a solid state culture, it is necessary to extract the enzyme and then proceed further with its concentration and purification if so required. The amount of the extractant used for the enzyme extraction should be sufficient to extract as much of the enzyme as possible from the solid state culture. However, that amount should not be too high as only a little more enzyme would be extracted in that case, the enzyme extract would be too diluted and would require an additional concentration step.

Various ratios of $CaCl_2$ solution : canola meal culture were used to test the effect of the amount of extractant ($CaCl_2$ solution) on the release of phytase for a single step extraction of the enzyme from a solid state culture. The following Michaelis-Menten type of equation was used to describe the relationship between the amount of extractant used for extraction (V_E) and the amount of extracted enzyme (Q):

$$Q = \frac{Q_{max} V_E}{K_V + V_E} \tag{3.43}$$

where K_V is the volume of extractant which can extract one-half of the maximum amount of the enzyme being extracted, Q_{max}, when $V_E \gg K_V$.

3.2.6 Time of Extraction Versus Phytase Activity

From a technological point of view, it is interesting to determine the extractant-solid state culture contact time during an enzyme extraction.

In this study the extraction of phytase was carried out for different periods of time, using an extractant:solid state culture ratio 5:1. It was also attempted to represent the relationship between the amount of the enzyme extracted, Q, and the time of extraction, t_E, with the following Michaelis-Menten type of equation:

$$Q = \frac{Q_{max} t_E}{K_t + t_E} \tag{3.44}$$

where K_t is the time of extraction at which the extracted phytase is half of the maximum amount of this enzyme that can be extracted under experimental conditions (Q_{max}) when $t_E \gg K_t$.

Chapter 4

Materials and Methods

This chapter describes the microorganism used in this study, the compositions and preparation of the cultivation media, and the analysis of the samples obtained during solid state fermentation, namely: phytic acid, available carbohydrates, protein content, phytase activity, and biomass determination. Methods for the measurement of biomass and glucose concentration in liquid medium will also be described.

4.1 Microorganism

Aspergillus carbonarius NRC 401124 was used to study the production of phytase and reduction of phytic acid content in canola meal during its growth in SSF process.

Nair and Duvnjak (1990) investigated the reduction of phytic acid content in canola meal using this microorganism.

4.1.1 Slants Agar

The microorganism was maintained in Roux bottles on a solid medium composed of 4.5% malt agar, 0.5% glucose, and 0.5% yeast extract and distilled water (all % are w/v). The medium was sterilized at 115°C for 15 min, inoculated and incubated at 30°C until sporulation. White micellium appeared over the whole surface after 36 h which converted completely to black color after 60 h incubation. Sterilized-distilled water was used to prepare a spore suspension which was kept at 4°C for further use.

4.1.2 Liquid Medium

The inoculum for SSF was prepared in a medium composed of 0.8% nutrient broth, 0.5% glucose and 0.5% yeast extract and distilled water. Erlenmeyer flasks (250 ml) with 100 ml of the medium in each were sterilized at 115°C for 15 min, cooled and inoculated with 1 ml spore suspension. Incubation was carried out in a rotary shaker at 30°C for 60 h. The microorganism grew in the liquid medium in the form of pellets.

4.1.3 Preparation of Solid Medium

The production of phytase was carried out in 500 ml Erlenmeyer flasks in a medium composed of 50 g of canola meal (commercial canola meal bought from Ritchie Feed and Seed Ltd., Ottawa) and 40 ml of distilled water. It was sterilized

at 121°C for 45 min (unless otherwise specified). After sterilization and cooling, the medium was inoculated with 20 ml of homogenized inoculum (unless otherwise specified) and incubated at 30°C. When the effect of glucose content, phosphate content and sodium oleate content were studied the medium was supplemented with various concentrations of these components required.

The biomass and enzyme productions, phytic acid content reduction, and protein and carbohydrates concentrations were followed during the SSF process.

4.2 Analysis of Samples

The phytic acid, carbohydrates, protein contents, phytase activity and biomass growth were evaluated in this study during the SSF process.

Determinations of biomass and glucose concentration were also performed in the liquid medium.

4.2.1 Biomass in Submerged Medium

A known volume of sample was centrifuged at 15000 × g for 20 min and the biomass was washed with distilled water. Centrifuging and washing were repeated twice. The biomass was then dried at 105°C for 24 hours. The amount of biomass was expressed in mg dry wt./mL of broth.

4.2.2 Sugar Determination

After centrifuging the sample the supernatant was taken directly for sugar determination using the dinitrosalicylic acid (DNS) method described by Weiner (1978). DNS reagent was prepared as follows: A) five grams of dinitrosalicylic acid were stirred continuously in 300 mL distilled water until dissolved; B) one gram phenol, 0.25 g sodium bisulfite and 100 g sodium potassium tartarate were dissolved in 200 mL distilled water. Solution B was added to solution A while stirring. The final solution had to be kept in the dark without exposure to light. One mL of the supernatant was mixed with 3 mL of DNS reagent and boiled for exactly 5 min in a water bath. After cooling, 20 mL of distilled water was added. The absorbance was read at 600 nm against a reference sample. The reference sample was prepared using 1 mL of distilled water in place of the diluted sugar sample and then subjected to the same treatment as the sample.

4.2.3 Phytic Acid

The Haug and Lantzsch method (1983) was used for rapid determination of phytic acid in canola meal.

Phytic acid was extracted from approximately 2 g of a sample using 33 mL of 2.4% HCl under continuous shaking (200 rpm) for 1 h. After extraction, the suspension was centrifuged (6000 × g, 15 min), and the supernatant collected. 5 mL of supernatant was mixed with 10 mL of ferric solution (0.2 g of $NH_4Fe(SO_4)_2.12H_2O$ in 100 mL of 2 N HCl and made up to 1 L with distilled water). The solution was

boiled for 30 min and then cooled in ice water for 15 min and allowed to reach room temperature. The solution was again centrifuged (3000 × g , 30 min). 2 mL of supernatant was treated with 3 mL of bipyridine-thioglycollic acid reagent (10 g bipyridine and 10 mL thioglycollic acid in distilled water and made up to 1000 mL) and the absorbance was read at 519 nm using distilled water as a reference. The concentration of phytic acid was calculated from a similarly prepared standard curve.

4.2.4 Protein

Approximately 2 g of a sample was boiled gently with 33 mL of 1 N NaOH. After cooling and centrifuging (6000 × g 15 min), the supernatant was diluted appropriately and proteins were determined according to the method described by Lowry et al. (1951). The following reagents were used for the analysis:

1. 2% sodium carbonate (Na_2CO_3) in 0.1 N sodium hydroxide (NaOH).
2. 0.5% copper sulphate ($CuSO_4.5H_2O$) in 1% sodium tartarate.
3. Reagent composed of 50 mL of reagent 1 mixed with 1 mL of reagent 2.
4. 1:2 (v:v) phenol reagent

One mL of diluted supernatant was mixed with 5 mL of reagent 3. The solution was allowed to stand for 15 min, and then 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 prepared in the same way using albumin.

4.2.5 Carbohydrate

Approximately 2 g of sample was boiled for 2 h with 33 mL water and 6.7 ml HCl. After centrifuging, an amount of supernatant was appropriately diluted. 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 boiled for 20 min. The solution was cooled in an ice bath. The absorbance was read at 625 nm against water treated similarly as the reference. The carbohydrate content was calculated from the standard curve prepared using sucrose (AOAC, 1975).

4.2.6 Phosphorus

The method adopted for phosphorus measurement was 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 100 mL flask and made up to volume with 10 N H_2SO_4. Five grams of 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 the absorbance read at 660 nm immediately using water similarly treated similarly to the reference. Phosphorus concentration was calculated from the standard curve prepared using potassium dihydrogen phosphate (KH_2PO_4).

4.2.7 pH Measurement

Approximately 2 g of sample was taken and mixed thoroughly with 20 mL distilled water. The mixtures were allowed to stand fo 1 h. The pH was measured using Fisher Accumet pH meter (model 805 MP).

4.3 Phytase Activity and Assay Procedure

The enzyme was extracted from the canola meal culture using 2% aqueous solution of $CaCl_2.2H_2O$ while shaking the suspension for 1 h (unless otherwise stated) on a rotary shaker at 200 rpm. The meal to extractant ratio was 1:5 (w:v) (unless otherwise stated). The suspension was squeezed through a double layer of cheese cloth and centrifuged (20000 $\times$ g , 10 min). The clear supernatant was designated as crude enzyme extract. Phytase activity was assayed using sodium phytate as substrate (Harland and Harland, 1980) and measuring the inorganic phosphorus released. A reaction mixture consisted of 5 mL of 0.2 M acetate buffer (pH 4.7), 1 mL of 2.4 mM phytic acid and 0.1 mL of crude enzyme extract. The reactions were carried out at 53°C for 10 min (unless otherwise stated), and then stopped by adding 5 mL of 10% TCA. Spectrophotometric determination of the

released phosphorus was done using the Taussky-Schoor reagent as described before.

One unit of enzyme activity is defined as the amount of enzyme preparation required to release 1 mg inorganic phosphorus per hour at the given temperature and pH.

4.4 Biomass in Solid Culture and Assay Procedure

Biomass in the solid culture was measured indirectly by determining the glucosamine content in the SSF samples by the method adopted from Sakurai et al. (1976). About 0.5 g of a sample was extracted with 2 mL of 60% H_2SO_4 for 24 h at 25°C. The mixture was then diluted with distilled water to decrease the concentration of H_2SO_4 to 1 N in a 100 mL Erlenmeyer flask, and the top of the flask was sealed with four layers of polyvinylidene wrap and a rubber band. The flask was autoclaved at 101.325 kPa for 1 h. The mixture was neutralized with 1 N NaOH to about pH 7, and diluted to a definite volume (100 to 250 mL was found adequate).

The glucosamine liberated after hydrolyzing the biomass was determined colorimetrically by Blix's method (1948). 2 mL of acetylacetone solution (1.5 mL acetylacetone + 50 mL 1.25 N Na_2CO_3 - freshly prepared) was added to 2 mL of sample. The mixture was heated at 96°C in a closed tube for 60 min in a

water bath and cooled to room temperature. 20 of mL of 96% ethyl alcohol was added t the mixture and thoroughly mixed. 2 mL of Ehrlich's reagent (1.6 g p-dimethylamino benzaldehyde + 30 mL conc. HCl and 30 mL of 96% alcohol) was also added. The resulting system was allowed to stand for 45-60 min, absorbance was read at 530 nm using similarly treated water as the reference.

According to this method, the range of glucosamine concentrations should be between 0.03 and 0.14 mg/2 mL of glucosamine solution. Biomass was estimated from the relationship between glucosamine content and biomass in the biomass produced in a liquid medium.

Chapter 5

Results and Discussion

Solid state fermentation was carried out to reduce the phytic acid content in canola meal using *A. carbonarius*. Bearing in mind that the composition of canola meal depends on the variety of canola seeds and their processing, the initial composition of the canola meal which was used in this study was determined prior to its utilization in the SSF processes. Table 5.1 gives the analytical results compared with the reported values.

Table 5.1: Compositions of canola meal

Components	This work %	Literature values %	References
Moisture	9.5	8-11	1,27, 42
Crude protein	35.5	34-38	1, 6, 27, 60
Carbohydrates	13.0	9-15	9, 56
Phytic acid	6.0	3-7	1, 6, 27, 36, 43, 52

5.1 Submerged Culture

Aspergillus carbonarius NRC 401124 was grown in the liquid medium containing glucose as the carbon source to study the influences of some parameters on the biomass and phytase production in submerged culture.

Fig. 5.1 shows the changes in glucose concentration, biomass, phytase production and pH in a batch initially containing 0.8 g/L glucose. The glucose was entirely consumed. A decrease in pH was associated with this process. It was noticed that the time of minimum glucose concentration corresponded to the minimum pH. The biomass increased with the fermentation process, and after the consumption of glucose it started to autolyze. This was associated with an increase in pH level. The phytase production decreased with an increase in biomass and then started decreasing with the autolysis of the biomass.

In order to study the effect of initial glucose concentration on the biomass production, flasks were filled with media containing different initial glucose concentrations. The biomass formed was measured when the glucose was entirely consumed. The results obtained show that an increase in glucose concentration resulted in the production of a larger amount of biomass (Fig. 5.2). Sometimes excessive concentration of nutrient can partially or even completely inhibit cell growth. According to Fig. 5.2, inhibition of the growth rate was not observed even with the glucose concentration of 25 g/L.

It is well known that some micoorganisms require oxygen for their growth.

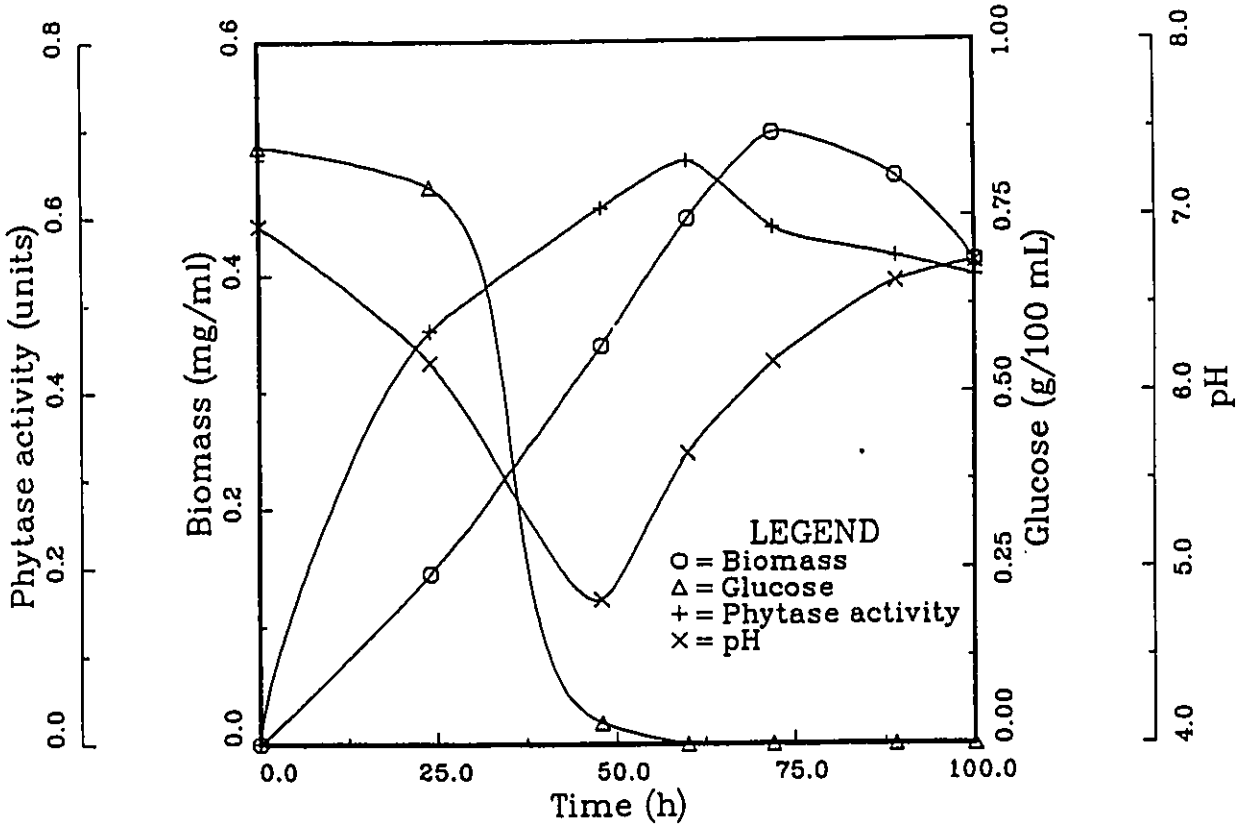


Figure 5.1: Changes in biomass, phytase activity, glucose concentration and pH in liquid medium

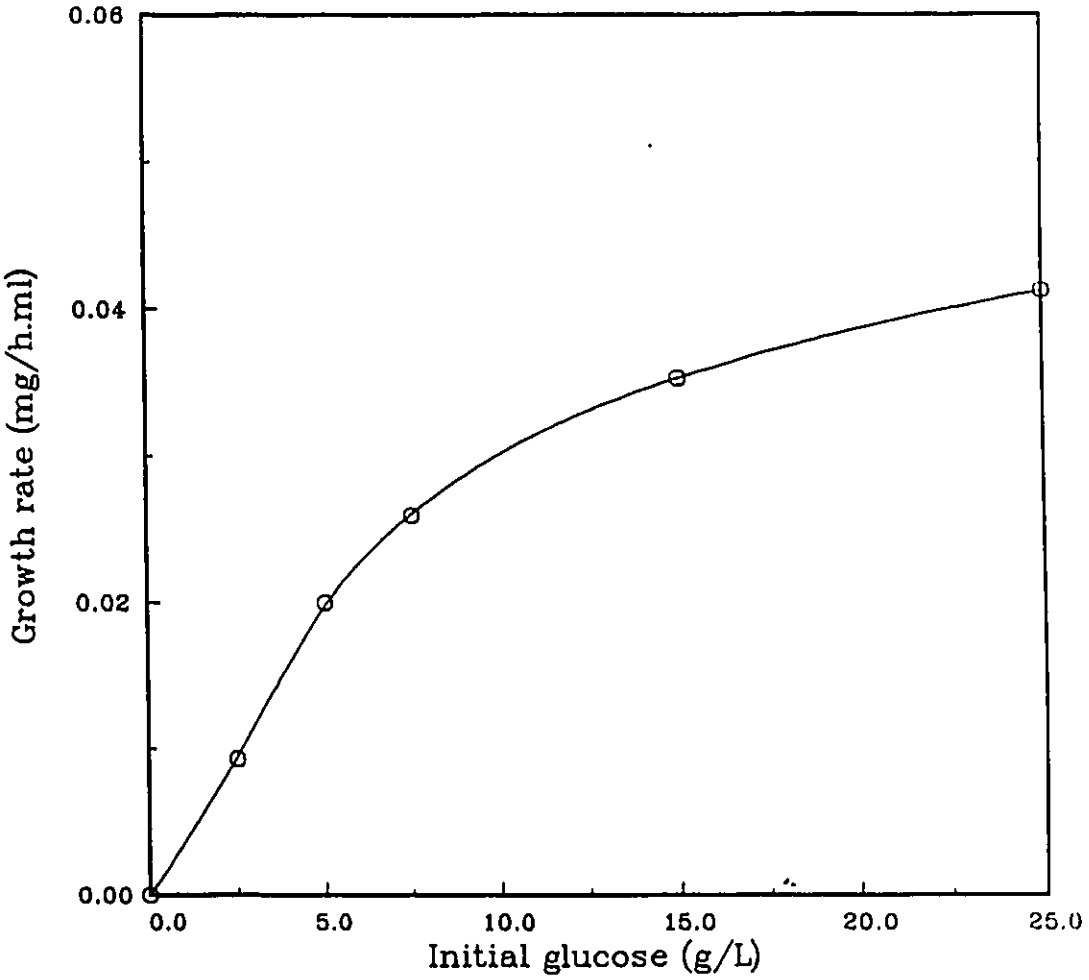


Figure 5.2: Effect of initial glucose concentration on the final biomass production in liquid medium

To establish the response of *A. carbonarius* to various oxygen concentrations, 250 mL Erlenmayer flasks were filled with two different amounts of the liquid medium containing 0.8% glucose. During this process, the concentrations of biomass and glucose were followed and the results are given in Figs. 5.3 and 5.4. The obtained data shows that the growth rate of biomass and its final concentration was higher in the flask which was filled with smaller amount (50 mL) of the medium (Fig. 5.3). The rate of glucose consumption is in good agreement with the biomass results; it was higher in the flask containing 50 mL of medium (Fig. 5.4).

The results shown indicate that oxygen has a positive effect on the growth of this microorganism which should be taken into consideration in the fermentation process when *A. carbonarius* is used as a producer of valuable products.

5.2 Characteristics of Phytase

In the utilization of enzymes, it is necessary to know their characteristics. It is very common that the same enzyme from various sources displays different characteristics.

In order to study characteristics of phytase from *A. carbonarius*, this microorganism was grown on a solid medium composed of canola meal and water. During its growth it produced the enzyme which was extracted from the solid state culture using 2% $CaCl_2.2H_2O$. The aim of this study was to determine the relationship between the parameters which are important in the utilization of the enzyme and its activity.

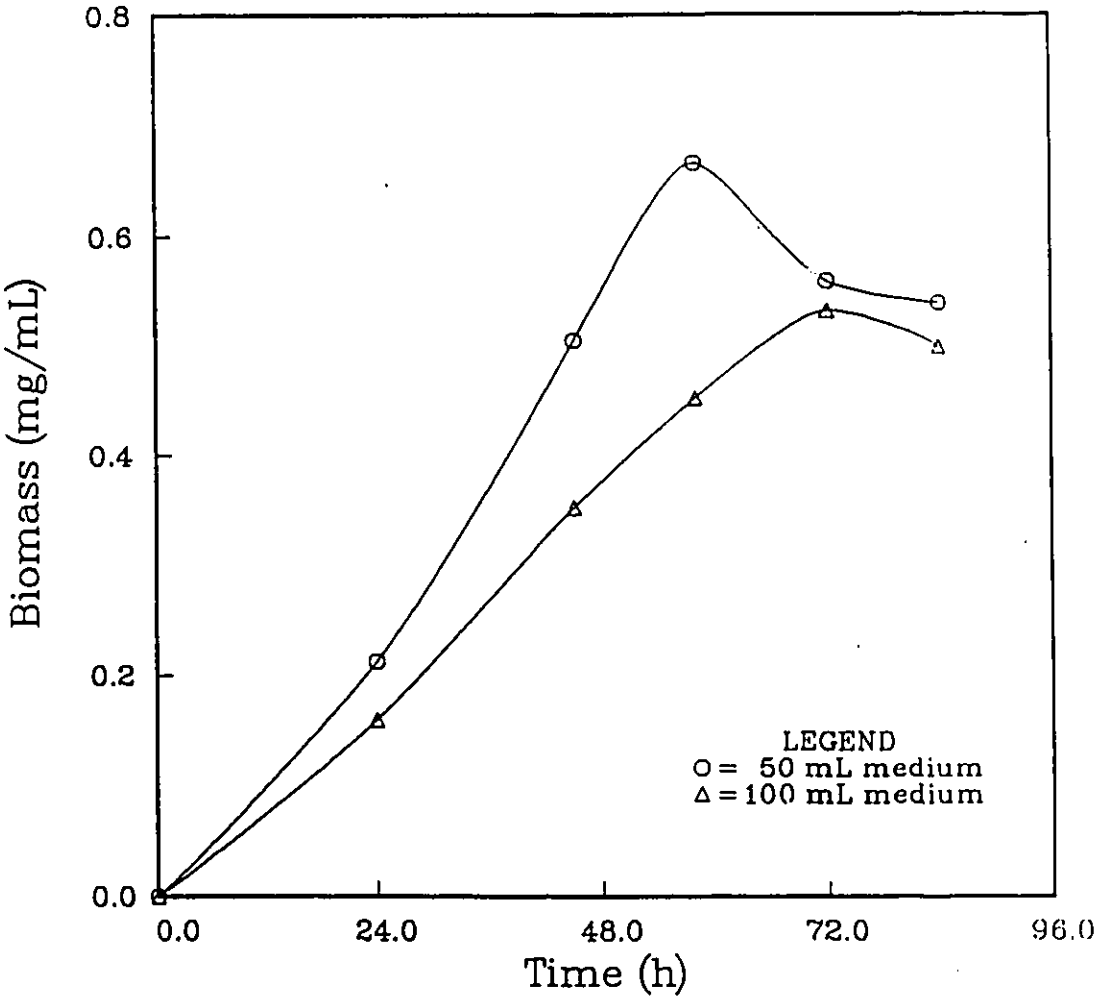


Figure 5.3: Effect of aeration rate on biomass production in liquid medium

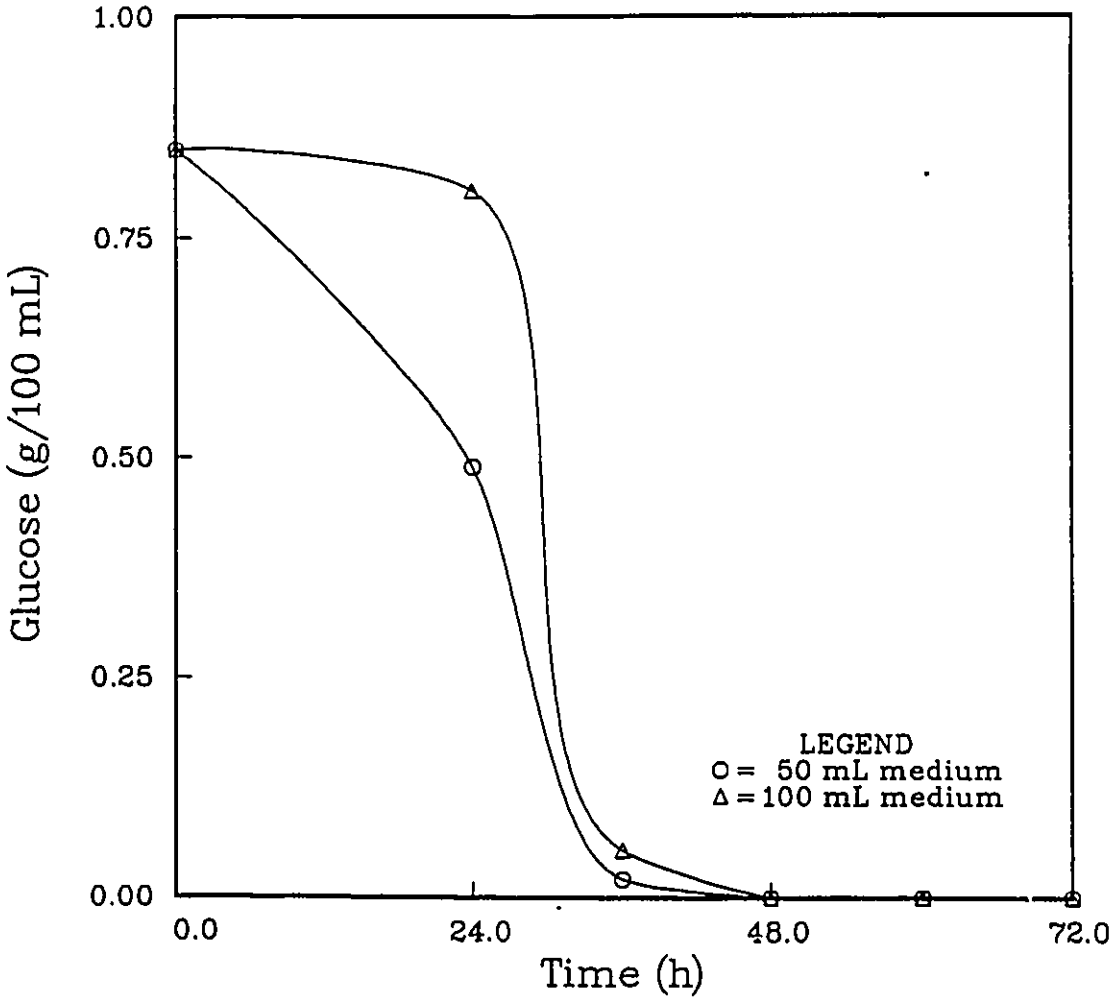


Figure 5.4: Effect of aeration rate on glucose consumption in liquid medium

The effect of the substrate concentration on the phytase activity is shown in Fig. 5.5. The K_m and v_{max} values calculated from Eq. 3.20, using least square technique (Marquardt algorithm), are 0.345 mM and 0.807 units respectively.

Different values of K_m and v_{max} have been reported in the literature for the phytases from various origins. Peers (1953) reported the K_m value of *A. ficuum* phytase to be 0.33 mM. Lolas and Markakis (1977) determined the K_m value of the navy bean phytase to be 0.018 mM. Shieh and Ware (1968) reported the K_m of *A. ficuum* phytase to be 1.25 mM. Nair and Duvnjak (1991) found the K_m and v_{max} values to be 0.27 mM and 0.46 units respectively, for *A. ficuum* phytase produced in canola meal culture.

The K_m and v_{max} values obtained in this work are within the range of the reported values for phytase of other origins.

In order to evaluate the relationship between the concentration of enzyme and its activity, various dilutions of a stock solution of the extracted phytase from a solid state culture were tested; a linear relationship was found between the enzyme concentration and its activity (Fig. 5.6). Bearing in mind that the stock solution used in this study was not very concentrated, such a relationship was expected because such linearity has been noticed for corn phytase up to 98 units of this enzyme (Chang, 1967).

The results of phytase activities versus the reaction time are shown in Fig. 5.7. The hydrolysis of the substrate was carried out up to 60 min. When Eq. 3.42 was used to calculate the Pi_{max} and $K_{t_{inc}}$ the values of 0.1375 mg/(ml) and 8.253

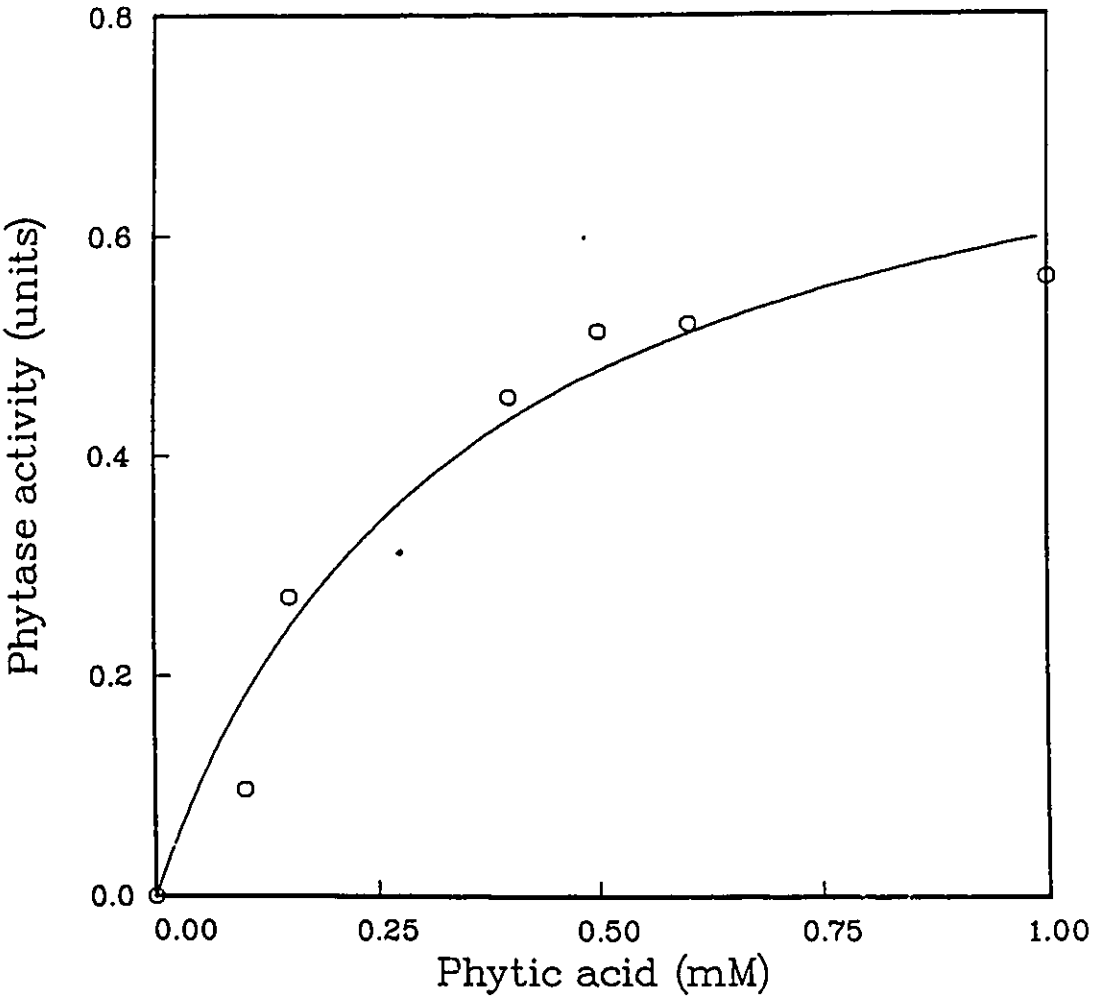


Figure 5.5: Effect of phytic acid concentration on phytase activity at 53°C and pH 4.7

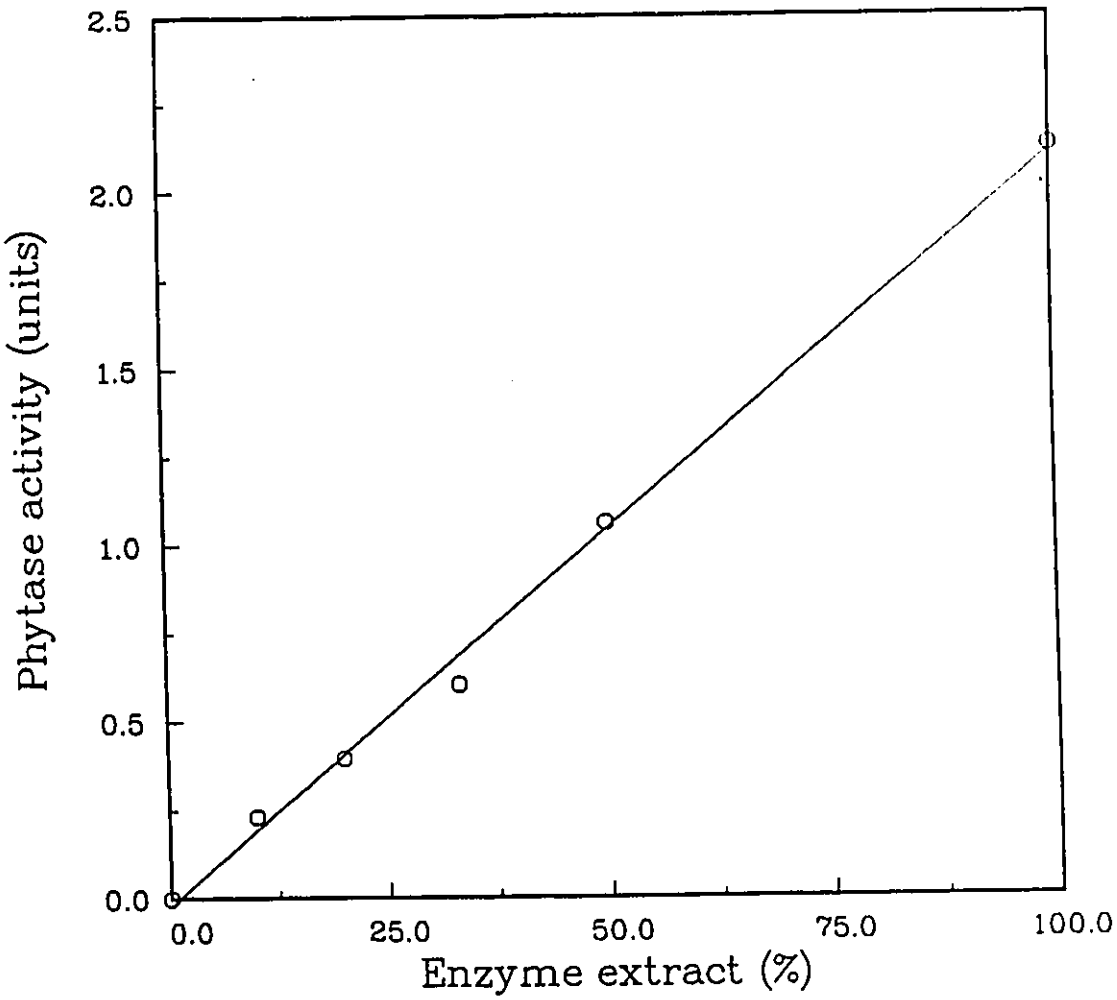


Figure 5.6: Effect of enzyme concentration on phytase activity at 53°C and pH 4.7

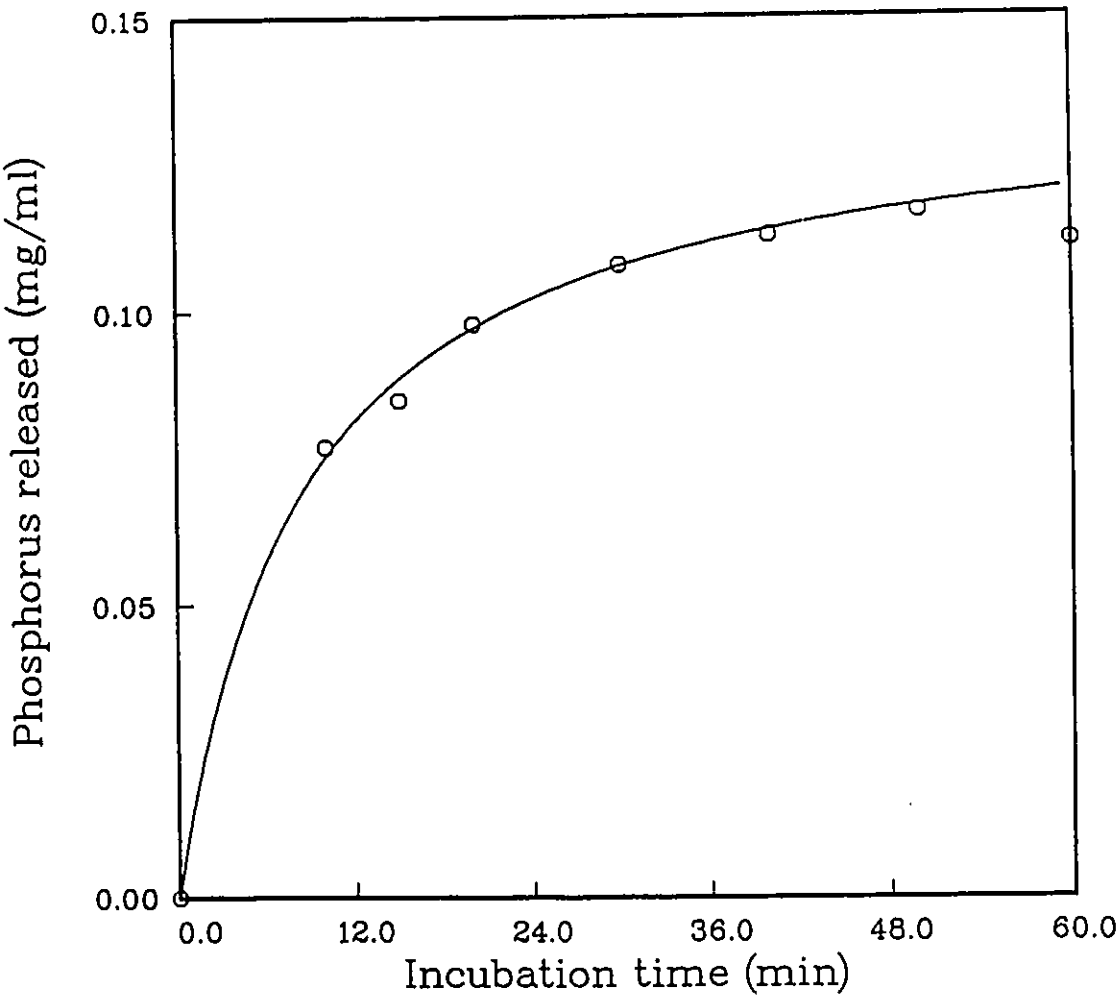


Figure 5.7: Effect of time of incubation on phytase activity at 53°C and pH 4.7

min respectively were obtained.

Studying the effect of temperature on the phytase activity of *A. carbonarius* it was determined that 53°C is the optimum temperature for this enzyme (Fig. 5.8). Peers (1953) reported an optimum of 55°C for the wheat phytase, while Mandal et al. (1972) found 57°C to be the optimum temperature for the phytase of mung beans. The optimum temperature of the navy beans phytase obtained by Lolas and Markakis (1977) was 50°C. Nair and Duvnjak (1991) determined an optimum temperature of 60°C for phytase of *A. ficuum* grown in canola meal.

In this work, the energy of activation (E), entropy change (ΔS_d) upon deactivation and enthalpy of deactivation (ΔH_d) were calculated for this enzymatic reaction. The energy of activation, E, computed from Eq. 3.27 is approximately 7800 cal/mole between 30-53°C for this enzyme. Lolas and Markakis (1977) reported the activation energy of the hydrolysis of phytic acid by navy bean phytase to be 11500 cal/mole. Nagai and Funahashi (1962) reported an energy of activation of about 12000 cal/mole for the wheat bran phytase, whereas Mandal et al. (1972) found the activation energy to be 8500 cal/mole between 37°C and 57°C for phytase of the germinating mung bean.

The entropy change upon deactivation, ΔS_d, of this enzyme and its enthalpy of deactivation, ΔH_d, as computed from Eq. 3.27, are 74 cal/(mole.K) and 24000 cal/mole respectively. The ΔS_d and ΔH_d values for phytase have not been reported in the literature. But some of these values for other enzymes are published. Baily and Ollis (1986) reported that the value of entropy of deactivation for trypsin

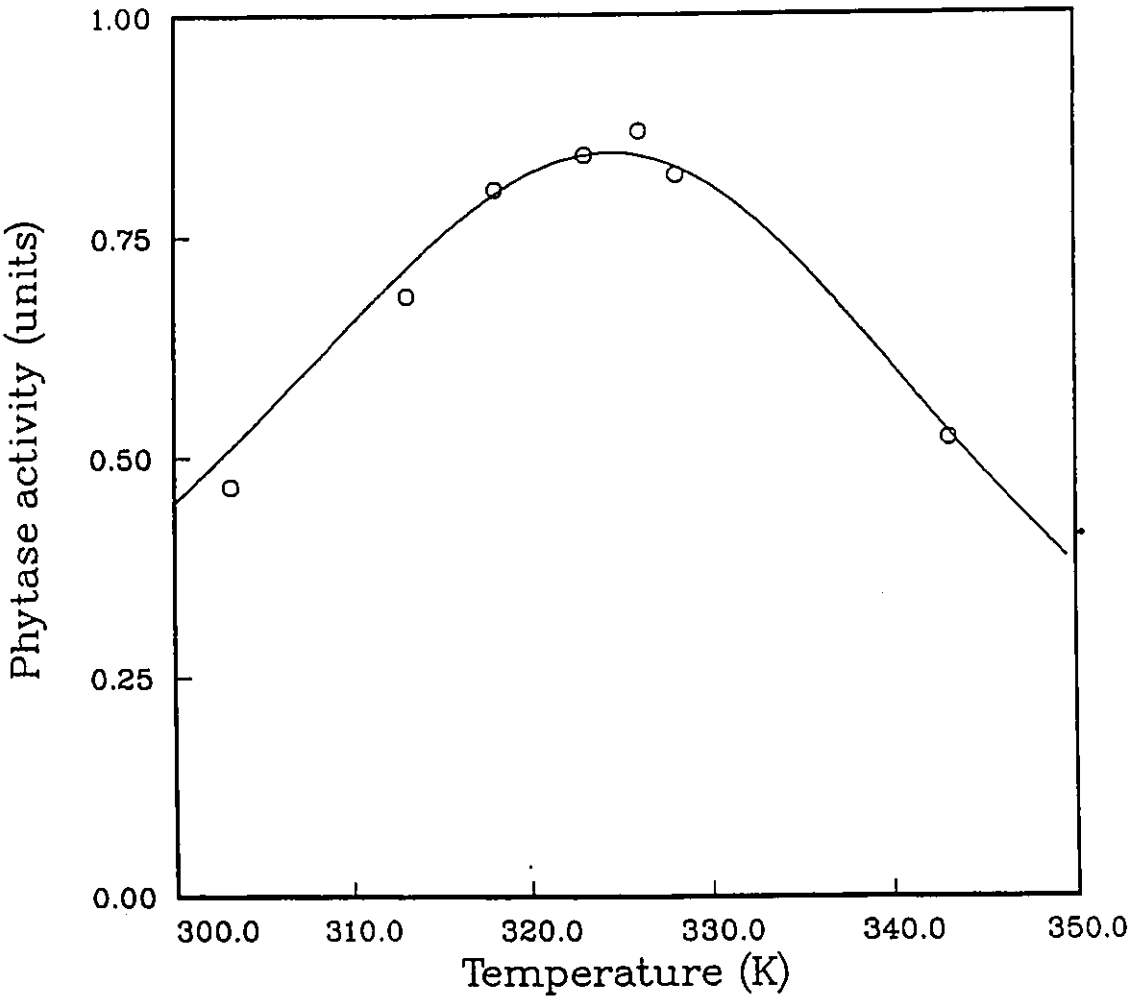


Figure 5.8: Effect of temperature of incubation on phytase activity at pH 4.7

and hen egg white lyozyme is 213 cal/(mole.K), which differs significantly from our estimated value. The same authors reported that the enthalpy of deactivation of enzymes is quite high: 68000 and 73000 cal/mole for trypsin and hen egg white lysozyme respectively.

Using the thermodynamic values calculated in this work for this enzyme and Eq. 3.27, the relationship between the temperatures and enzyme activities were calculated and shown together with the experimental results in Fig. 5.8; the results indicate a good agreement between the experimental and predicted values.

When the thermostability of phytase was studied, the preparation of this enzyme was preincubated at 50°C, 60°C, 70°C and 90°C. After the preincubation the enzyme activity was determined at 50°C (Fig. 5.9). The results show that during the preincubation, the activation of the enzyme occured. An increase in the activity was noticed with an increase in the preincubation time up to a certain point where a maximum was attained, and then, with a further increase of the time of preincubation, a decrease in the activity was measured. The highest activation was noticed when the enzyme was preincubated at 70°C. To attain the maximum activity for a particular temperature, longer preincubation times were required for lower temperatures of preincubation than for higher ones.

In this study, the phytase from *A. ficuum* was also preincubated at 70°C and 90°C for various periods of time to compare the results with those just mentioned for *A. carbonarius*. It was noticed that the phytase from *A. ficuum* was not activated at all during the preincubation (Fig. 5.9). In addition, the results showed

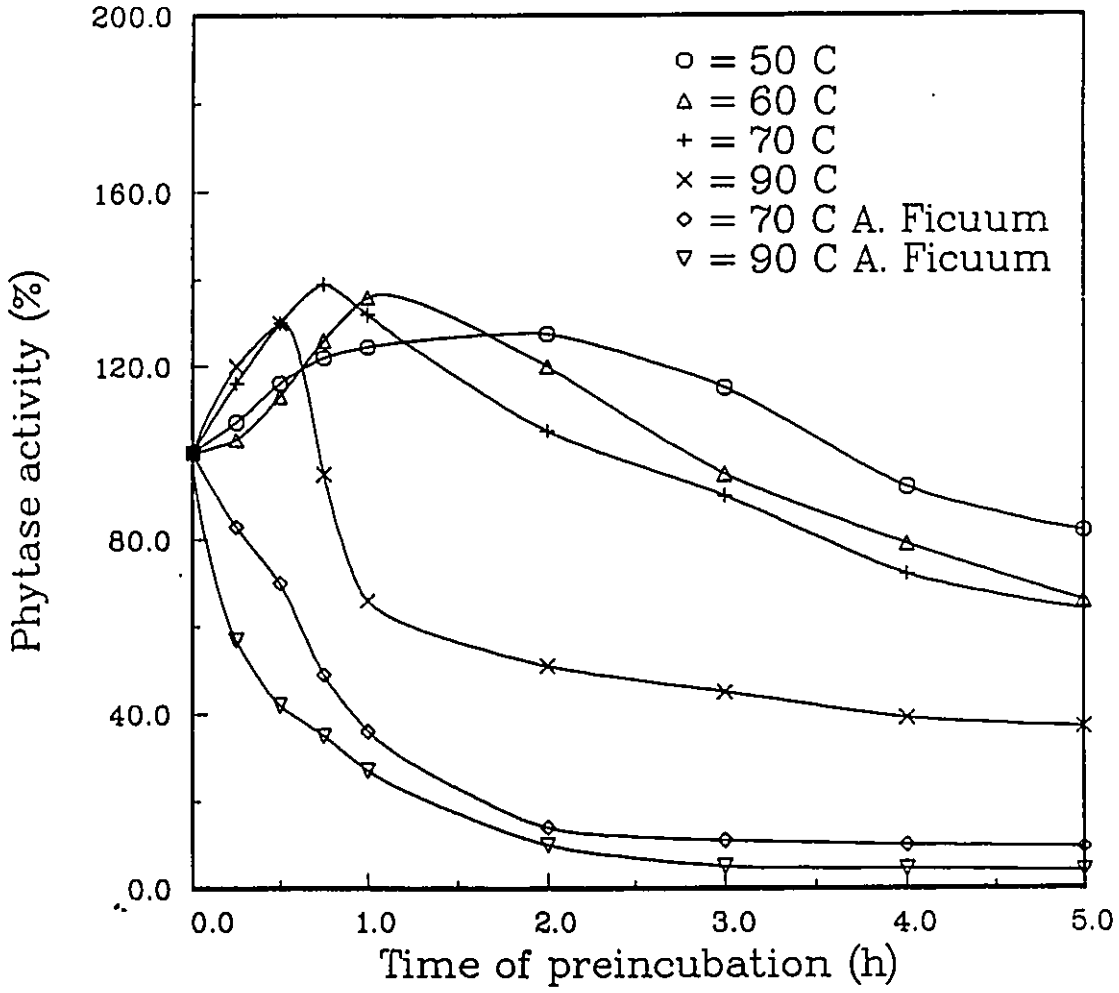


Figure 5.9: Effect of preincubation temperature (preheating) on phytase activity

that this enzyme is much less thermostable than *A. carbonarius* phytase.

This characteristic of *A. carbonarius* phytase is very important for its application. Most researchers have not been reported any activation of phytases from other origins during the preincubation. Mandal et al. (1972) preincubated the mung bean phytase at 57°C for 1 h; about 90% of the enzyme activity was lost. Lolas and Markakis (1977) reported that heating of the navy bean phytase up to 50°C for 10 min did not decrease the enzyme activity, but at 65°C the activity decreased to about 45% of the control value, and at 80°C the enzyme was almost completely inactivated in 10 minutes. Peers (1953) found that the phytase activity of whole wheat meal did not diminish by heating up to 80°C for 10 min, but an aqueous extract of the same meal entirely lost its phytase activity during heating at 70°C for 10 min. Chang (1967) found that preheating of the corn phytase up to 50°C for 10 min did not depress the enzyme activity. However, at 60°C the activity was reduced to about 40% of the control value and at 80°C, 90°C and 100°C the enzyme was practically completely inactivated. Nair and Duvnjak (1991) preincubated the phytase of *A. ficuum* grown in canola meal; they reported that preincubation at 60°C for half an hour did not cause a significant reduction in phytase activity. However, almost 70% of the activity was lost during the preincubation for 3 h, and a preincubation at 50°C for 3 h resulted in the reduction of only 5% of the activity.

Different values of optimum pH were reported in the literature for phytase from various origins. Peers (1953) reported a pH of 5.2 for the wheat phytase,

Chang (1967) found 5.6 to be the optimum pH for corn phytase, and Irving and Gosgrove (1972) claimed that the acid phosphatase preparation has a single pH optimum at 2.2, while phytase preparation has optima at pH 2.5 and 5.3; they also suggested a third optimum in the region of about 2.8. Mandal et al. (1972) reported an optimum pH of 7.5 for the mung bean phytase, while the value of Lolas and Markakis (1977) for the navy bean phytase was 5.3. Shieh and Ware (1968) reported a pH value of 5.5 for the *A. ficuum*, while Nair and Duvnjak (1991) reported a pH value of 5.0 for *A. ficuum* grown in canola meal. The optimum pH value for *A. carbonarius* phytase determined in this work is 4.7 (Fig. 5.10).

The values of the equilibrium constants K_1 and K_2, computed from Eq. 3.41 are 1.12×10^{-4} M and 1.29×10^{-6} M respectively. Using these values and the activity at the optimum pH value for this enzyme, the activities at other pH values were calculated applying Eq. 3.41. These are shown in Fig. 5.10 together with the experimental results. Although in general it can be said that Eq. 3.41 fits the experimental results reasonably well, it is noticeable that the peak obtained using this equation differs slightly from the optimum pH measured experimentally and has a value of 4.9.

The effect of extractant to meal ratio on the enzyme extraction from a solid state culture is shown in Fig. 5.11. The extraction was carried out for 1 h at room temperature. The results indicate that it is unnecessary to use higher than a 3.5 extractant:meal ratio because the amount of the extracted enzyme would not be much higher even if that ratio is significantly higher than the mentioned

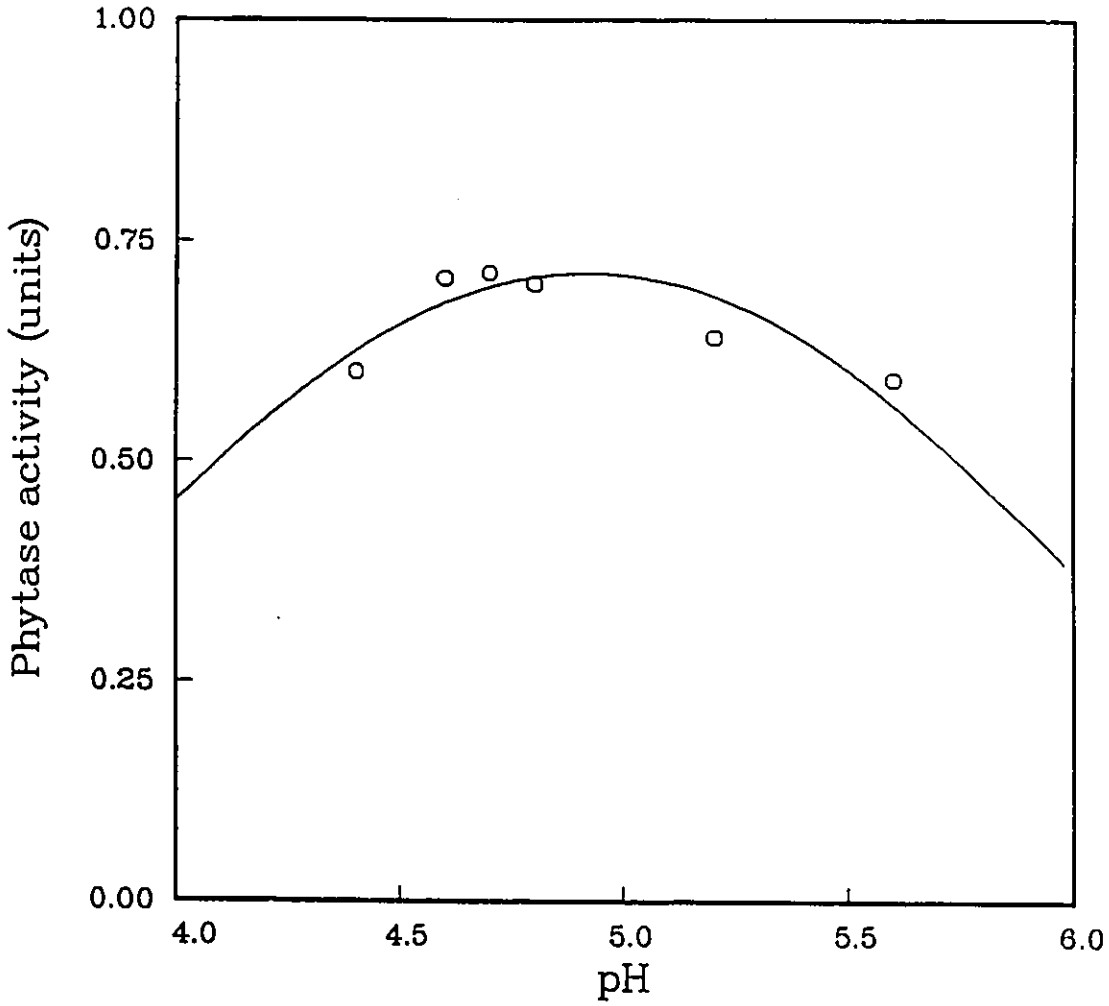


Figure 5.10: Effect of pH on phytase activity at 53°C

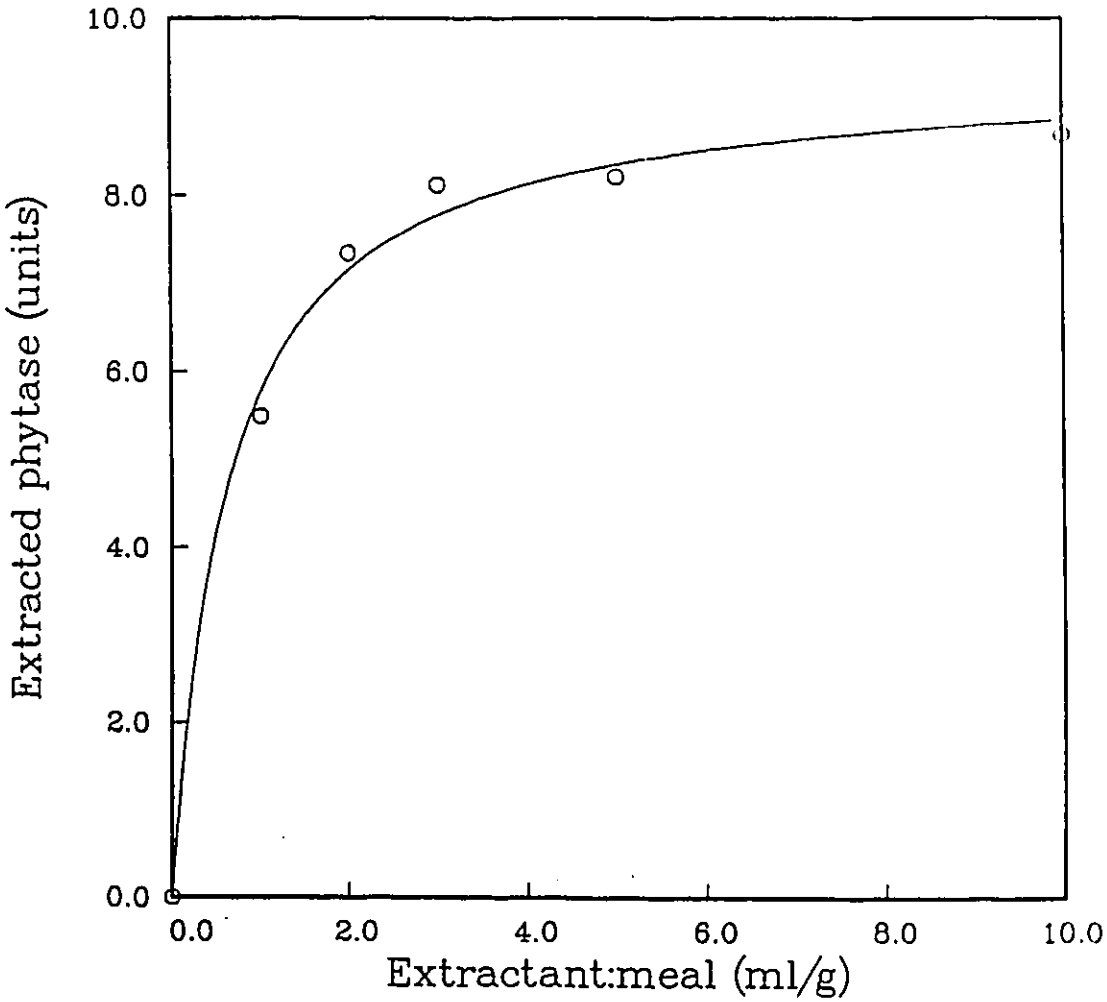


Figure 5.11: Effect of extractant to meal ratio on phytase activity

value. The values of Q_{max} (for a very high extractant:meal ratios) and K_V (for the extraction of a half of the maximum amount of the enzyme that can be extracted under the experimental conditions), estimated from Eq. 3.43, are 9.416 units/g and 0.6324 ml/g respectively. Knowing these values, Eq. 3.43 can be used to calculate the amount of the enzyme which can be extracted for any extractant:solid state culture ratio. A good agreement between the experimental values and those calculated by Eq. 3.43 was obtained (Fig. 5.11).

In studying the effect of the time of extraction on the amount of extracted phytase from a solid state culture, the extractant:solid state culture ratio used was 5. The extraction was carried out at room temperature under constant shaking of the extractant-solid state culture mixture. The obtained results (Fig. 5.12) show that a major fraction of the total amount of the enzyme in the solid state culture was extracted in a short period of time. The K_t and Q_{max} values are 1.5 min and 2.1 units/g respectively, and were estimated by means of a Michaelis-Menten type of equation as for μ_m and K_s using the experimental data and Eq. 3.44. These values and Eq. 3.44 can be used to compute the amount of enzyme which can be extracted for a period of time of extraction under experimental conditions.

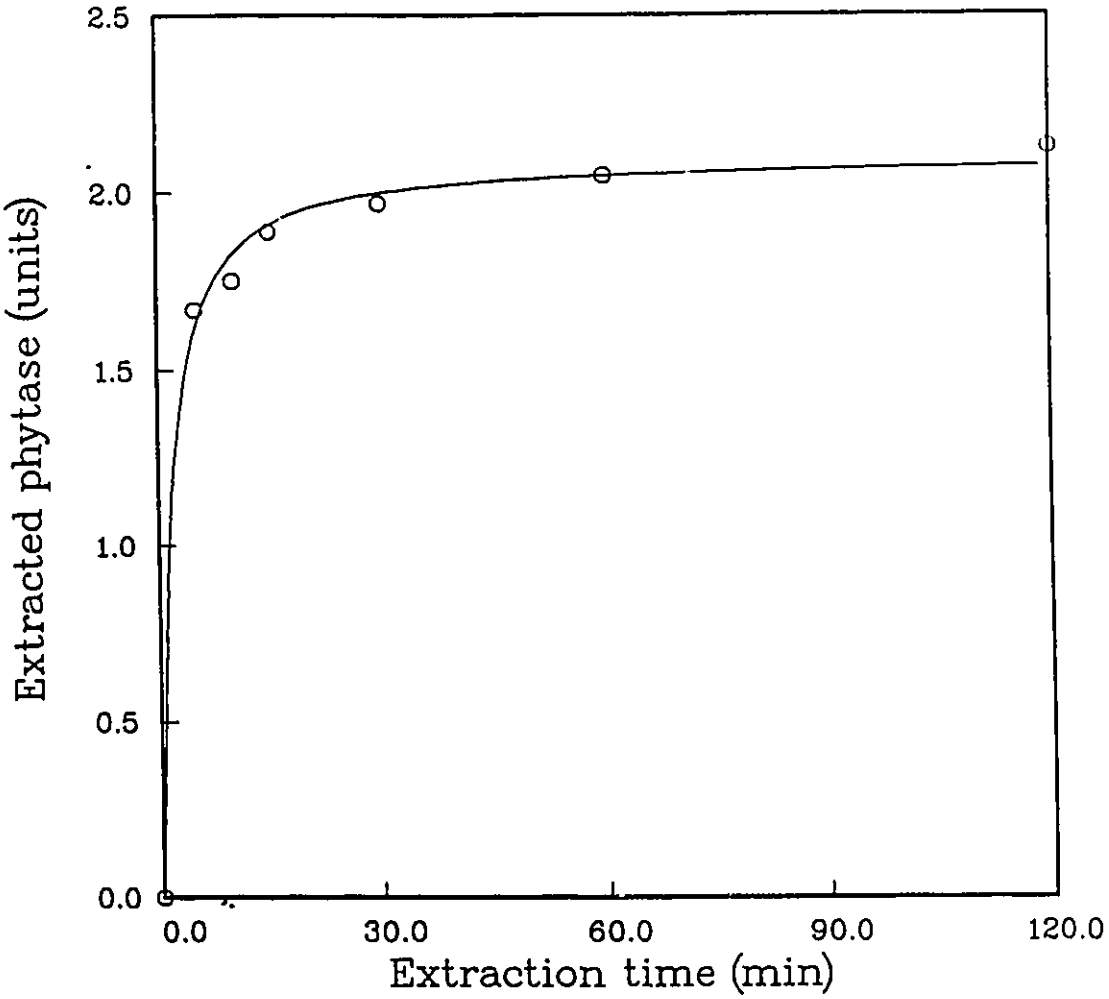


Figure 5.12: Effect of time of extraction on phytase activity

5.3 Changes in Phytic Acid and Protein Contents, Available Carbohydrates, Phytase Activity and Biomass Production

Measurements showed that the canola meal used in this study contained 6% phytic acid. To decrease the phytic acid content, the meal was used as a medium in a solid state fermentation with *A. carbonarius* NRC 401124 as a producer of phytase.

Changes in the phytic acid and protein contents, available carbohydrates, phytase activity and biomass production during SSF process are shown in Fig. 5.13. After inoculation, biomass started growing without a noticeable phase lag. The increase in the biomass concentration was followed by the increase in the phytase activity. In both cases the increase was slower during the first 24 h than in the next 24 h. The phytic acid was hydrolyzed entirely in canola meal in 40 hours of the fermentation. The carbohydrates concentration dropped to 75% of their initial value in two days, while the apparent protein concentration increased by about 25% and attained its maximum when the phytate concentration was reduced to zero. Following this the protein concentration started declining. Similar trends were noticed during a solid state fermentation with *Aspergillus ficuum* (Nair and Duvnjak, 1990). It is appropriate to mention that biomass is difficult to measure in solid-state culture directly because of the penetration of the fungal hyphae in the solid-substrate; as a result of this, an indirect method was adopted. Glucosamine content of cell has been found to be an indicator of the biomass concentration and

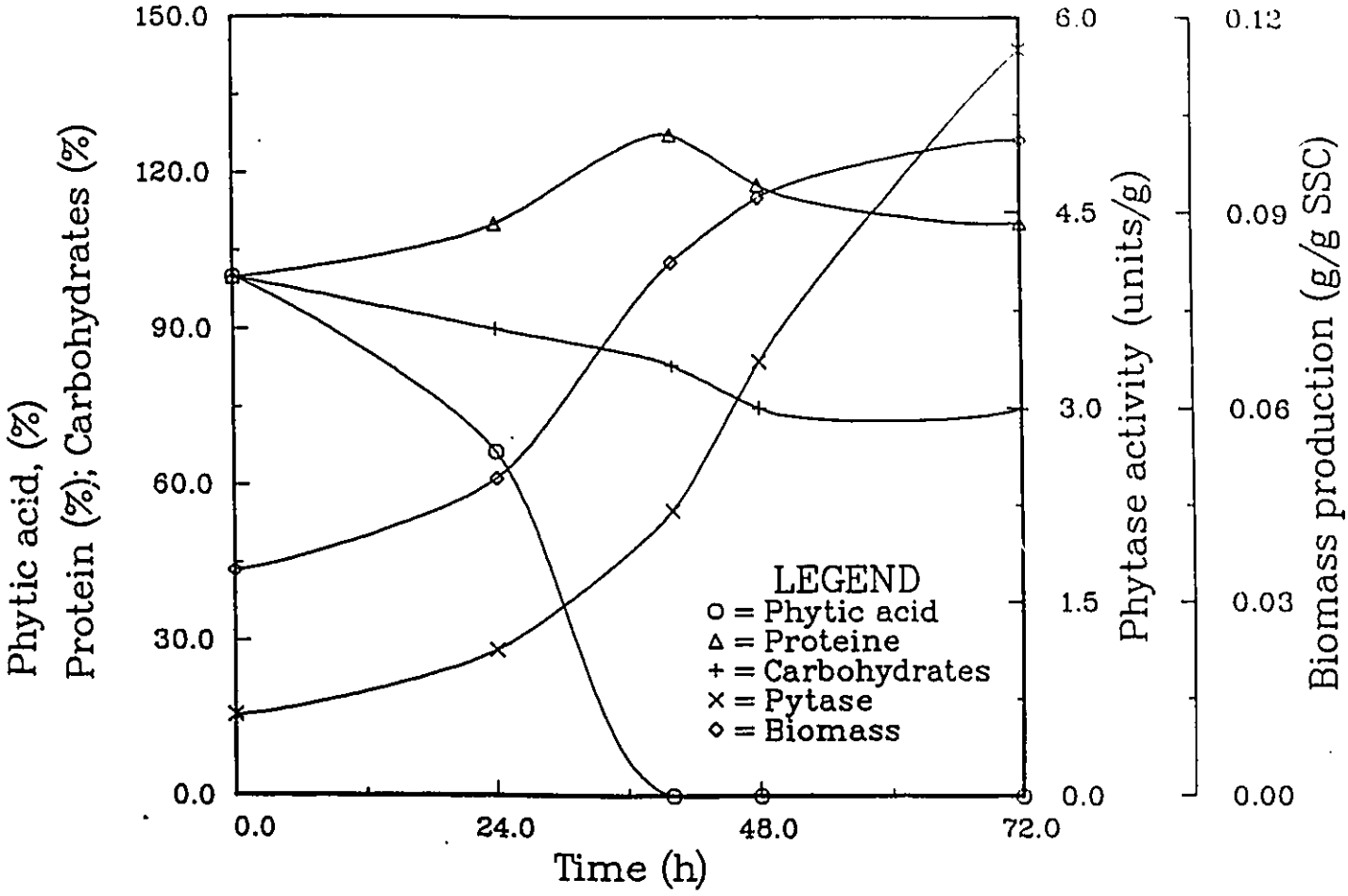


Figure 5.13: Changes in phytic acid and protein contents, available carbohydrates, phytase activity and biomass production during SSF process

the biomass:glucosamine ratio is constant throughout fungal growth and remains the same under different conditions of growth (Desgrandes et al., 1991).

5.4 Effect of Physical and Chemical Factors on SSF

In this section some physical and chemical factors that influece the rate of phytic acid content, anzyme activity and biomass production will be discussed.

5.4.1 Effect of Moisture Content

Addition of water causes swelling of the substrates and facilitates its utilization by the microorganisms. However, the optimum amount of water (moisture content) required varies, and must be determined for each system and microorganism (Cannel and Moo-Young, 1980) . Therefore, tests were carried out with the meal containing between 29% and 65% moisture to study the effect of moisture on the phytic acid content reduction. The result shows (Fig. 5.14) that the optimum moisture content for this system is 53%. A lower level of water affects the utilization of substrate by the microorganism and subsequently a lower amount of enzyme is produced. This results in a decrease in the rate of phytic acid consumption; the system with a moisture content of 29% was found to contain 63% of the phytic acid after 72 h fermentation. With higher moisture content than 53%, the medium was like mud, and this caused difficulties in oxygen transfer which is very important for the growth of the microorganism as it was shown in the submerged fermentation test in the begining of this study. A similar conclusion was found

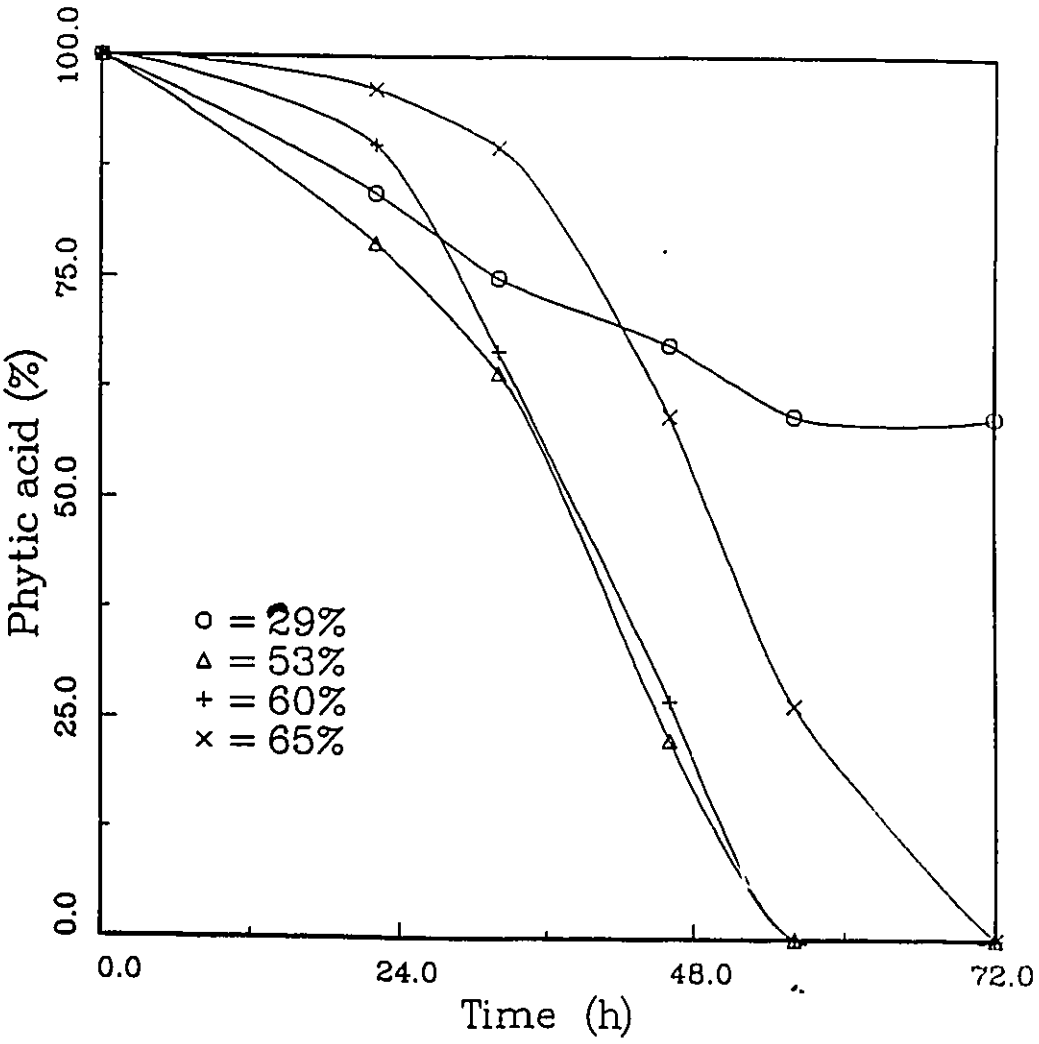


Figure 5.14: Effect of moisture content on the reduction of phytic acid content in canola meal during SSF process

by Han and Gallagher (1987), when they studied the production of phytase by *Aspergillus ficuum* on several cereal grains and legume seeds.

5.4.2 Effect of Inoculum Concentration

The effect of the biomass amount in the inoculum on the reduction of phytic acid content is shown in Fig. 5.15. The system with the largest amount of biomass has the highest rate of phytic acid content reduction. It is important to minimize the wastage of inoculum and also to optimize enzyme production so that we can avoid the use of high amount of inoculum which affects the cost. For example, a medium with 240 mg biomass and a medium with 150 mg biomass can bring phytic acid content to zero for the same period of time, e.g. after 48 h, but each with different initial rate. A similar trend was found in a solid-state fermentation for phytic acid reduction in canola meal using *Aspergillus ficuum* (Nair and Duvnjak, 1990).

5.4.3 Effect of Sterile and Nonsterile Media

Partial reduction of phytic acid content in canola meal occurred even when uninoculated non-sterile medium was incubated (Fig. 5.16); in this case about 48% of phytic acid was reduced after 50 h of incubation. This activity can be assigned to the microorganisms from canola meal. To see the effect of sterilization, both sterile and non-sterile media were inoculated with *A. carbonarius*. A complete reduction of phytic acid content was obtained in the sterile medium in 50 h, while for the same period of time only 62% of the phytic acid content was reduced in

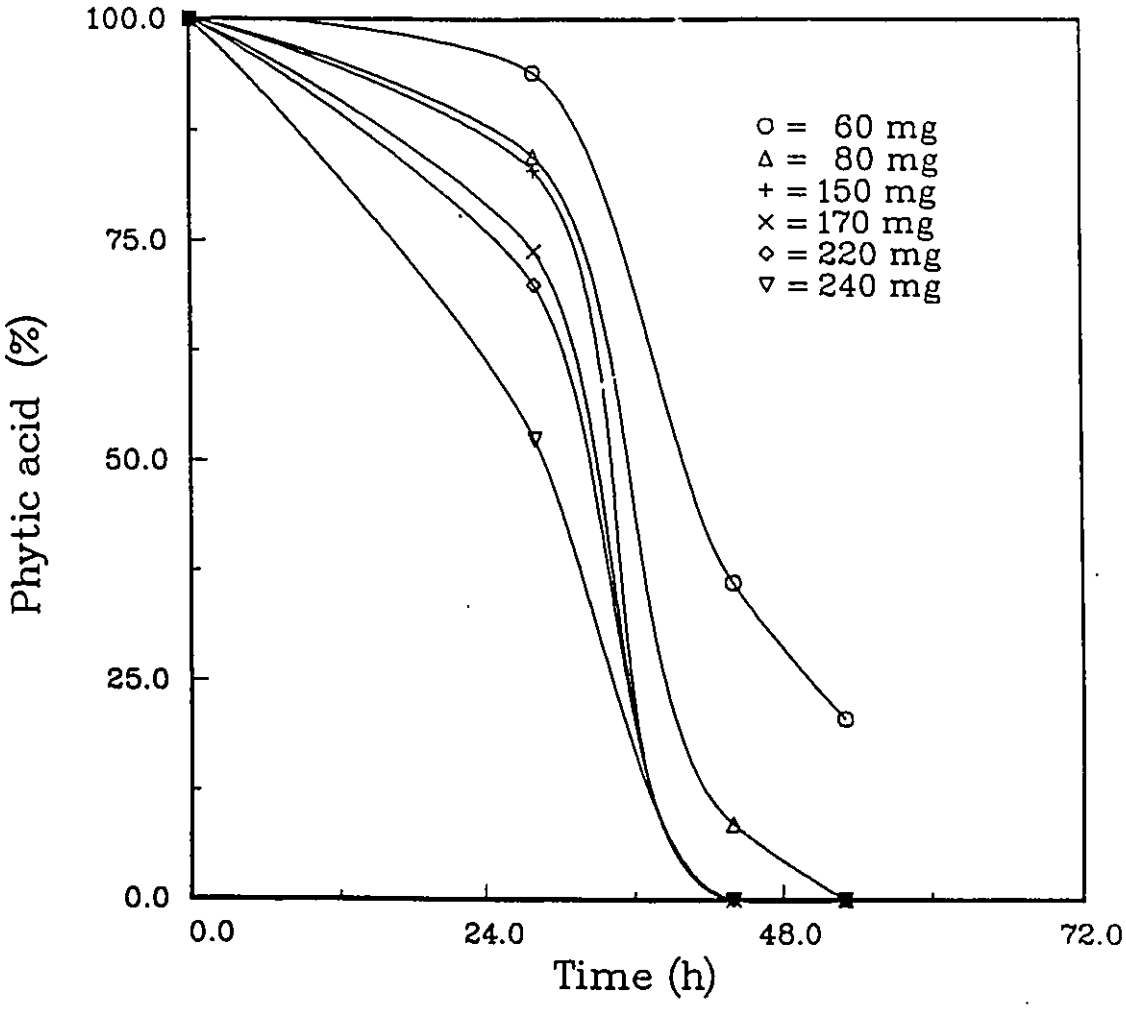


Figure 5.15: Effect of the amount of biomass in the inoculum on the rate of phytic acid content reduction in canola meal during SSF process

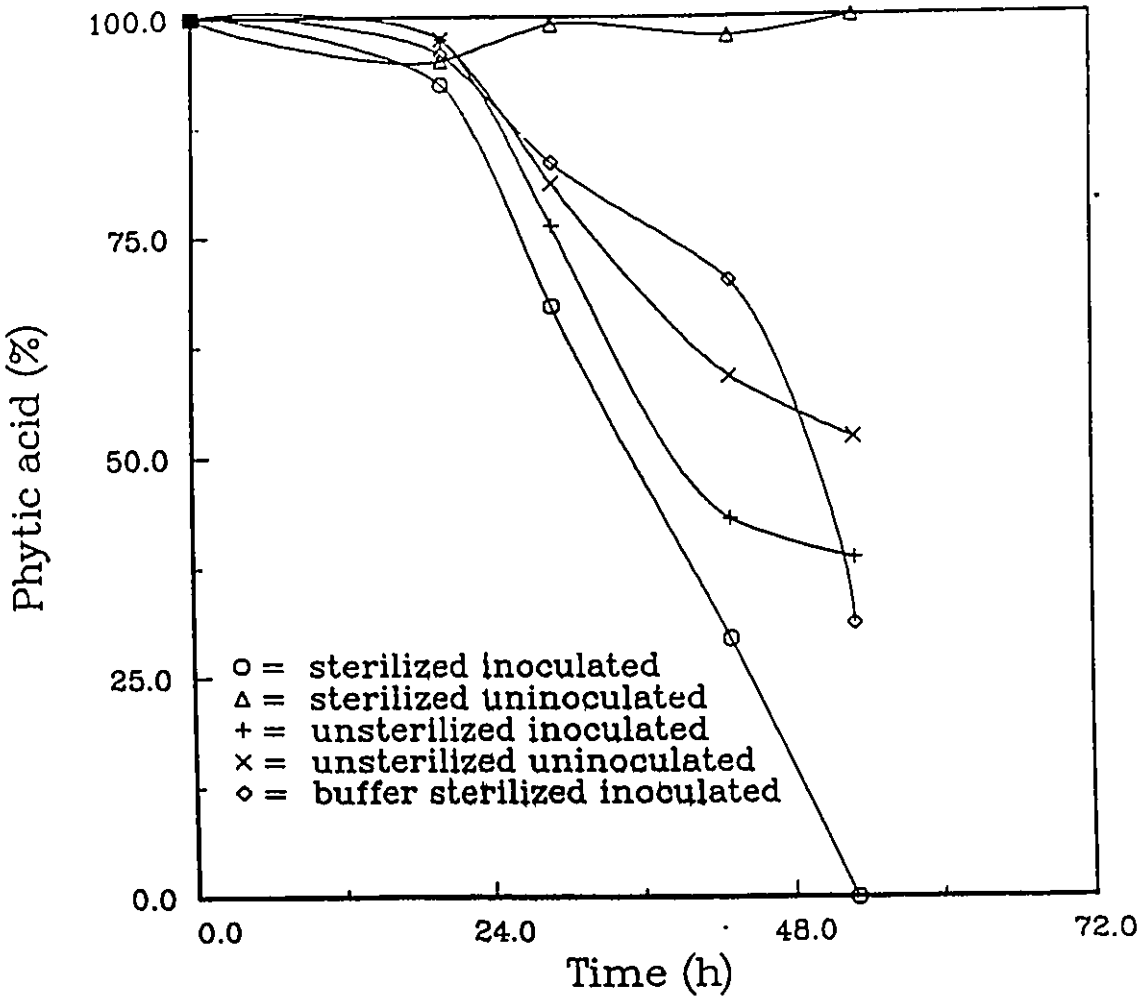


Figure 5.16: Effect of sterilization, inoculation and buffered media on the reduction of phytic acid content in canola meal during SSF process

the other one. The original microbial population in the canola meal, which altered conditions in the medium during the SSF process, was the reason for the poorer performance of *A. carbonarius* in the non-sterile medium.

Fig. 5.16 also shows the effect of the acetate buffer on the reduction of phytic acid content. The inoculated sterile buffered medium exhibited a slower rate of phytic acid content reduction than the unbuffered one. Nair and Duvnjak (1990) found that the growth in buffer medium is better than the water medium using *A. ficuum.*

5.4.4 Effect of Homogenization of Inoculum

The effect of homogenization of the inoculum on the productions of biomass and phytase, and the reduction of phytic acid content in canola meal was studied. *A. carbonarius* grew in the liquid medium for inoculum preparation in the form of pellets; it was difficult to distribute them evenly within the solid medium. Thus, the inoculum was homogenized in a blender prior to its utilization in order to increase the number of centers of biomass growth. The results show that an increase in the time of homogenization up to 120 s resulted in an increase of the biomass and phytase productions (Figs. 5.17 and 5.18).

Seventy two hours after the beginning of the growth, the biomass concentration was 35% higher in the medium inoculated with the inoculum homogenized for 120 s than in that inoculated with inoculum homogenized for 10 s. The phytase concentration was also higher by about 15% in the medium inoculated with

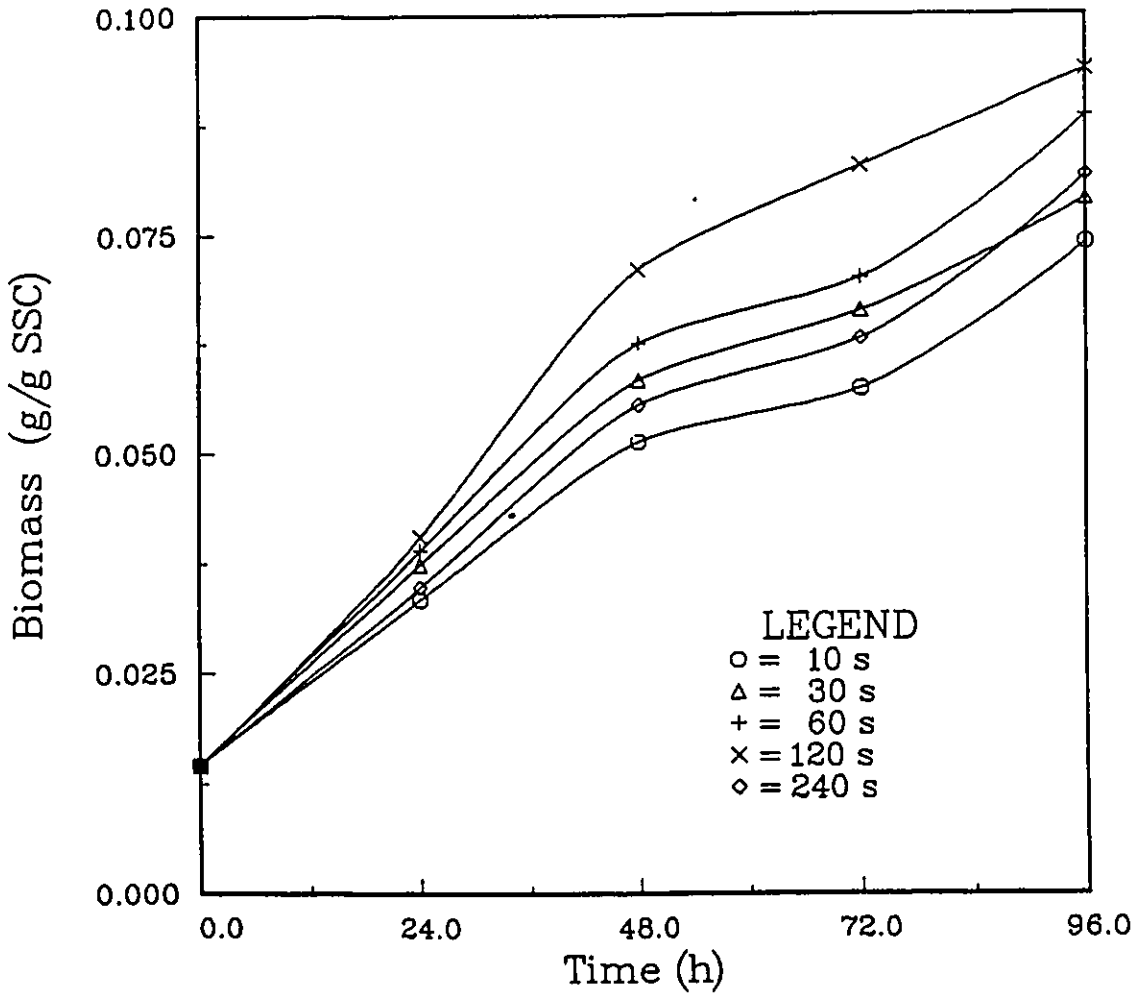


Figure 5.17: Effect of the time of homogenization of the inoculum on biomass production in canola meal during SSF process

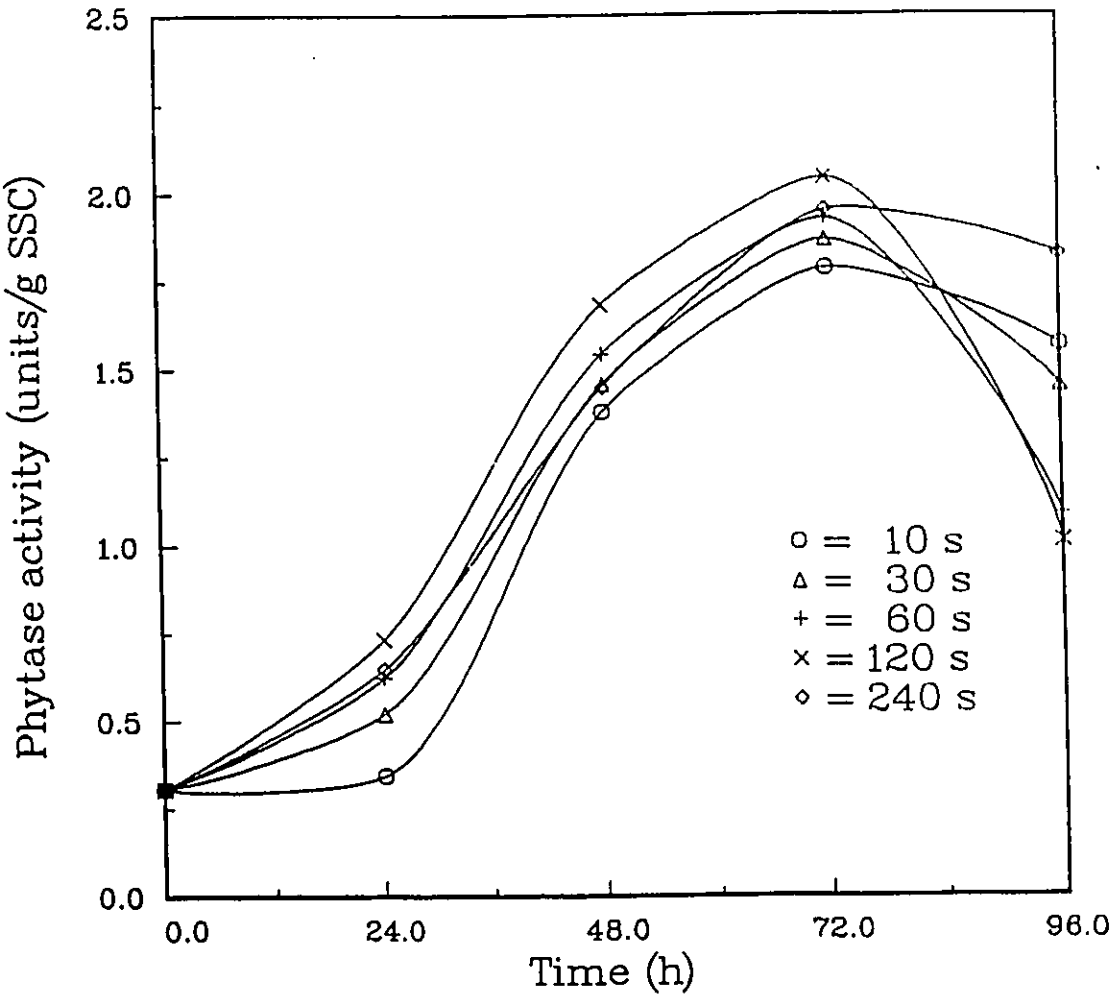


Figure 5.18: Effect of the time of homogenization of the inoculum on the phytase production in canola meal during SSF process

120 s homogenized inoculum. When the inoculum was homogenized for 240 s, a negative effect of the prolonged homogenization was noticed on the biomass and phytase productions. It is clear that the biomass from the inoculum was partially damaged during the extended homogenization time and, after that, its growth and the production of phytase were retarded.

Bearing in mind that the production of phytase is directly related to the biomass growth, it was normal to expect the reduction of phytic acid content in the medium to be affected. Taking into consideration the rates of phytic acid content reduction in the medium between the inoculation time and 48 hours of incubation, it is noticeable that an increase in the times of inoculum homogenization up to and including 120 s resulted in an increase of the rate of the reduction of phytic acid content in the medium. The rate was much slower when the inoculum was homogenized for 240 s (Fig. 5.19). The results show that the phytic acid content in all media was zero 72 hours after inoculation, regardless of the time of homogenization of the inoculum, because each of the media contained a sufficient amount of the enzyme and enough time to react with the total amount of phytic acid in the medium.

Although the amounts of biomass and enzyme were affected by the length of homogenization the specific enzyme productivity did not change much (Table 5.2).

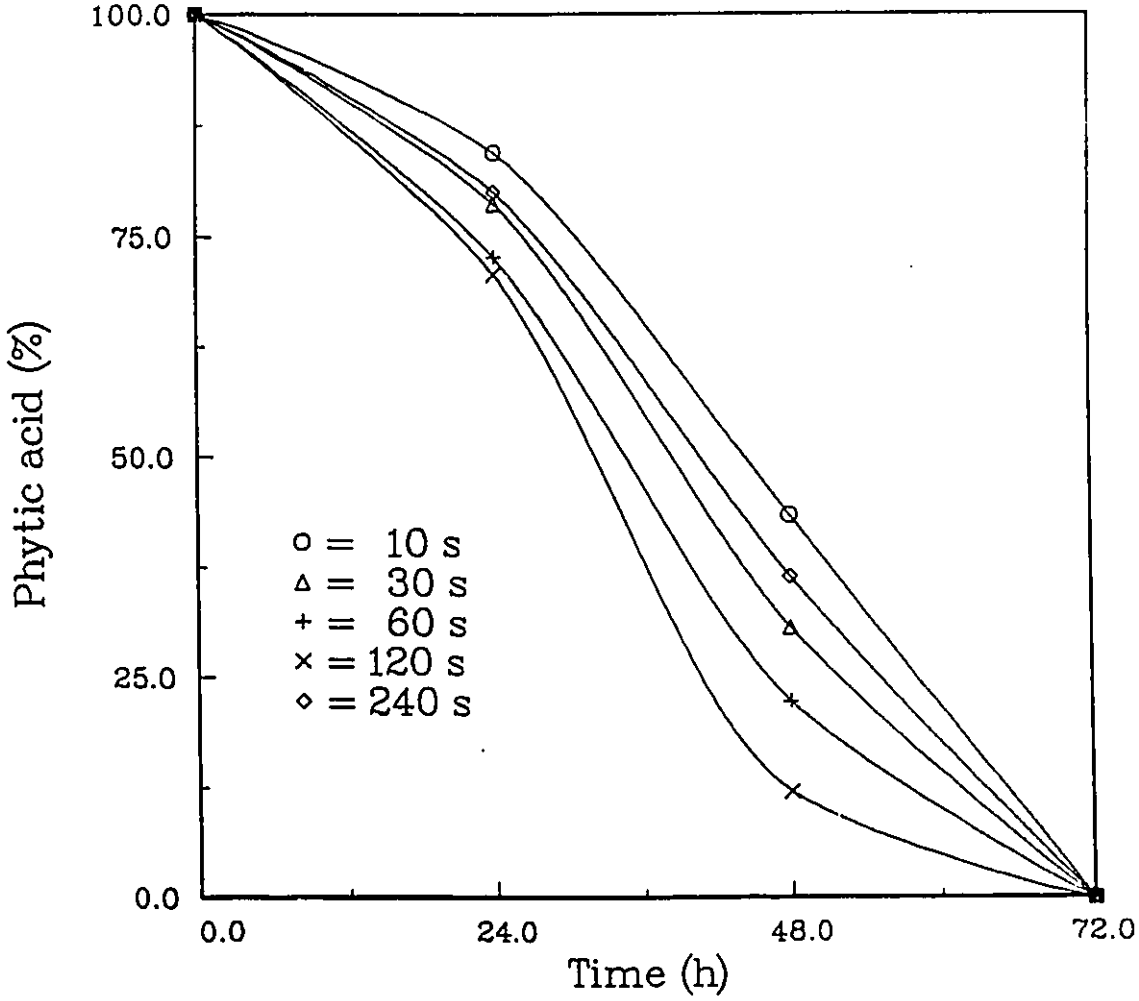


Figure 5.19: Effect of the time of homogenization of the inoculum on the reduction of phytic acid content in canola meal during SSF process

Table 5.2: The effect of the time of homogenization of the inoculum on the biomass and enzyme productions, and phytic acid content reduction after 48 h incubation.

Time of homo-genization (s)	Biomass produced (mg/g SSC)[1]	Enzyme produced (units/g SSC)[2]	Phytic acid content reduced (%)	Specific enzyme productivity (units/mg)[3]
10.0	53.12	1.38	43.42	0.0259
30.0	58.33	1.46	30.96	0.0250
60.0	64.32	1.54	22.30	0.0239
120.0	71.09	1.68	12.00	0.0236
240.0	55.52	1.45	36.50	0.0261

[1] mg of biomass per g of SSC.
[2] units of enzyme per g of SSC.
[3] units of enzyme per mg of biomass.

5.4.5 Particle Size of Canola Meal

A certain amount of canola meal (500 g) was used for screen analysis. Table 5.3 shows a typical screen analysis of canola meal as a substrate for the SSF process. Each of these fractions was used as a substrate in the SSF process in order to

Table 5.3: Particle size analysis of canola meal

Particle number and size	Mass fraction retained
S-1 smaller than 0.180 mm	0.0978
S-2 0.180-0.425 mm	0.2706
S-3 0.425-0.710 mm	0.2166
S-4 0.710-1.000 mm	0.1829
S-5 1.000-1.400 mm	0.1680
S-6 bigger than 1.400 mm	0.0638

study the effect of particle size of canola meal on biomass and phytase production

and phytic acid content reduction.

In solid culture, there are many factors which affect the microbial growth, among them the void space and the surface area of the particles. Substrate with finer particles has very small void space and is not sufficient for oxygen transfer, and this results in a reduction of microbial growth since oxygen is very important for the growth of the microorganism. Also substrates with finer particles showed improved degradation due to an increase in the total surface area per unit volume. With higher particle size, the void space increases, so the oxygen transfer increases, but the total surface area decreases. Therefore, considering these two factors there is an optimum particle size of the meal for the growth of the microorganisms.

Fig. 5.20 shows that the smaller particle substrate gives the lower amount of biomass, and the highest amount of biomass is obtained from a substrate of particles 1.0-1.40 mm. However, for particles higher than 1.4 mm a decrease in biomass production was noticed. Similar trends were observed with the phytase production; Fig. 5.21 shows the phytase activity obtained in media made of different particle size substrate. The smaller particle substrate gave the lower rate of phytase production. Maximum rate of phytase production was obtained with substrates containing particles of 1.0-1.40 mm; while a decrease in phytase production was obtained with substrates containing particles higher than 1.40 mm.

Pandey (1991) studied the effect of particle size of the wheat bran and whole corn flour on glucomylase production during SSF process using *Aspergillus niger*.

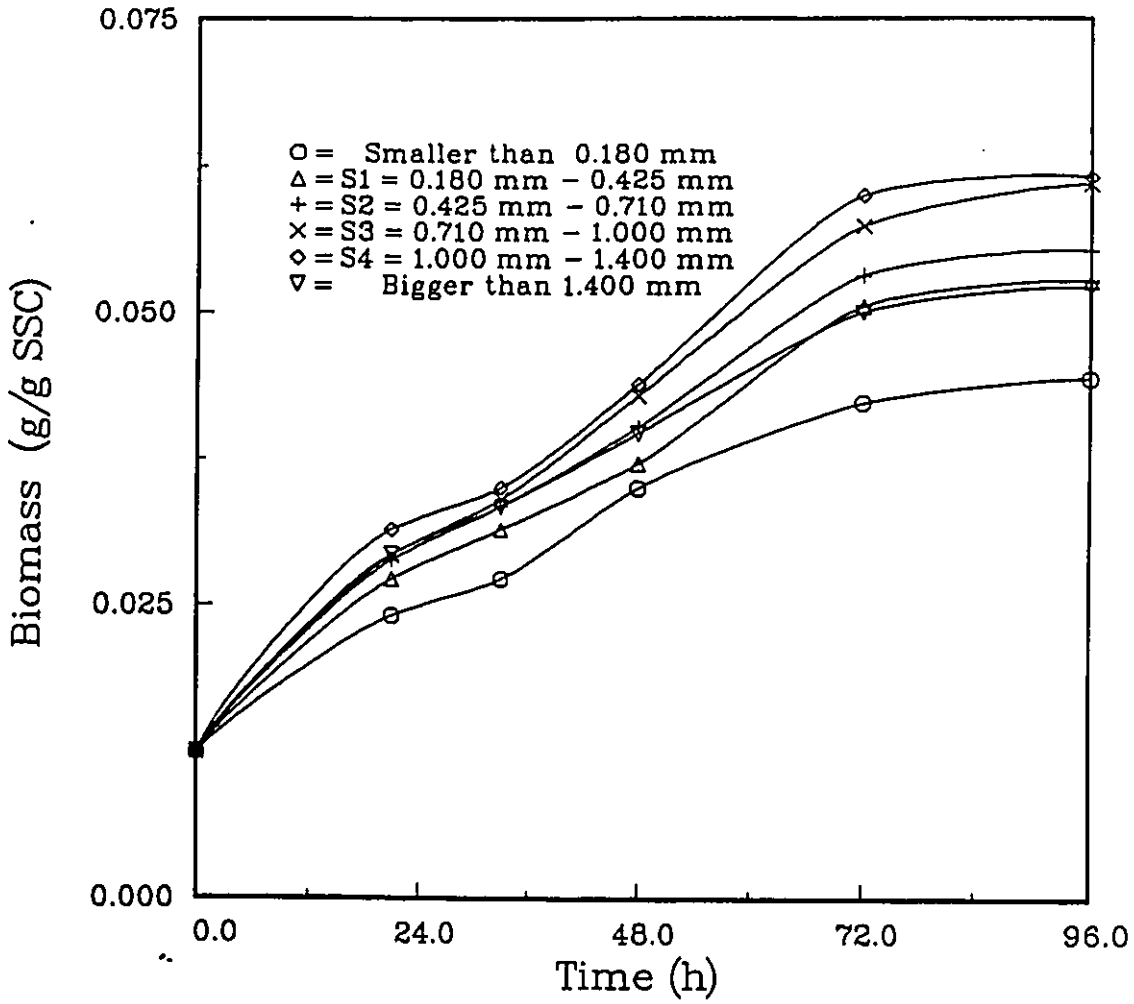


Figure 5.20: Effect of particle size of canola meal on biomass production during SSF process

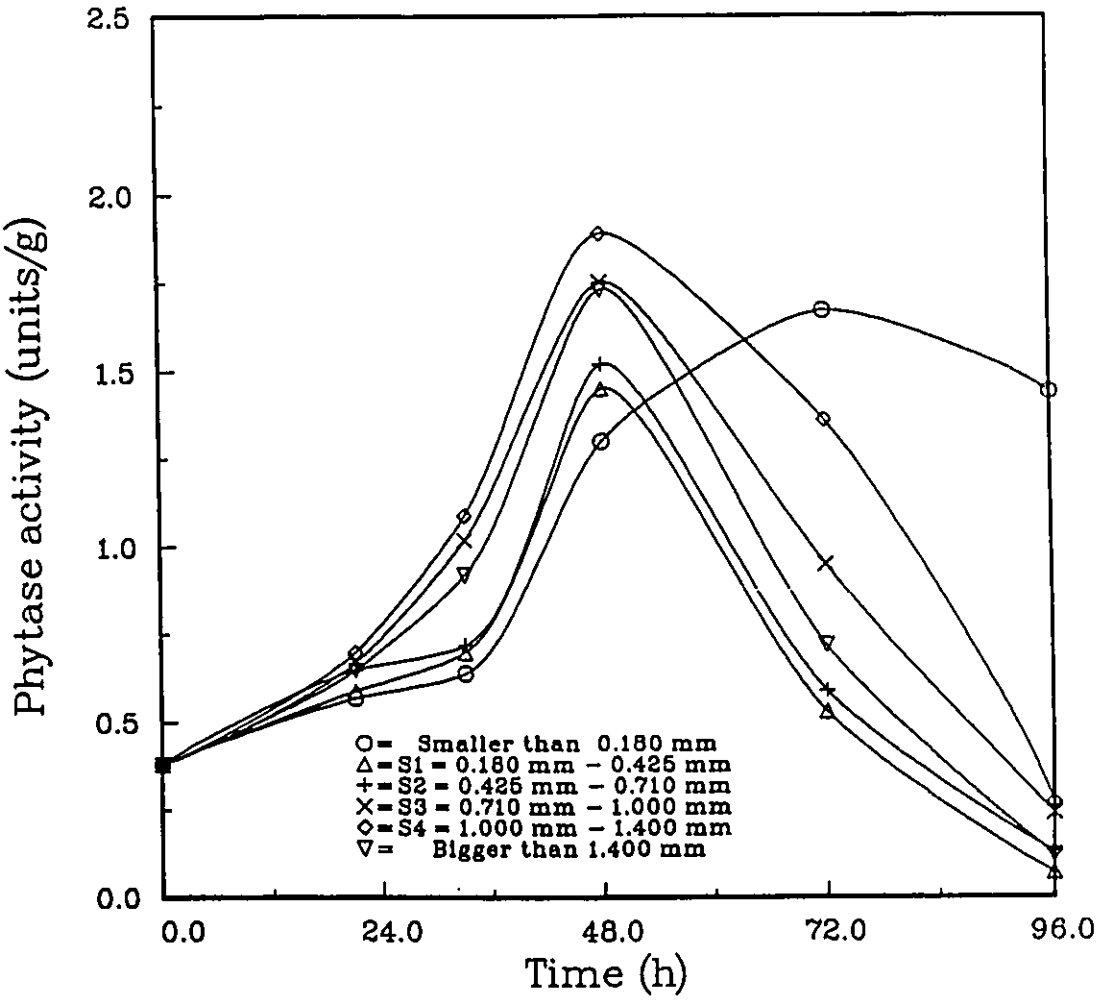


Figure 5.21: Effect of particle size of canola meal on phytase production during SSF process

He obtained results similar to those obtained in this work. He also found that substrates with smaller particles resulted in a high degree of solubilization.

Consequentely, the lower particle sizes results in a lower rate of phytic acid content reduction (Fig. 5.22) while the particles which yield the higher amount of phytases result in a higher rate of phytic acid content reduction.

5.4.6 Effect of Initial pH

Buffer can affect the medium positively or negatively. It has been shown previously that a medium with distilled water yield better phytase production than a medium with acetate buffer (pH 4.7).

This section discusses the effect of using different kinds of buffers, each having different initial pH, on phytase production and phytic acid content reduction. Regardless of the type of buffer the level of phytase production was found to be dependent on the initial pH of the medium.

Media were prepared for SSF with acetate buffer in the range of pH 3.8-5.6 and one with distilled water. The results obtained are shown in Figs. 5.23 and 5.24.

Fig. 5.23 shows that a system with distilled water gave the higher phytase production in the first 72 h of the process than the other systems regardless of the initial pH. For a process time greater than 72 h, the phytase activity decreased in the system with distilled water while it continued to increase in the other systems with acetate buffer. The phytase activity of the systems with pH 5.2 and 5.6 has

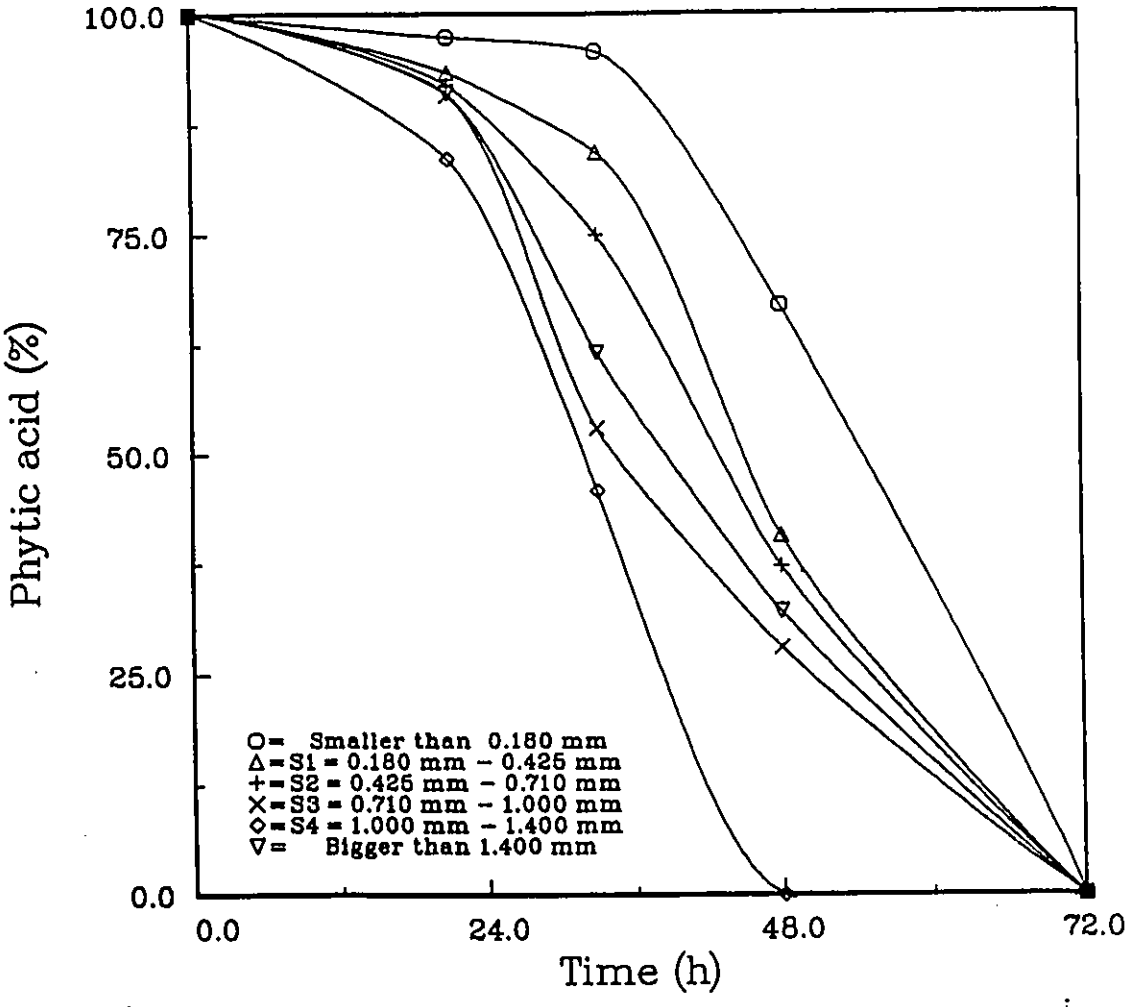


Figure 5.22: Effect of particle size of canola meal on phytic acid content reduction during SSF process

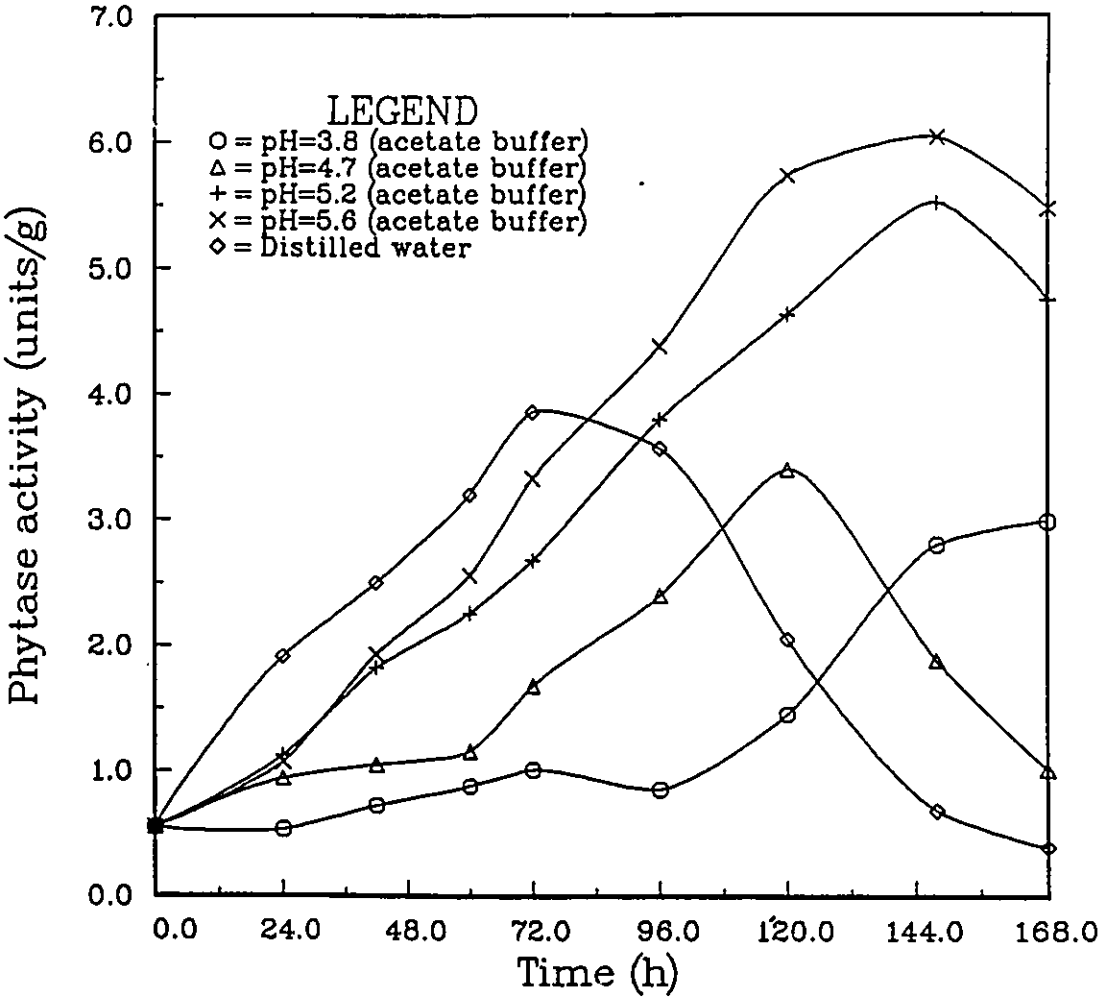


Figure 5.23: Effect of initial pH of acetate buffer on phytase activity during SSF process

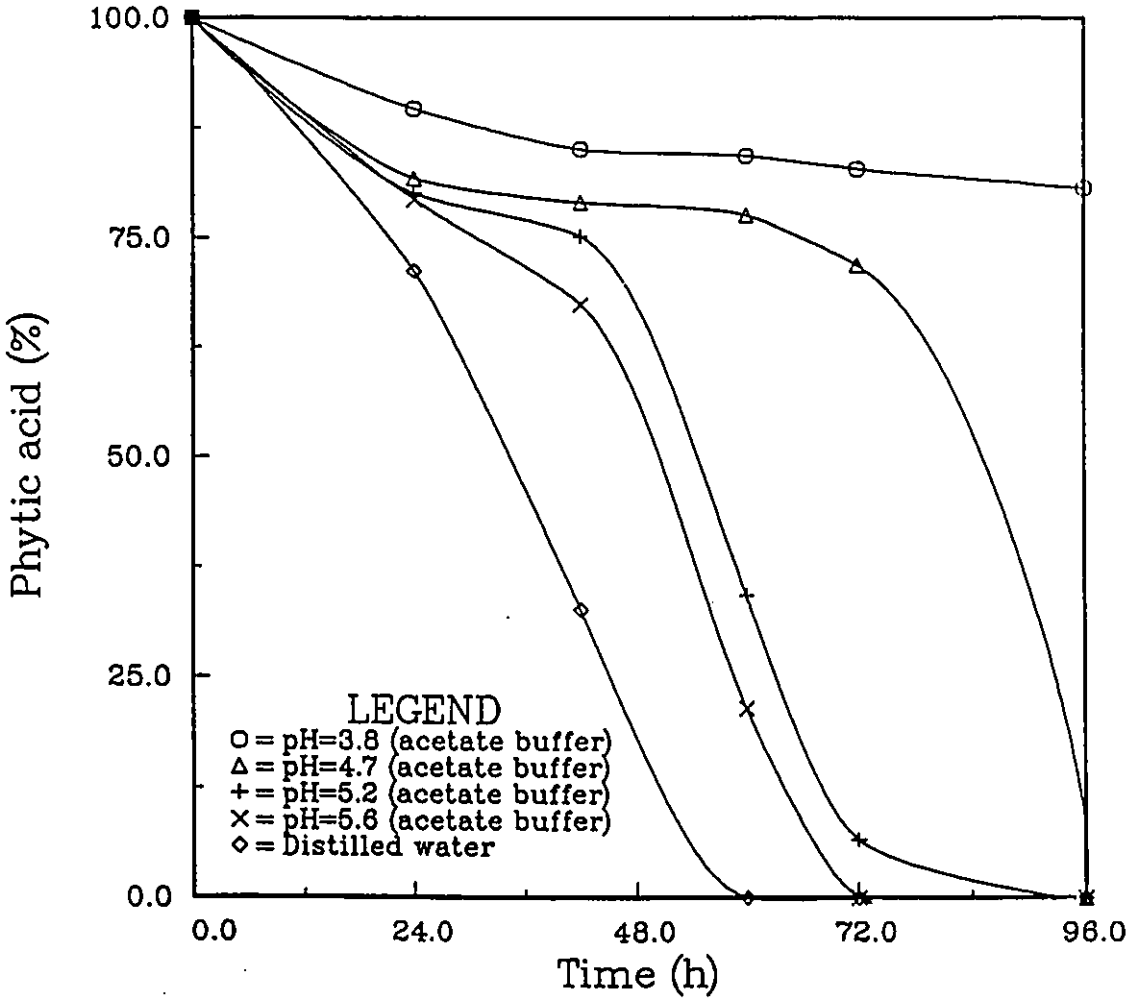


Figure 5.24: Effect of initial pH of acetate buffer on phytic content reduction during SSF process

a maximum value which is higher than the maximum phytase acitivity in the case of distilled water. Fig. 5.23 also shows that the phytase production increased with an increase in the initial pH of the acetate buffer, and a very slow rate of phytase production was obtained with the pH 3.8 system. Maximum phytase activity was attained with the system of the maximum pH (5.6) when using this buffer.

The effect of initial pH of acetate medium on phytic acid content reduction is shown in Fig. 5.24. Systems containing distilled water and acetate buffer of pH 5.6 had completed the reduction in phytic acid content in 60 h and 72 h of fermentation, respectively. It is obvious that the rate of phytic acid content reduction increased with an increase in the initial pH of the medium. The system with pH 3.8 was found to reduce only 20% of phytic acid content after 96 hours of incubation.

The effect of acetate buffer having pH greater than 5.6 has not been studied in this work, because this buffer doesn't work with pH greater than 5.6. Therefore, other kinds of buffers that work at high levels of pH were investigated to test their effect on the process.

Fig. 5.25 shows the effect of phosphate buffer of the pH range of 5.6-6.5 on phytase activity. It shows that maximum phytase activity can be attained using the system with a pH of 5.6 (Fig. 5.25) while inhibition occurs with the other systems of higher pH (6.0 and 6.5). Here, also the system with pH 5.6 had completed phytic acid content reduction in 60 hours of fermentation (Fig. 5.26) like the one with acetate buffer having the same pH, but each has different rate

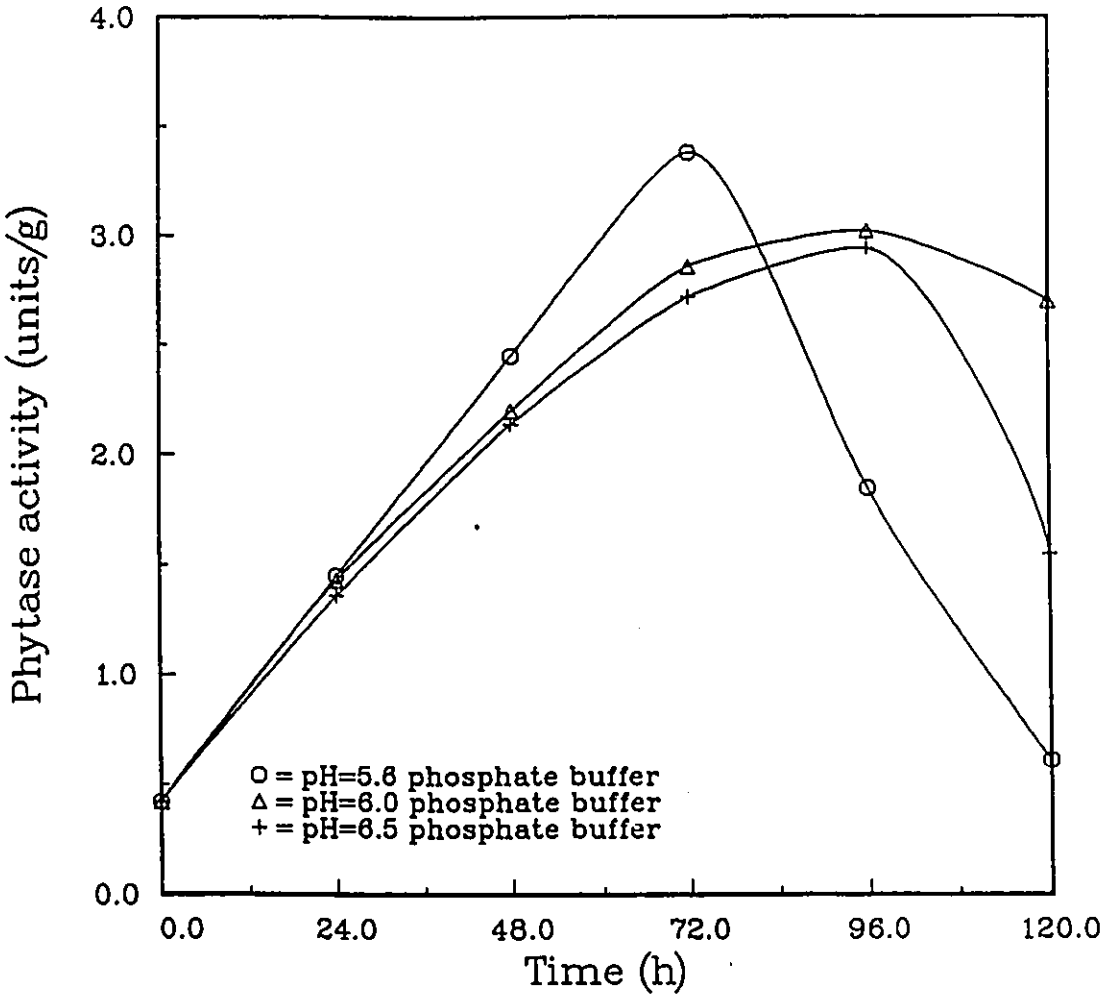


Figure 5.25: Effect of initial pH of phosphat buffer on phytase activity during SSF process

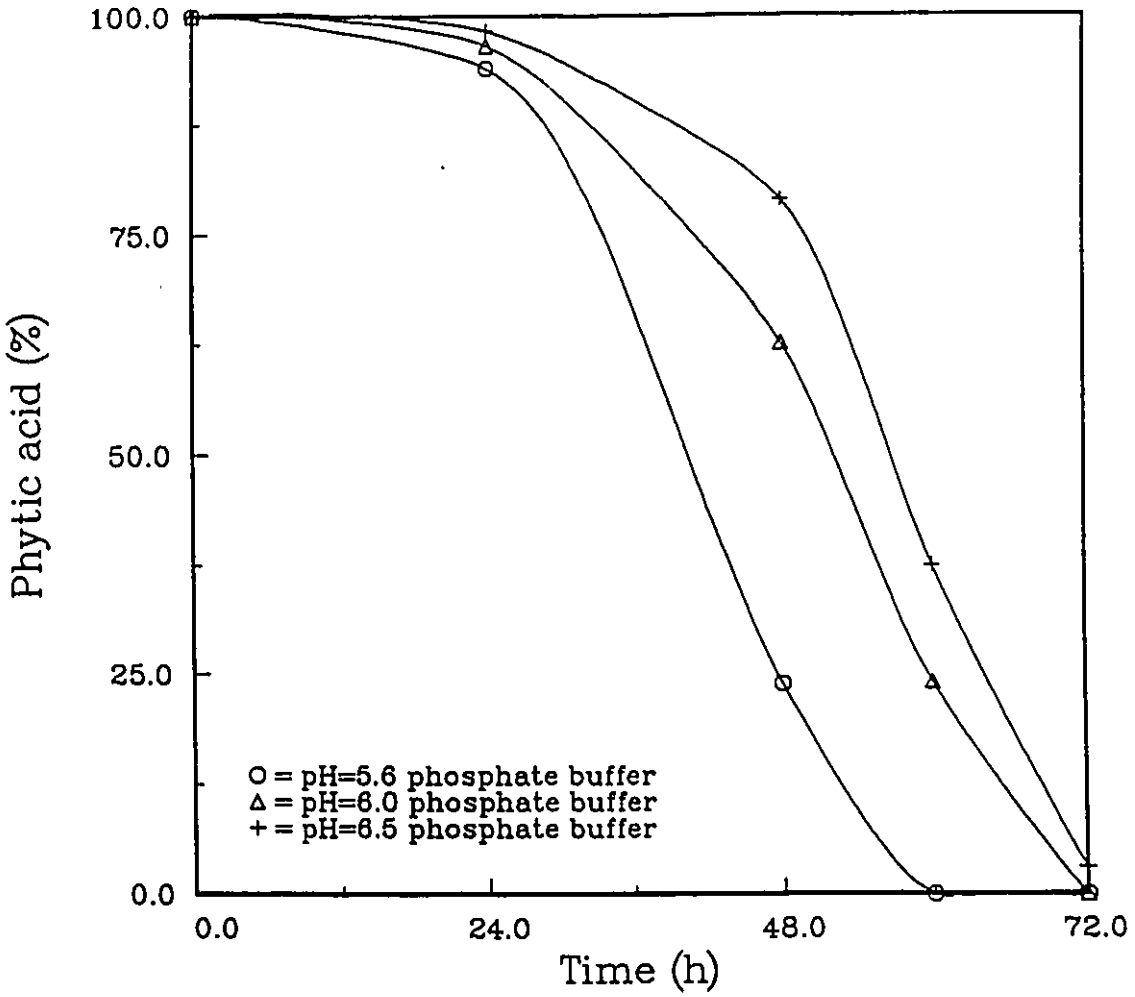


Figure 5.26: Effect of initial pH of phosphate buffer on phytic content reduction during SSF process

of reduction. The system with pH 6.0 had diminished the phytic acid content to zero after 72 h of incubation, while the system with pH 6.5 still had 3% of phytic acid content.

Citrate buffer has also been tested. It gave results completely opposite to the data obtained in the presence of phosphate buffer. Fig. 5.27 shows that maximum phytase activity was attained with the system having a pH value of 6.5; while the minimum phytase activity was obtained with the system having a pH value of 5.6.

Studying the effect of citrate buffer on the phytic acid content reduction, it has been found that the system with pH 6.5 has the highest rate of phytic acid content reduction, and completed the reduction in 60 h of incubation while the other systems, with pH 5.6 and 6.0, required 72 h to reduce phytic acid content to zero (Fig. 5.28). It was seen that the system with acetate buffer of pH 5.6 had the higher phytase activity than the systems of phosphate buffer and citrate buffer having the same pH.

5.5 Nutritional Factors

In this section, the effect of some nutritional components added to the medium on the biomass and phytase productions and phytic acid content reduction, will be discussed.

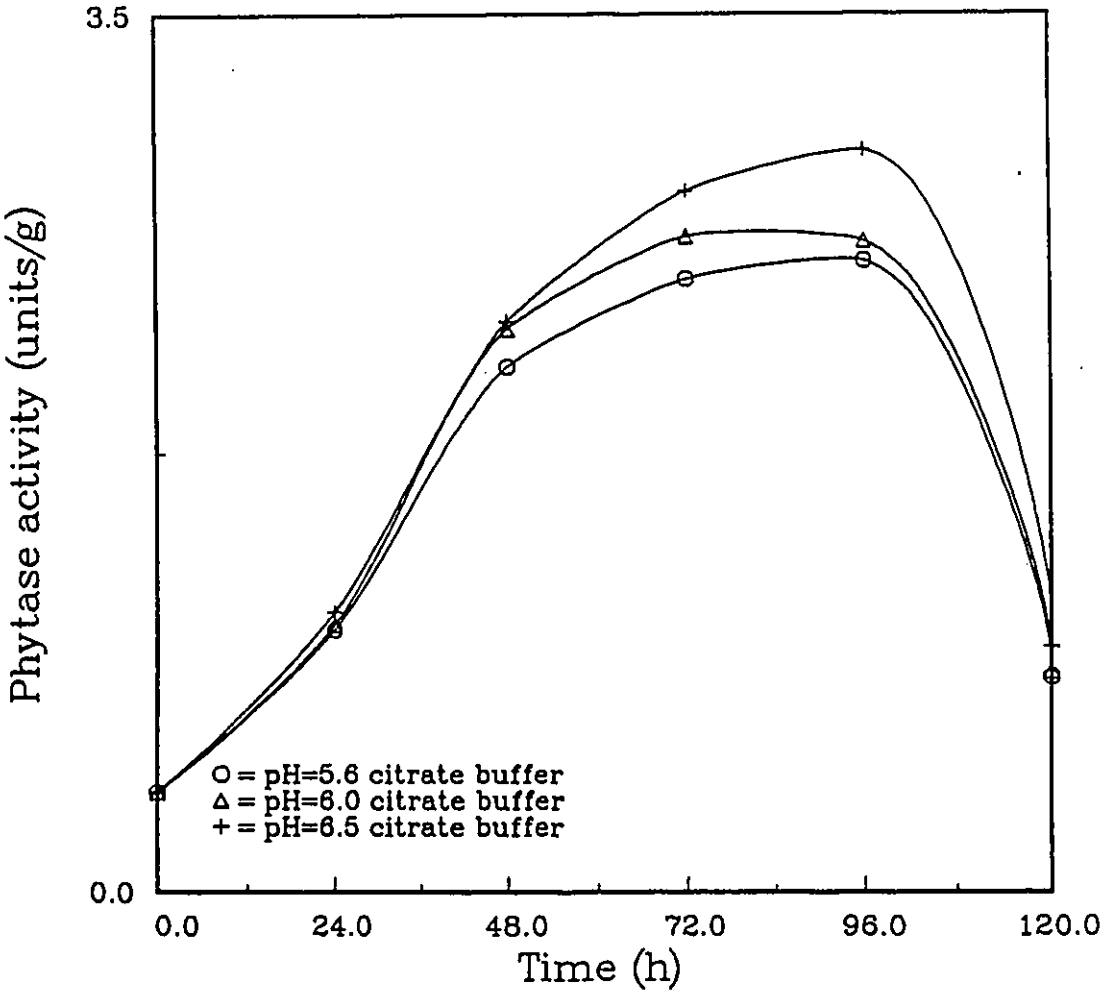


Figure 5.27: Effect of initial pH of citrate buffer on phytase activity during SSF process

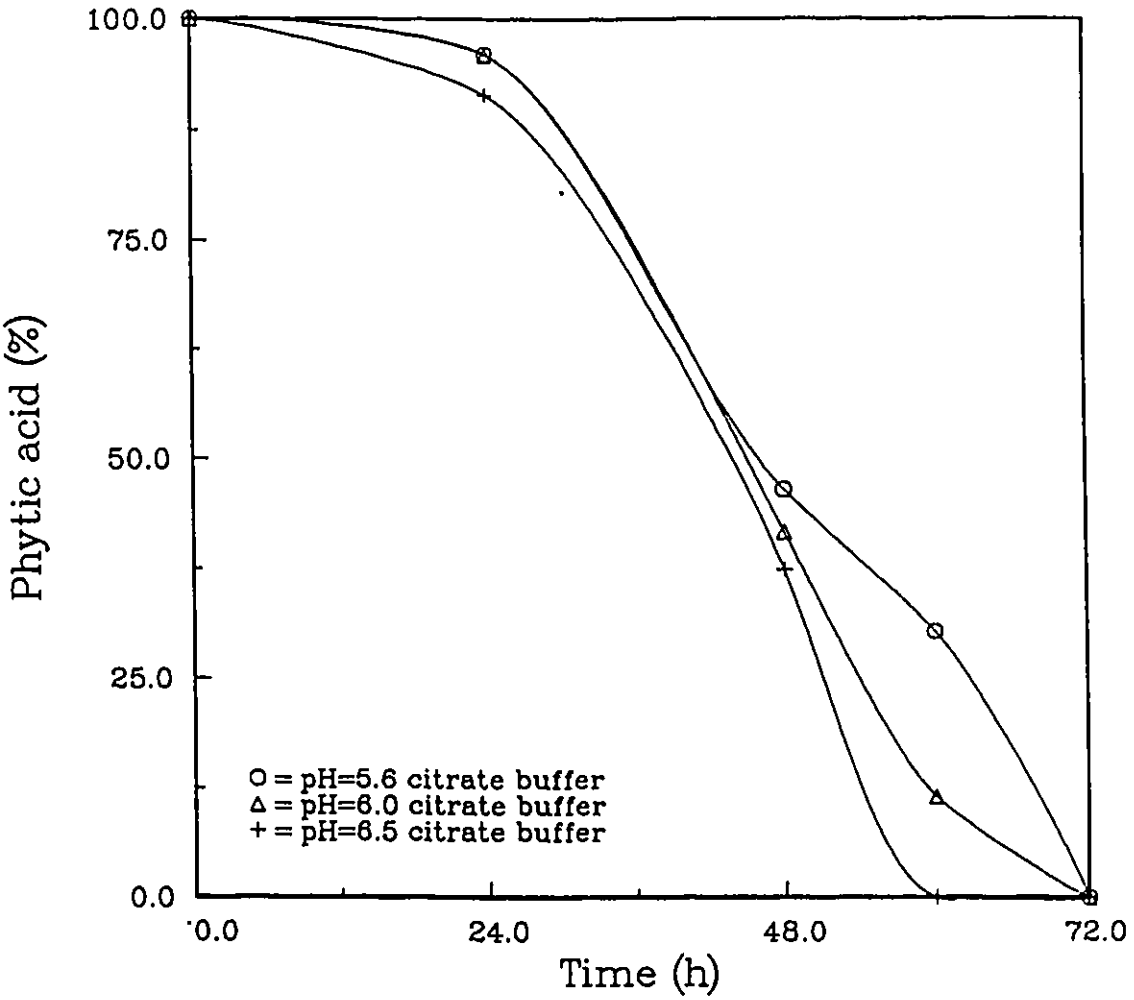


Figure 5.28: Effect of initial pH of citrate buffer on phytic content reduction during SSF process

5.5.1 Effect of Added Glucose

Since canola meal is very scarce in free carbohydrates, the purpose of this section is to study the effect of added glucose to canola meal on the biomass growth, phytase production and the reduction of phytic acid content in the meal during SSF process. Mathematical models were applied which related the glucose concentrations with the followed parameters.

Media were prepared for SSF process each having a concentration of glucose in the range of 0-24 g per system.

The effect of added glucose to canola meal systems on biomass growth is shown in Fig. 5.29. Compared with the control which was not supplemented with this component, a faster growth and larger amount of biomass were observed in the first 120 h of the process in the systems containing added glucose when its amount did not exceed 6 g per system. The systems containing 12 g and 24 g of added glucose showed slower biomass growth than the control.

It is well known that the difference between submerged and solid cultures is that in submerged culture no microbial growth appear with large concentration of glucose, like these concentrations used in solid state culture.

The effect of added glucose on enzyme production showed a trend similar to that noticed for the biomass growth (Fig. 5.30). Increase in the amount of supplemented glucose up to and including 6 g increased the rate of the enzyme production, while the systems with 12 and 24 g of glucose resulted in lower rate

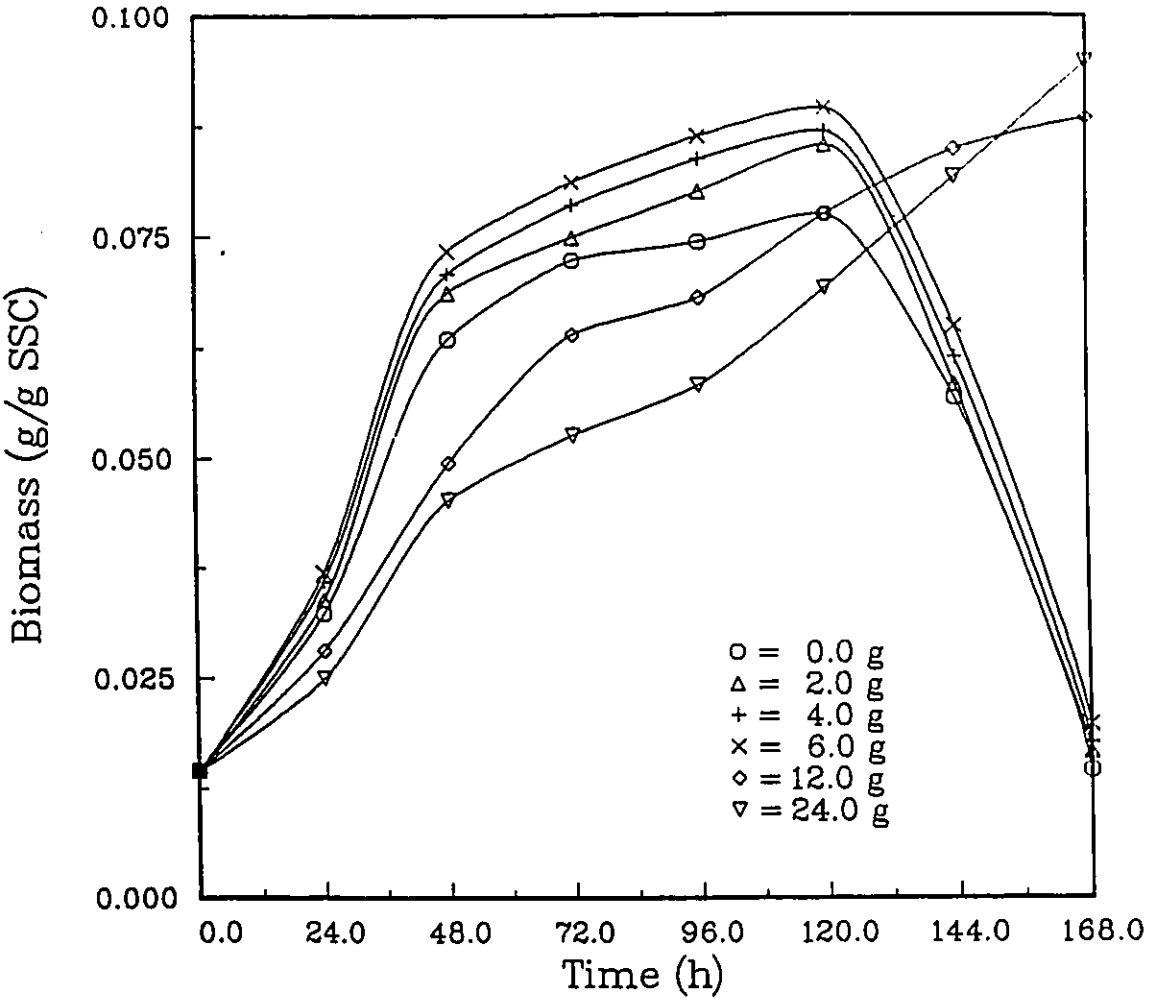


Figure 5.29: Effect of added glucose to canola meal systems on the biomass growth during the SSF process

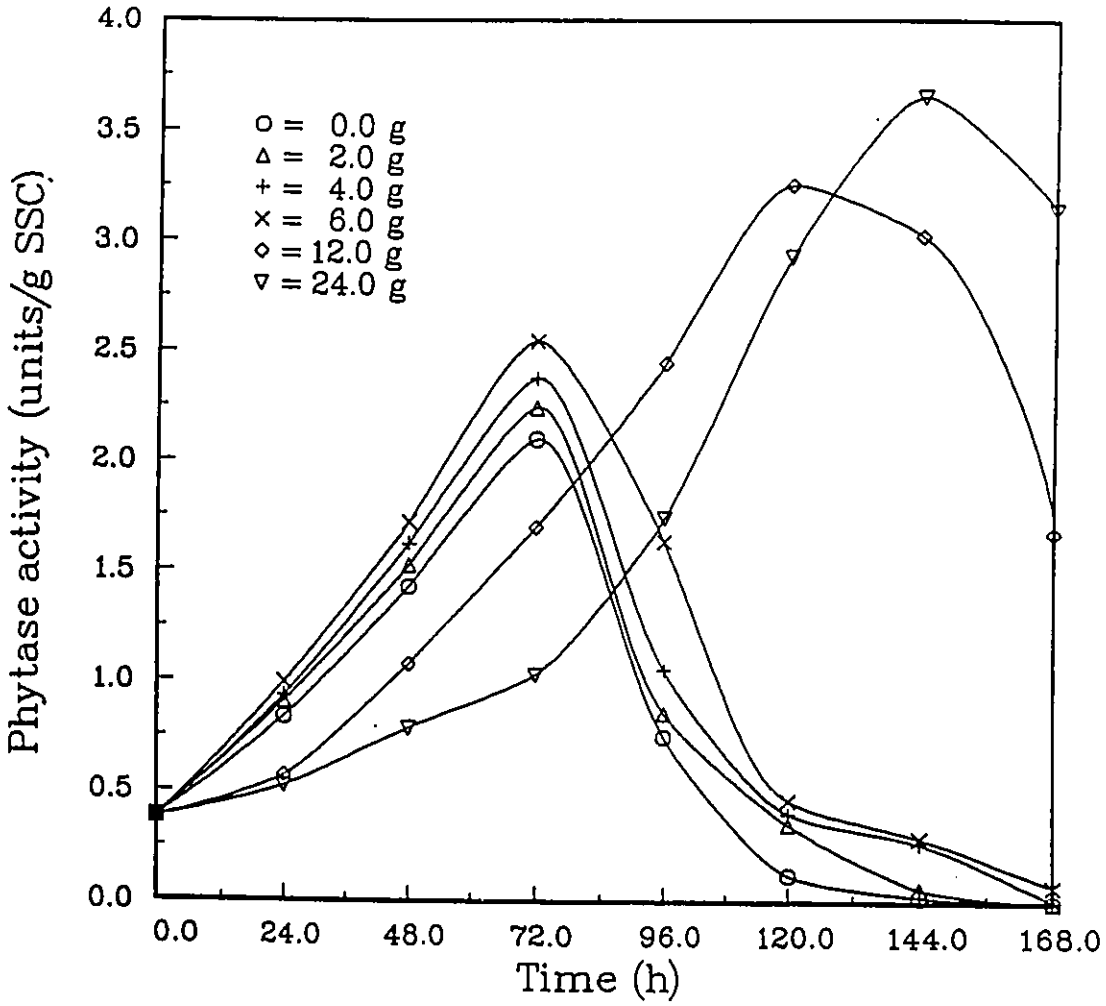


Figure 5.30: Effect of added glucose to canola meal systems on the production of phytase during the SSF process

of the enzyme production than in the control system.

The maximum enzyme activity was attained in 72 hours of the process in the systems containing 6 g (or less) of glucose per system (Fig. 5.30); after that time, it started decreasing, although the biomass continued growing for a while. The decrease in the enzyme activity may be due to the production of other enzymes which hydrolyzed phytase.

The biomass concentrations in these systems started decreasing after 120 h (Fig. 5.29), while those in the systems containing 12 g and 24 g glucose were still in the increasing phase at that time and in 160 h of growth were the same or little higher than in the system with 6 g of glucose respectively.

Longer growth phases of the last two systems resulted in the extended periods of phytase production and higher maximum activities of the enzyme (Fig. 5.30).

Taking into account the increase of the production rates of the enzyme activities with the increase in the glucose concentration, it was possible to expect the rate of phytic acid content reduction also to be affected by the added glucose. The system with 6 g of added glucose had the highest rate of the phytic acid reduction, and the phytic acid content in the meal was reduced to zero in 48 h, while the systems with 12 g and 24 g of added glucose even after 72 h still had 5.6% and 36% of the initial phytic acid content, respectively (Fig. 5.31); this observation is consistents with the trend of enzyme activity during the SSF process.

To model these processes, the values of the parameters X_m and μ_m for different glucose concentrations were calculated (Table 5.4) using Eq. 3.5 and applying

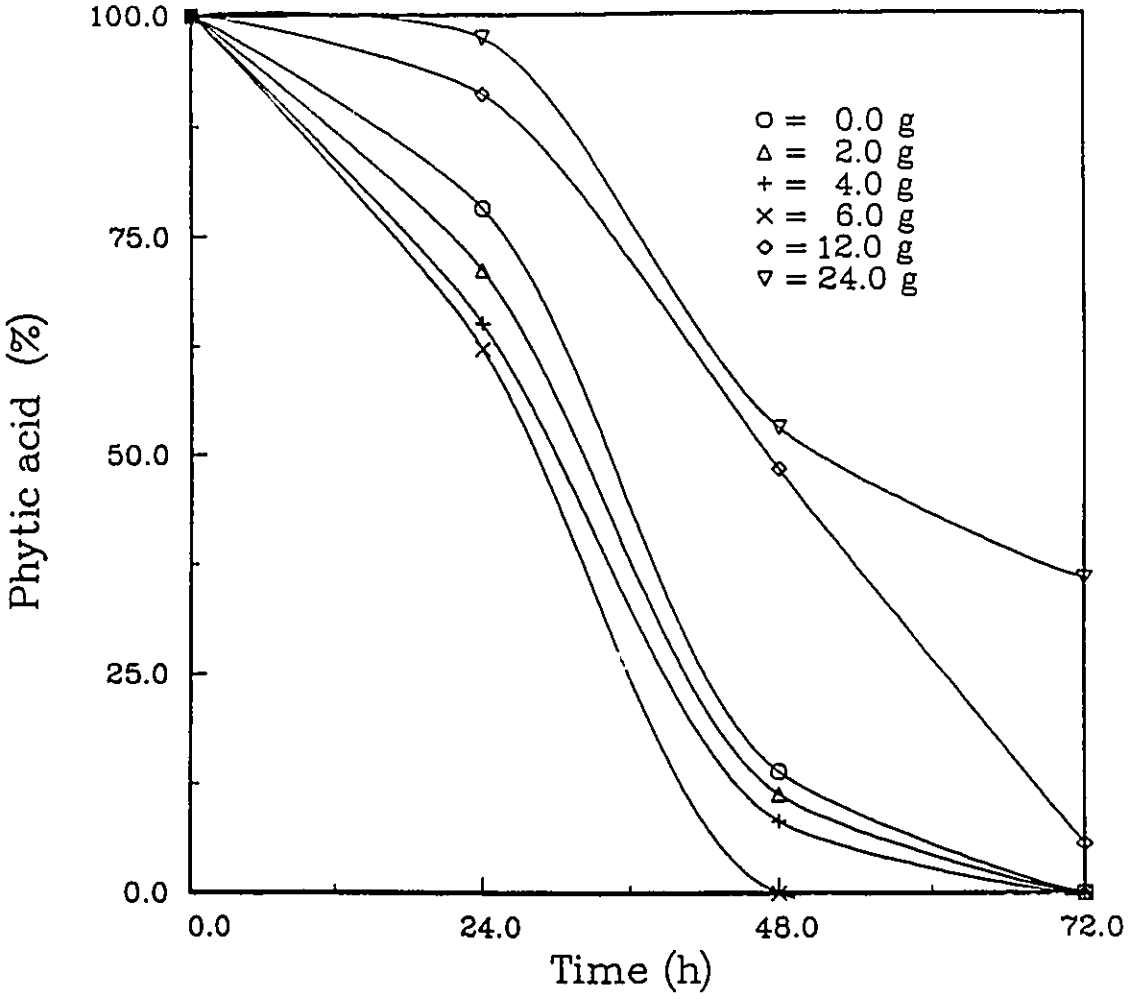


Figure 5.31: Effect of added glucose to canola meal systems on their phytic acid content reduction during the SSF process

the least square technique (Marquardt algorithms) for the set of experimental data for each glucose concentration. The correlation between X_m and μ_m with glucose

Table 5.4: Maximum biomass concentrations and maximum biomass specific growth rates for various glucose concentration.

Glucose concentartion, C_g (g)	Maximum biomass concentrations, X_m (g/gSSC)	Maximum biomass specific growth rate, μ_m (h^{-1})
0.0	0.0780	0.0560
2.0	0.0836	0.0575
4.0	0.0862	0.0595
6.0	0.0887	0.0610
12.0	0.0777	0.0410
24.0	0.0705	0.0350

concentration, C_g, can be expressed by the following fourth order polynomials:

$$X_m = 10^{-3}(78.116 + 2.65C_g - 0.0137C_g^2 - 0.0304C_g^3 - 0.00107C_g^4) \quad (5.1)$$

$$\mu_m = 0.056 - 3.57 \times 10^{-4}C_g + 6.55 \times 10^{-4}C_g^2 - 9.34 \times 10^{-5}C_g^3 + 2.71 \times 10^{-6}C_g^4 \quad (5.2)$$

Fig. 5.32 shows that the logistic law expression (Eq. 3.5) represented very well the experimental biomass data for the increasing part of the curve of its production.

To use the model (Eq. 3.10) for the calculation of the enzyme activity during the SSF process, the k_v constant for each glucose concentration was calculated (Table 5.5) using experimental data from this work, and the X_m and μ_m values

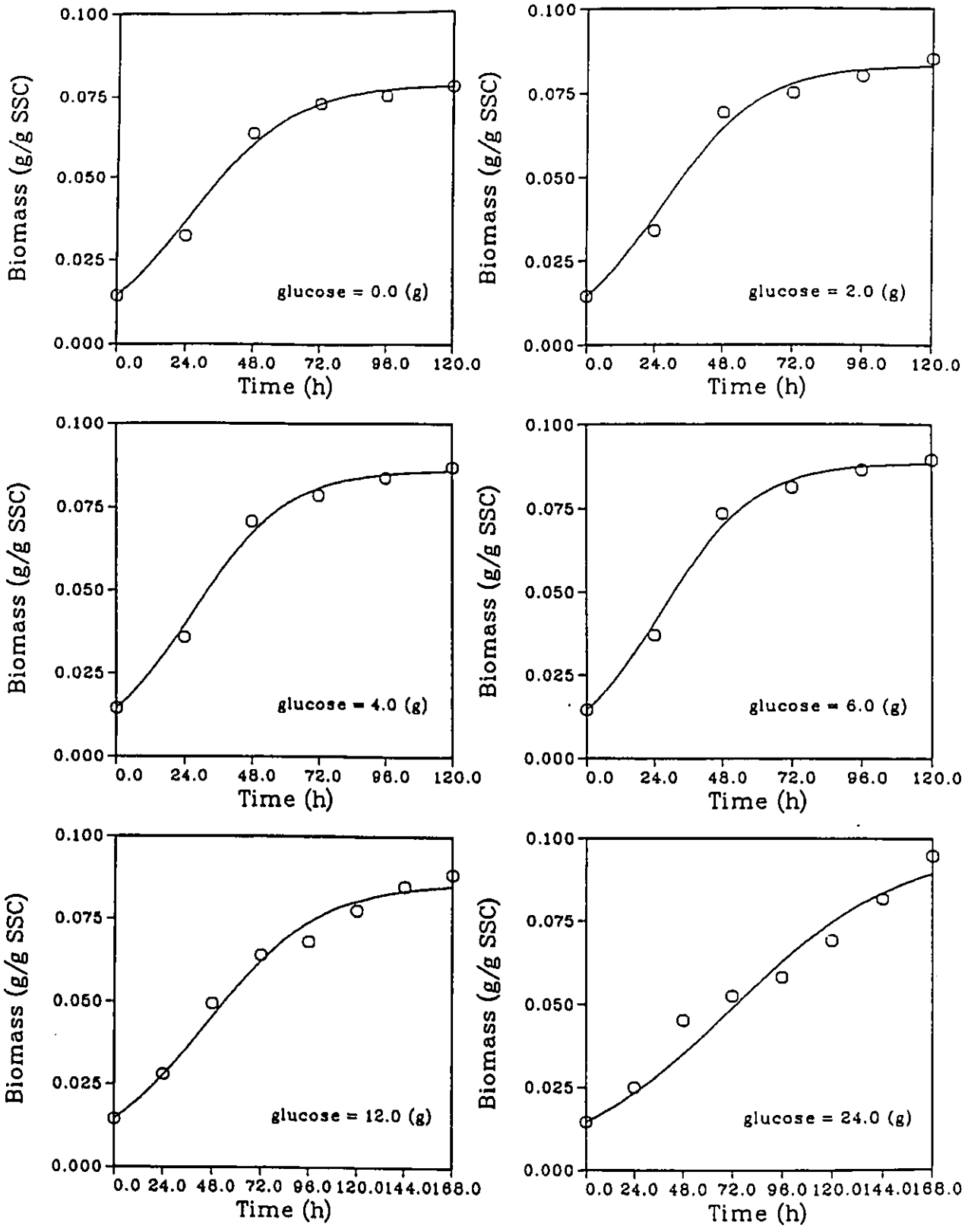


Figure 5.32: Correlation between the experimental biomass data (symbols) and predicted using Eq. 3.5 (lines) for the systems with various glucose concentrations

from the logistic law. The values of this constant can also be correlated with

Table 5.5: Rate constant, k_v, for various glucose concentrations for the model (Eq. 3.10) representing the increasing phase of phytase production.

Glucose concentration, C_g (g)	Rate constant, k_v (units/g.h)
0.0	0.5309
2.0	0.5468
4.0	0.5651
6.0	0.5938
12.0	0.4633
24.0	0.4293

the glucose concentrations by the following fourth order polynomial:

$$k_v = 0.5321 - 4.61 \times 10^{-3} C_g + 6.48 \times 10^{-3} C_g^2 - 8.17 \times 10^{-4} C_g^3 + 2.28 \times 10^{-4} C_g^4 \quad (5.3)$$

The empirical constants a and b for the empirical model (Eq.3.11) for the increasing part of the phytase formation curve were also calculated (Table 5.6) for various glucose concentration.

The constant a was increasing with increasing glucose concentration up to and including 6 g glucose, and then it was decreasing with further increase in glucose concentration; there was no specific trend for the constant b with glucose concentration.

Table 5.6: Emperical constants a and b for the empirical model (Eq. 3.11) representing the increasing phase of enzyme production for various glucose concentrations.

Glucose concentration, C_g (g)	Constant a (units/g)	Constant b (h^{-1})
0.0	0.414	0.1000
2.0	0.487	0.0800
4.0	0.522	0.0805
6.0	0.571	0.0777
12.0	0.181	0.2966
24.0	0.147	0.1324

The constants, k_d (Table 5.7), for the decay of enzyme activity after t_{max}, were calculated by fitting Eq. 3.12 to the experimental data for different glucose concentrations.

The constant k_d was decreased with increasing glucose concentration.

Table 5.7: Empirical constant k_d for various glucose concentration for the empirical model (Eq. 3.12) representing the declining phase of the enzyme production.

Glucose concentration, C_g (g)	Constant k_d (day^{-1})
0.0	1.1357
2.0	0.9728
4.0	0.8333
6.0	0.6636
12.0	0.2450
24.0	0.1500

Comparison between the experimental data for the enzyme production and predicted values using Eq. 3.10 and Eq. 3.11 (for the increasing phase of the enzyme production), and Eq. 3.12 (for the decreasing phase of the enzyme production) depicted in Fig. 5.33 for various glucose amounts shows that all these suggested models fit the experimental data reasonably well. Fig. 5.33 also demonstrates that the empirical model which is used to represent the increasing phase of the enzyme production, is slightly better than the model derived from the logistic law.

To use the model for the phytic acid content reduction, the values of the rate constant,k_p, were calculated (Table 5.8) from Eq. 3.17 for different glucose concentration using the values of X_m and μ_m obtained previously from the biomass data for each glucose concentration. The highest value of the constant was

Table 5.8: Rate constant, k_p, for various glucose concentrations for the model (Eq. 3.17) representing the reduction of phytic acid content.

Glucose concentration, C_g (g)	Rate constant, k_p (h^{-1})
0.0	22.284
2.0	23.630
4.0	26.085
6.0	29.423
12.0	16.008
24.0	12.561

obtained for the system with 6 g glucose. The k_p values can be correlated with

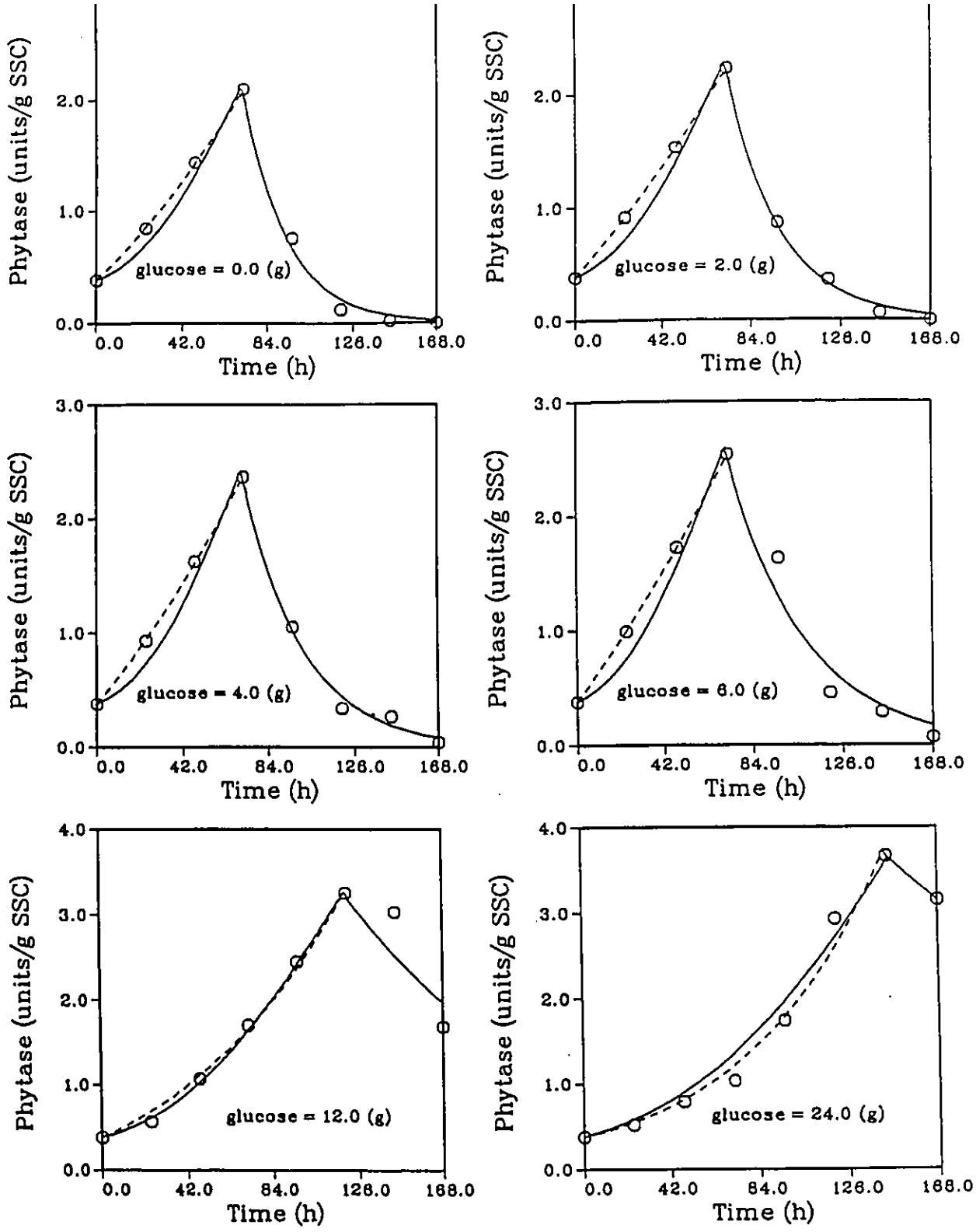


Figure 5.33: Correlation between the experimental data for the production of phytase (symbols) and predicted using Eq.3.10 (solid lines, for increasing phase), Eq. 3.11 (interepted lines, for increasing phase) and Eq. 3.12 (solid lines, for decreasing phase) for the systems with various glucose amounts

glucose concentration by the following fourth order polynomial:

$$k_p = 22.378 - 0.760C_g + 0.784C_g^2 - 0.0935C_g^3 + 0.00257C_g^4 \qquad (5.4)$$

A comparison between experimental data for the reduction of phytic acid content during SSF process, and the calculated phytic acid concentrations using Eq. 3.17 is shown in Fig. 5.34 for various glucose concentrations.The results show that the model is very good for the systems which do not contain more than 6 g of glucose. For the systems with higher glucose amounts, the model gives the values which are approximations of the experimental results.

5.5.2 Effect of Added Phosphate

A certain amount of phosphate in the growth medium is required for the production of phosphatase. In general, a high level of inorganic phosphate inhibits the synthesis of phosphatase; the type and amount of phosphatase synthesized are dependent on the concentration of the phosphate presented in the growth medium. Therefore, the optimum amount of phosphate in the growth medium should be carefully determined (Shieh et al., 1968). Han and Gallagher (1986) observed that for high level of phosphatase production low levels (1-5%) of initial phosphorus were necessary, and polyphosphate was the desired form rather than the monophosphate.

To study the effect of added phosphate to canola meal on the biomass growth, phytase production and reduction of the phytic acid content, phosphate (added

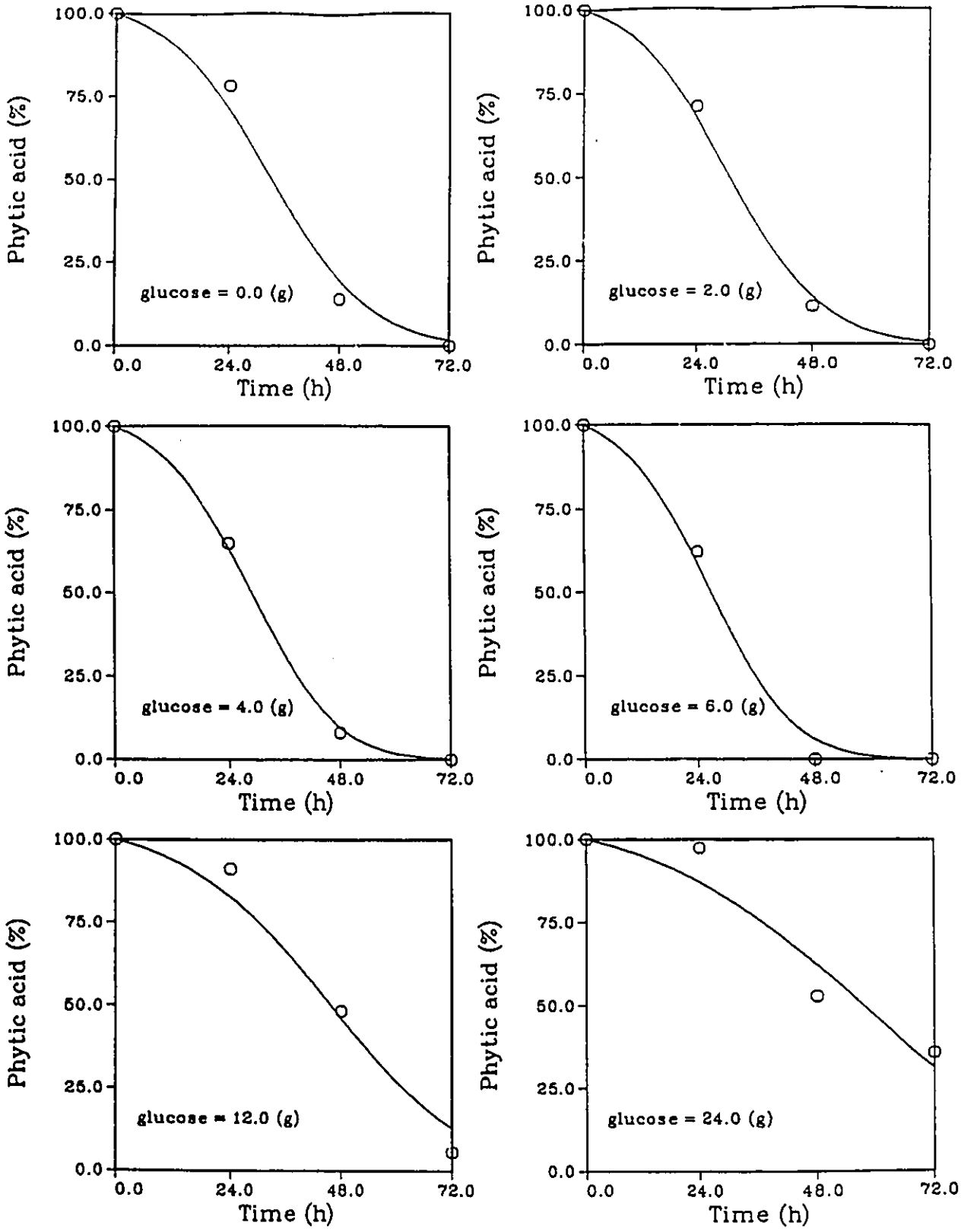


Figure 5.34: Correlation between the experimental data for the phytic acid content reduction (symbols) and predicted using Eq. 3.17 (lines) for the systems with various amounts of glucose

in the form of potassium dihydrogen phosphate) in the range 1-100 mg per system of inoculated solid medium was added to it. The results showed (Fig. 5.35) that a better growth was observed in the systems containing 1 mg and 5 mg of added phosphate than in the control which was not supplemented with it. On the other hand, the systems with 50 mg and 100 mg of added phosphate showed slower biomass growth and less biomass was produced than in the control, while the system with 10 mg of phosphate was comparable to the control.

The effect of added phosphate on enzyme production showed a trend similar to that noticed for the biomass (Fig. 5.36). The amount of phosphate of 1 mg and 5 mg improved enzyme production; while apparent inhibition is seen in the systems containing 50 mg and 100 mg of added phosphate respectively. The effect of phosphate trend was noticed by Han and Gallagher (1987) when they studied the phytase production by *Aspergillus ficuum* on a solid substrate using soybean. They found that *Aspergillus ficuum* required more than 8 mg/100 ml of phosphate for optimum cell growth, whereas 1-5 mg/100 ml of phosphate was needed for maximum phytase production. The presence of more than 10 mg/100 ml of phosphate in the growth medium severely repressed the phytase synthesis.

The addition of phosphate in the solid culture affected the rate of phytic acid content reduction as well Fig. 5.37. The systems supplemented with 1 mg and 5 mg of phosphate increased the rate of the phytic acid content reduction. The increase in the rate of phytic acid reduction is directly related to the biomass and enzyme productions in the systems.

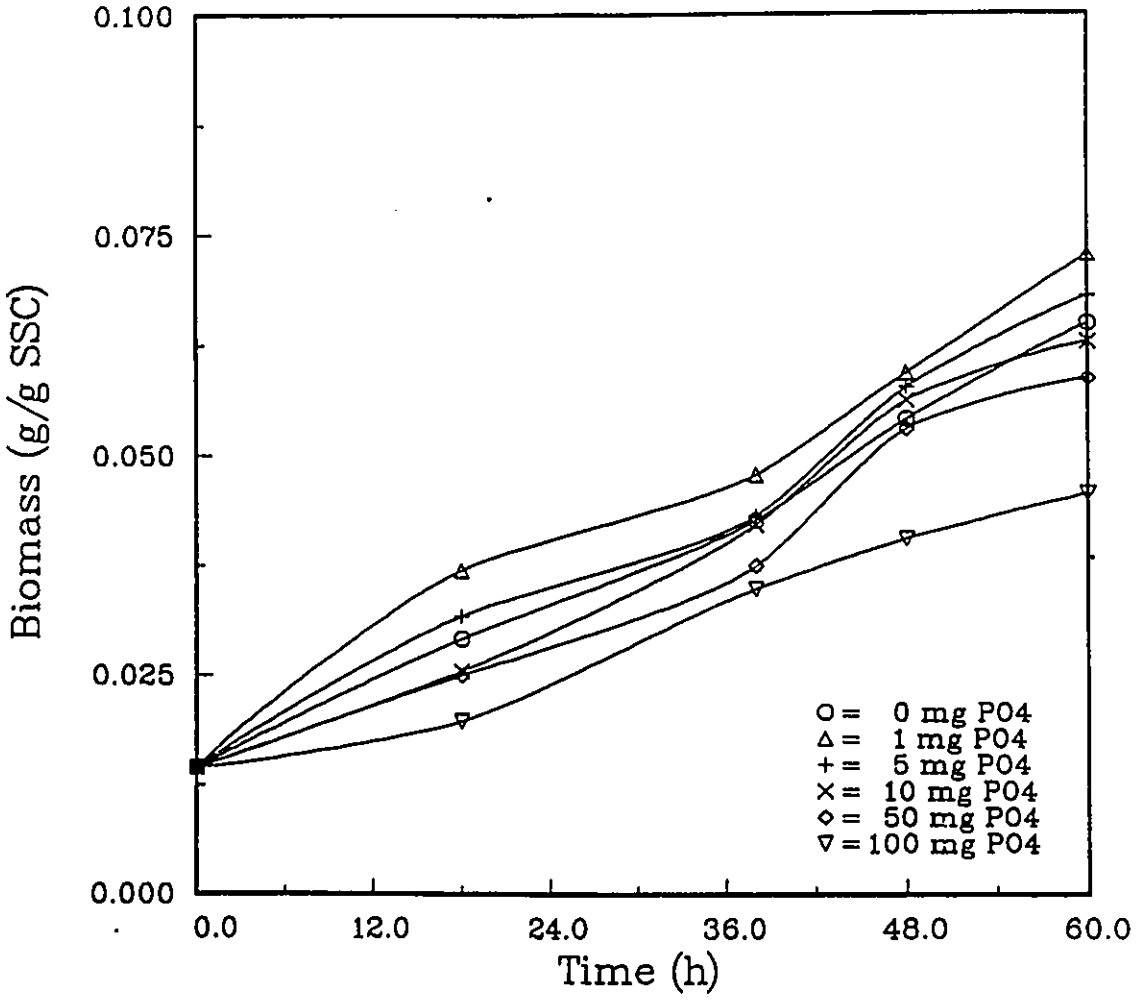


Figure 5.35: Effect of phosphate content on the biomass growth during the SSF process

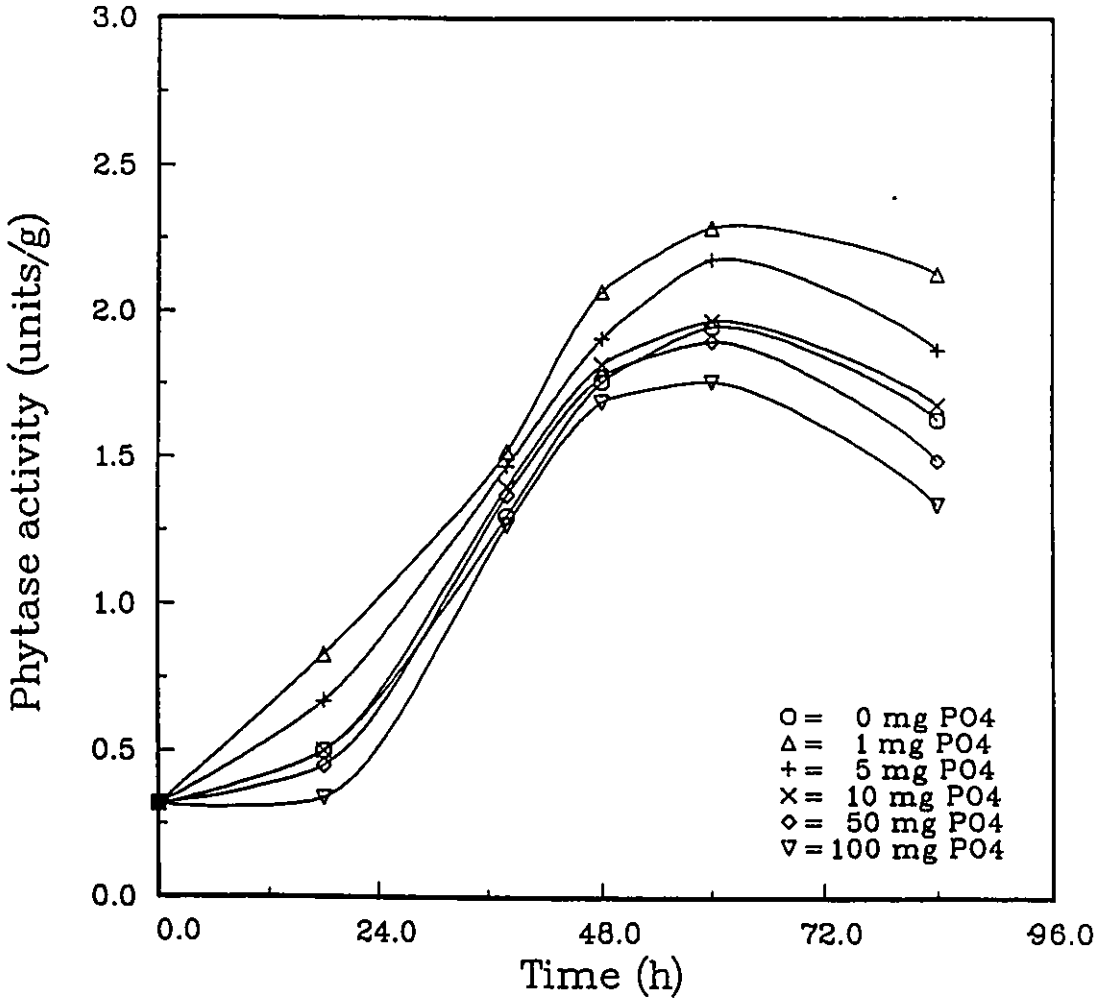


Figure 5.36: Effect of phosphate content on the phytase production during the SSF process

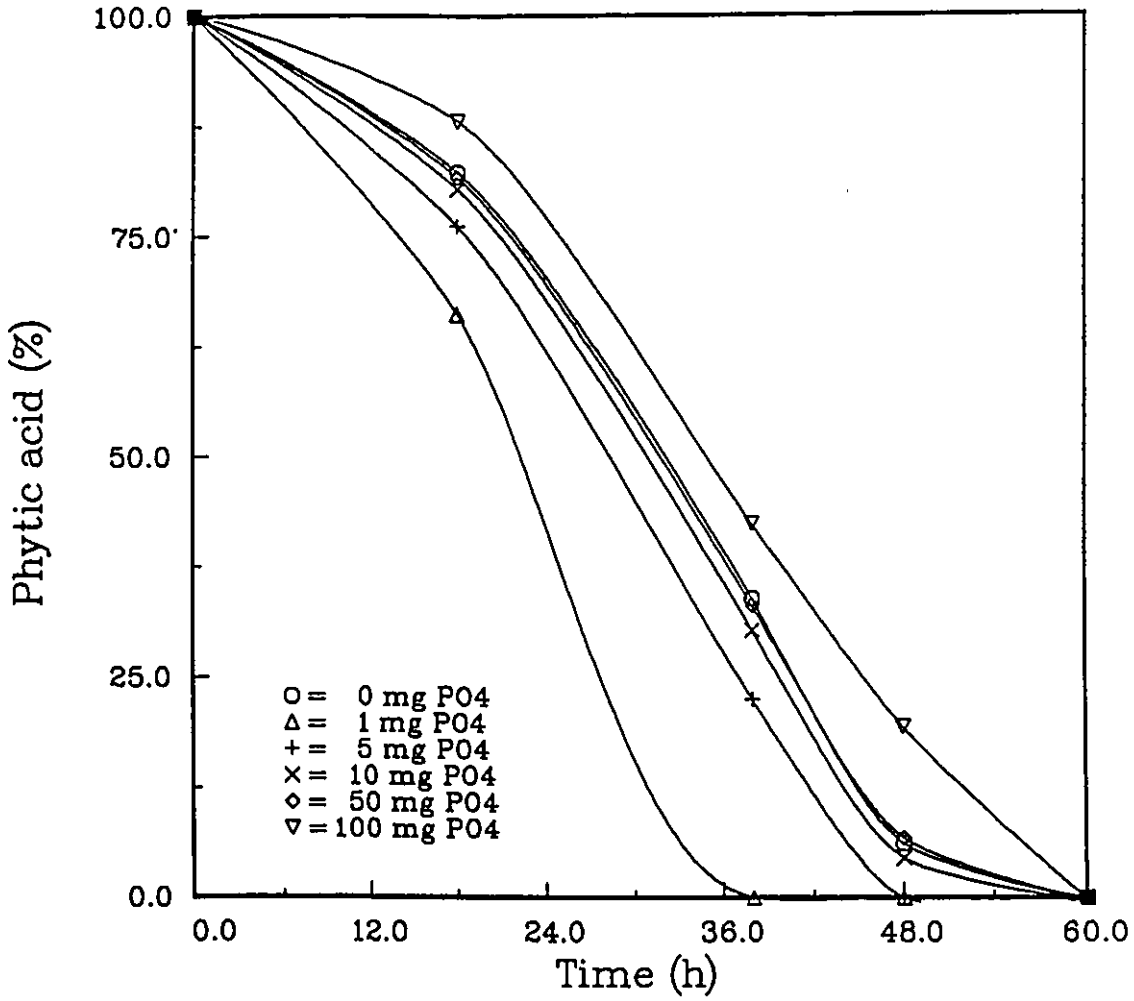


Figure 5.37: Effect of phosphate content on phytic acid content reduction during the SSF process

To model the productions of biomass and phytase, and the reduction of phytic acid content in canola meal supplemented with phosphate during solid state fermentation, the values of the parameters X_m and μ_m for different concentrations of phosphate were calculated from Eq. 3.5 using the method of least squares (Marquardt algorithms) by fitting the equation to a set of data for each phosphate concentration (Table 5.9).

It is seen from Table 5.9 that maximum rate of growth, corresponds to

Table 5.9: Maximum biomass concentrations and maximum specific growth rates for different phosphate concentrations

Phosphate concentration, C_p (mg)	Maximum biomass concentration, X_m (g/g SSC)	Maximum biomass specific growth rate, μ_m (h^{-1})
0.0	0.0929	0.0418
1.0	0.0863	0.0518
5.0	0.0943	0.0440
10.0	0.0923	0.0415
50.0	0.0974	0.0368
100.0	0.07266	0.0327

the phosphate concentration of 1 mg, and this is consistent with the experimental results. The comparison of the biomass concentrations predicted by Eq. 3.5 and the experimental biomass results for different phosphate concentrations is shown in Fig. 5.38; the presented results show good agreement between the experimental results and predicted data.

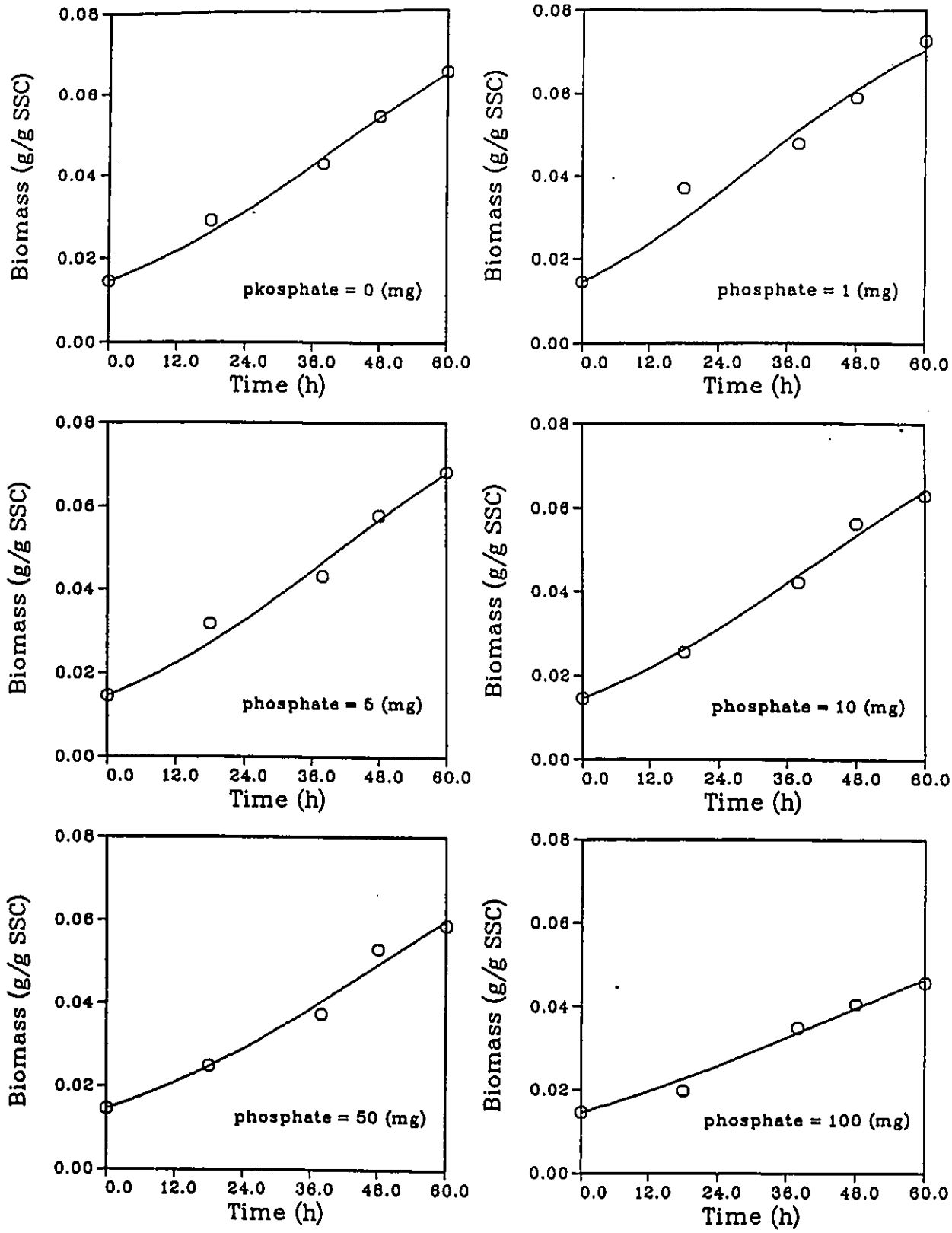


Figure 5.38: Comparison between predicted values of biomass (Eq.3.5) and the biomass produced in the SSF process for different phosphate concentrations

To use Eq. 5.10 to predict the enzyme concentration in the increasing phase of its production during solid state fermentation, the values of the parameter k_v for different phosphate concentrations had to be obtained. They were calculated by applying the previously obtained X_m and μ_m values for each phosphate concentration in Eq. 3.10 and fitting this equation to the experimental enzyme concentration data.

Generally, the maximum rate of increase in enzyme activity was acheived

Table 5.10: Rate constant, k_v, for various phosphate concentrations for the model (Eq. 3.10) representing the increase phase of phytase production

Phosphate concentration, C_p (mg)	Rate constant, k_v (units/g.h)
0.0	0.7127
1.0	0.8797
5.0	0.8771
10.0	0.8389
50.0	0.8607
100.0	0.7148

at the concentration of phosphate of 1.0 mg (Tables 5.10).

Comparison of the experimental and predicted values by Eq. 3.10 is shown in Fig. 5.39 for different concentrations of phosphate. Although a relatively good agreement between these sets of data was obtained, a correction of the equation should be done to have a better fit in the part of the production curve which

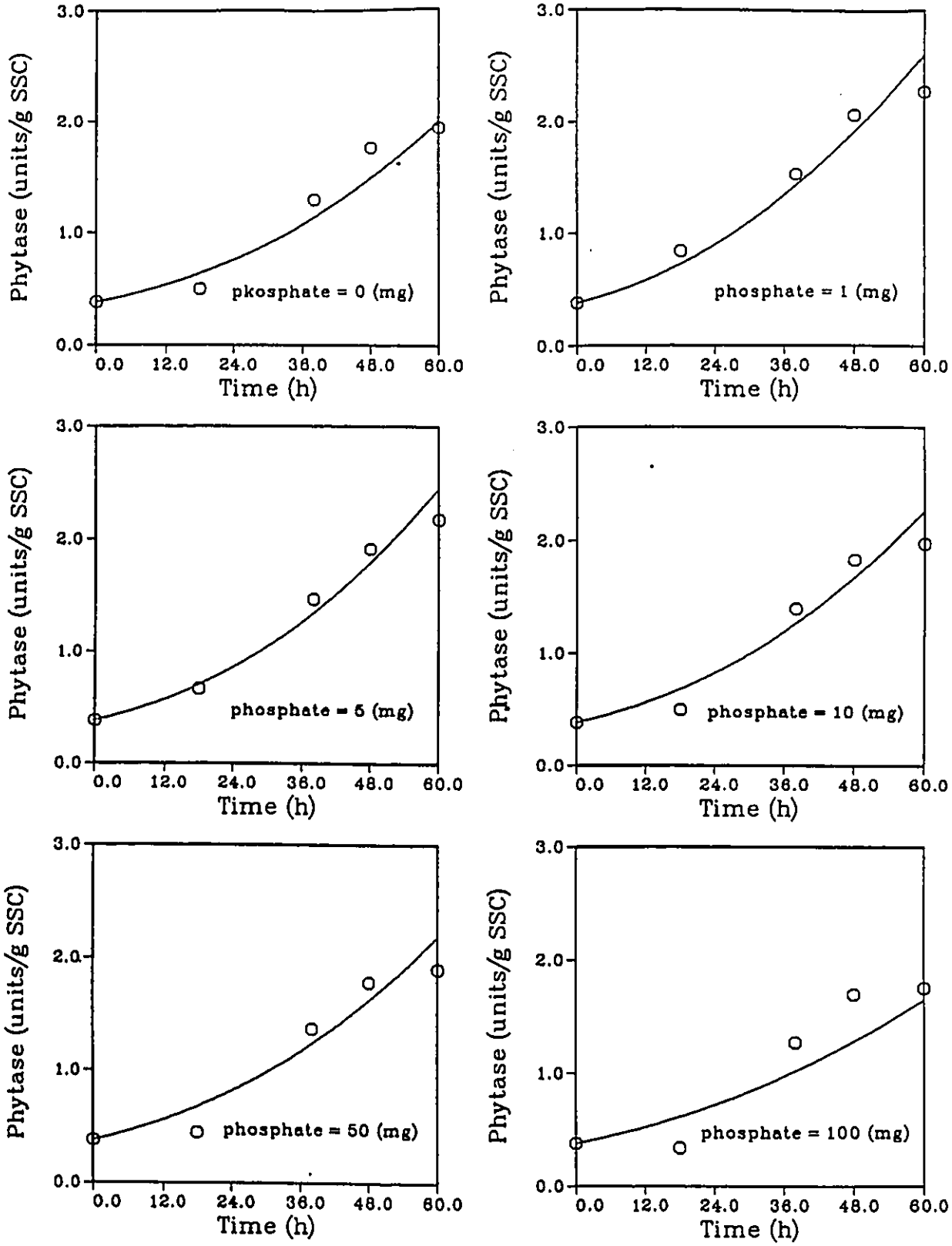


Figure 5.39: Comparison between predicted values of phytase activity (Eq.3.10 and 3.11) and the phytase activity produced in the SSF process for different phosphate concentrations

approaches its maximum and for phase lag for the systems with higher phosphate concentrations.

It was shown that the phytic acid content reduction in canola meal was also influenced by phosphate. For modelling of this effect, it was necessary to calculate the rate constants of these processes, k_p, from Eq. 3.17 for each phosphate concentration (Table 5.11).

In the evaluation of these constants, the X_m and μ_m values previously

Table 5.11: Rate constant, k_p, for various phosphate concentrations for the model (Eq. 3.17) representing the reduction of phytic acid content

Phosphate concentration, C_p (mg)	Rate constant, k_p (h^{-1})
0.0	35.7
1.0	59.2
5.0	43.2
10.0	39.1
50.0	41.2
100.0	41.4

obtained were used as well.

Using Eq. 3.17 the phytic acid concentration in canola meal was calculated for different times of fermentation and shown in Fig. 5.40 together with the experimental results. Inspecting the presented data, it can be seen that Eq. 3.17 fits the experimental data reasonably well.

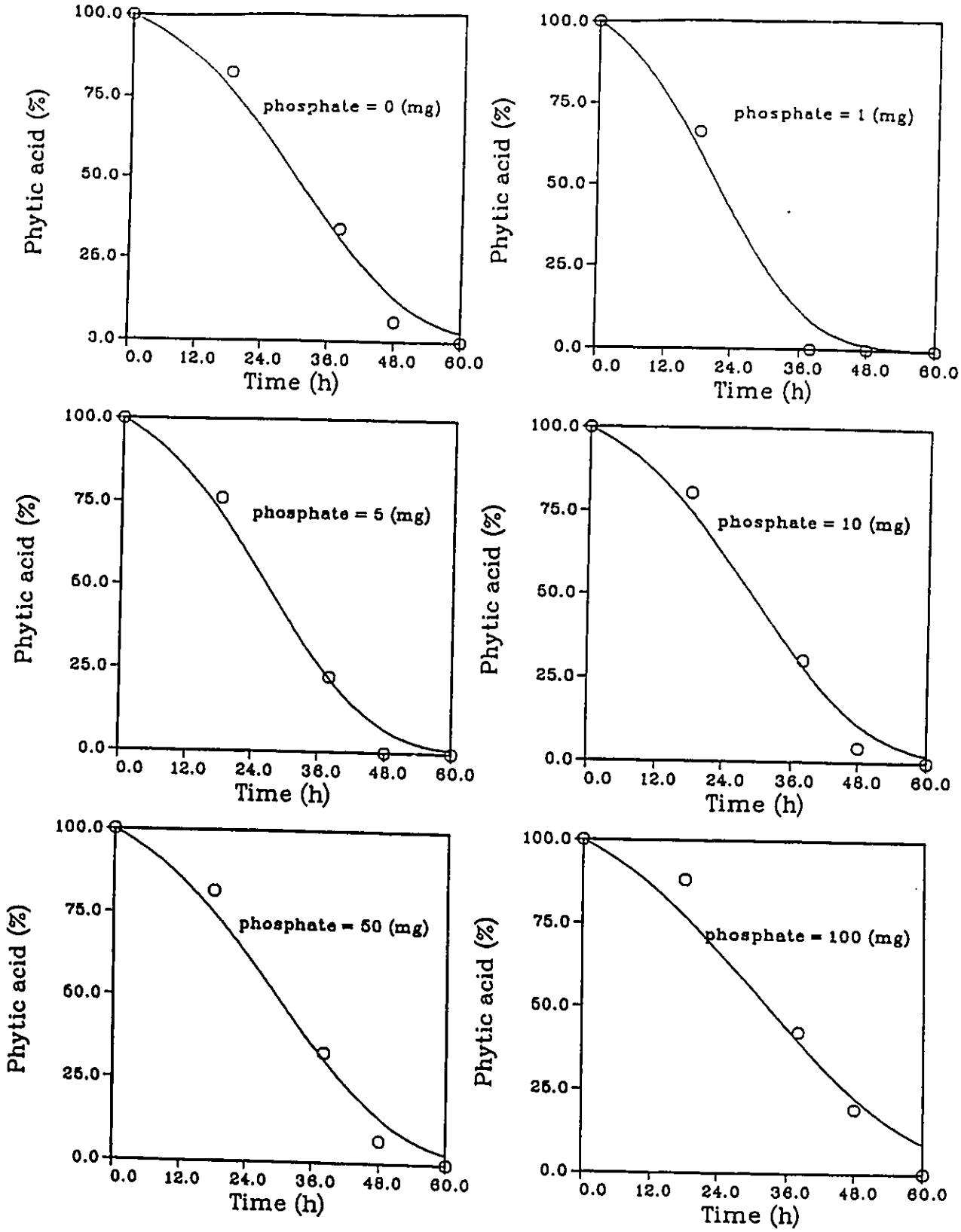


Figure 5.40: Comparison between predicted values of phytic acid content (Eq.3.17) and the phytic acid content determined experimentally in the SSF process for different phosphate concentrations

5.5.3 Effect of Surfactants

Various surfactants have been used in bacterial cultures to assist in cell growth and enzyme production. It is generally known that surfactants play a role in enzyme production and secretion, but the explanation of how the surfactants act to increase enzyme yield is largely conjectural (Rees and Maguire, 1969). Rees and Maguire (1969) investigated the production of nukleosidases by adding sucrose monopalmitate and Tween-80 to media containing the inducer and found that the enzyme production was increased as much as fivefold by the additives in some organisms, but was not affected in other organisms.

The results of Jager et al. (1985) showed that the addition of Tween-80 or Tween-20 permits development of ligninase activity of *Phanerochate chrysosprium* in agitated submerged culture; while in the absence of detergent, no ligninase activity was detected.

Amtual et al. (1988) studied the effect of surfactant concentration on the production of extracellular carboxymethyl cellulase (CMCase) and avicelase activities by a locally isolated *Cellulomonas* species identified as *Cellulomonas flavigena* in submerged culture and they found that Tween-80, when added to culture medium at a concentration of 0.1%, resulted in more than twofold increase in activities of both CMCase and avicelase in the culture supernatant. They explained that the surfactant increased the permeability of the bacterial cell wall, thus facilitated the release of the enzyme into the medium and hindered the immobilization of the

enzyme on the substrate by reducing the strength of adsorption.

In this study the effects of sodium oleate, Tween-80 and Triton x-100, which are separately added to the solid medium, on the biomass and phytase productions and phytic acid content reduction were examined. The solid medium was supplemented with 0.5% of the surfactant which was tested. The effect of surfactants on the biomass production is shown in Fig. 5.41. The systems with sodium oleate and Tween-80 produced more biomass comparing with the control, while Triton x-100 has negative effect on the biomass production. A trend similar to the biomass growth was also observed for the enzyme production. Maximum enzyme formation was noticed with the system containing sodium oleate, while the least enzyme was formed in the system with Triton x-100 (Fig. 5.42). Han and Gallagher (1987) also had a higher enzyme (the phytase of *Aspergillus ficuum*) level in the system containing 0.5% sodium oleate in the culture medium.

The enzyme levels in the canola meal cultures influenced the rate of phytic acid content reduction. The highest rate was observed in the system containing sodium oleate (Fig. 5.43) and in 36 h the phytic acid content was reduced to zero in that system. The other systems require 48 h for complete hydrolysis of phytic acid in canola meal.

Bearing in mind that the best results were obained with sodium oleate, the effect of concentration of this surfactant on the studied precesses was investigated.

The relatioship between sodium oleate concentration and the biomass production (Fig. 5.44) showed that the biomass had a profile response with an increase

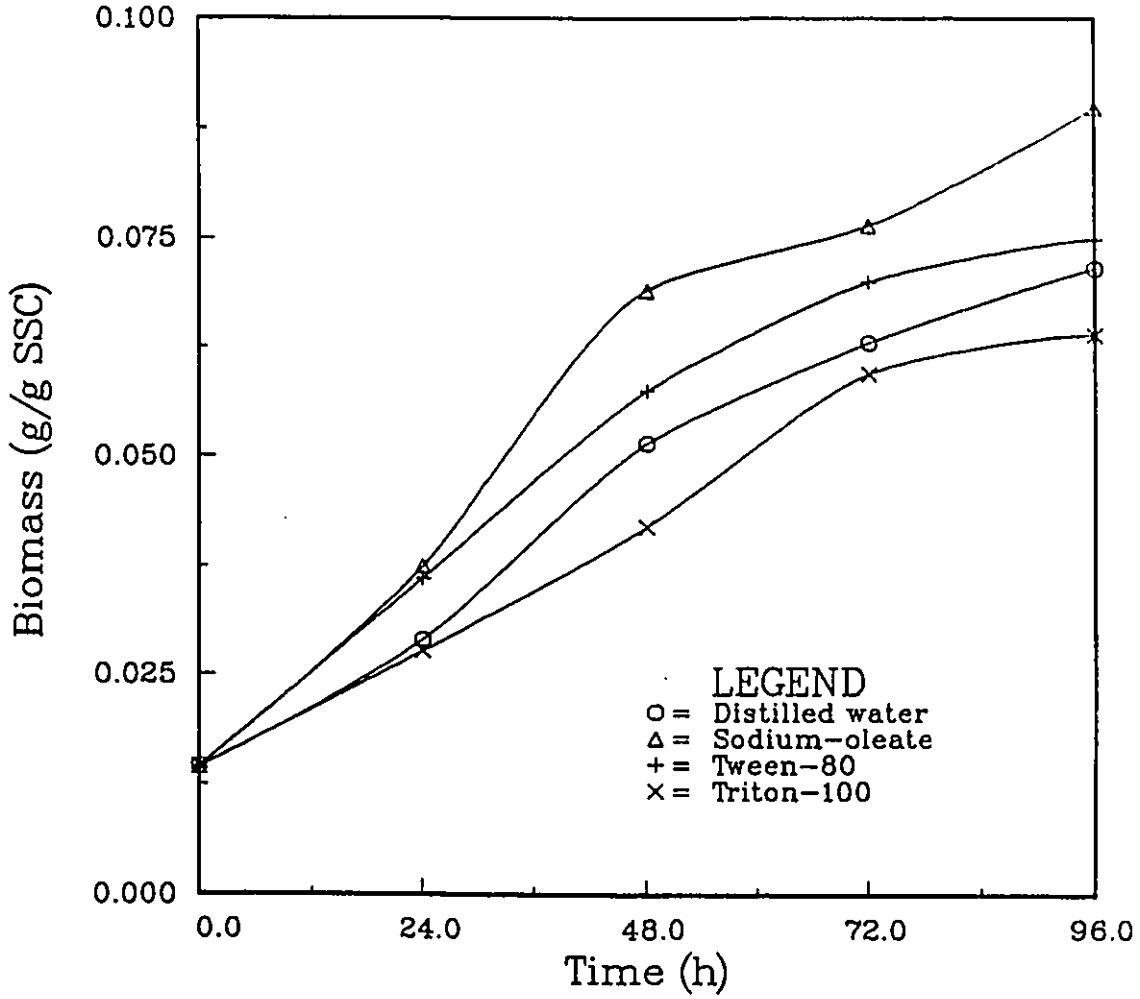


Figure 5.41: Effect of surfactants on the biomass growth during the SSF process

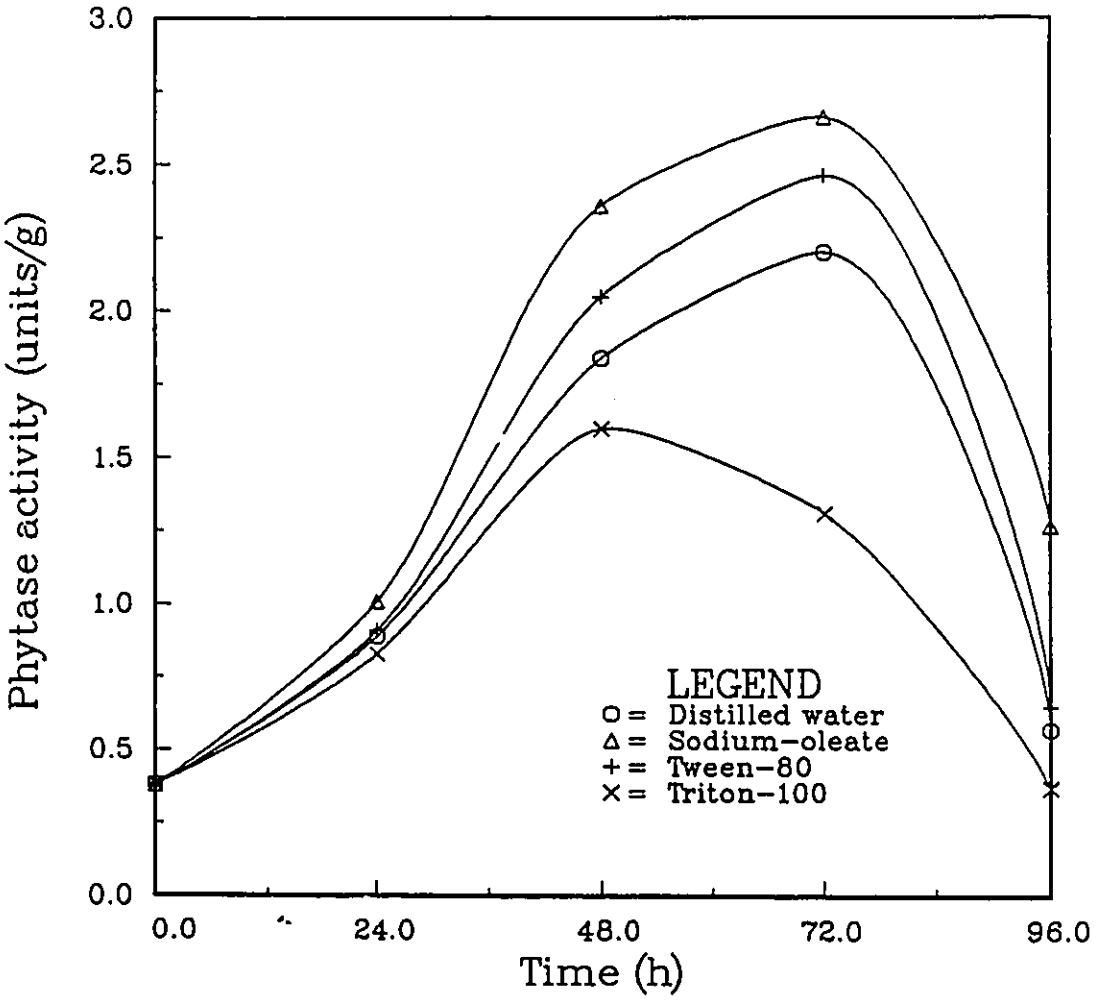


Figure 5.42: Effect of surfactants on the phytase production during the SSF process

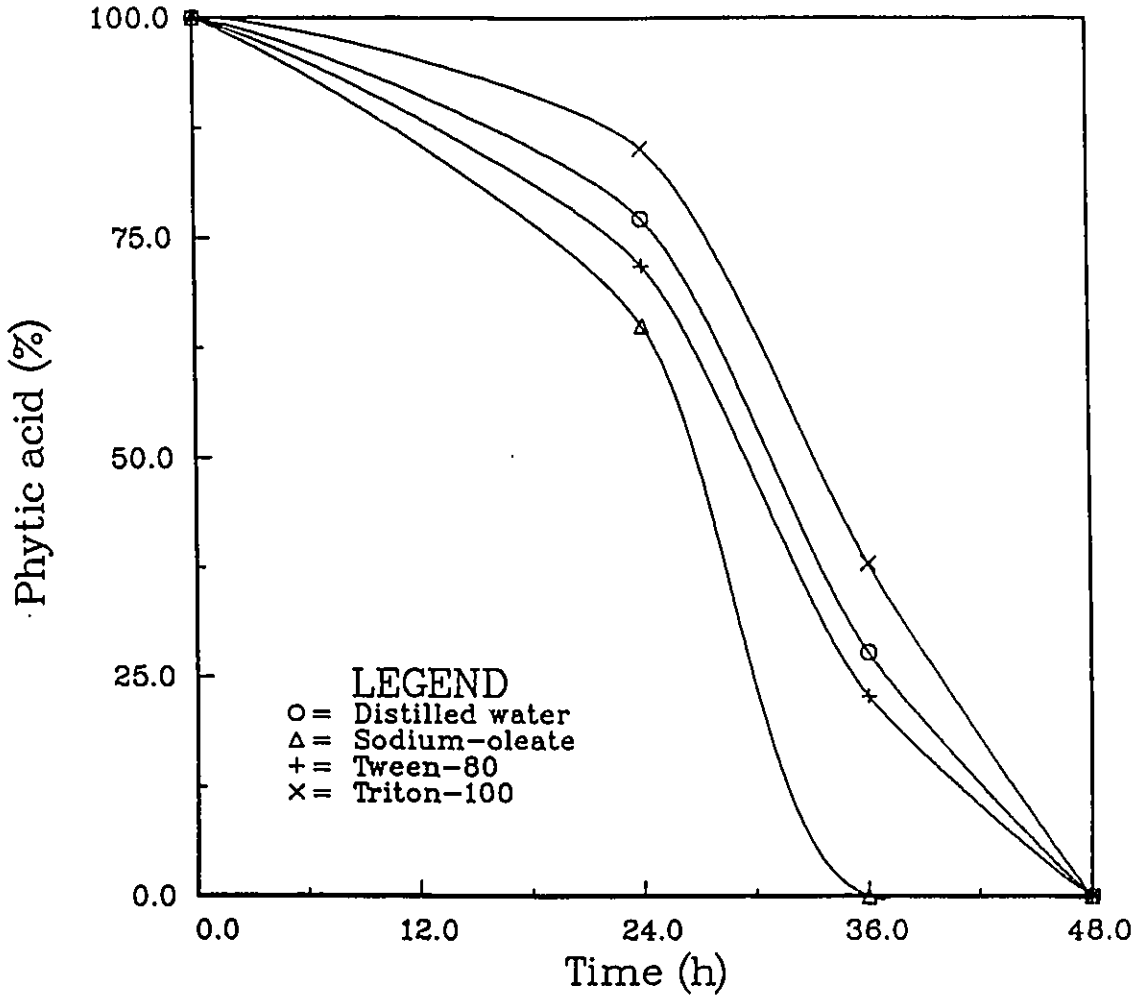


Figure 5.43: Effect of surfactants on phytic acid content reduction during the SSF process

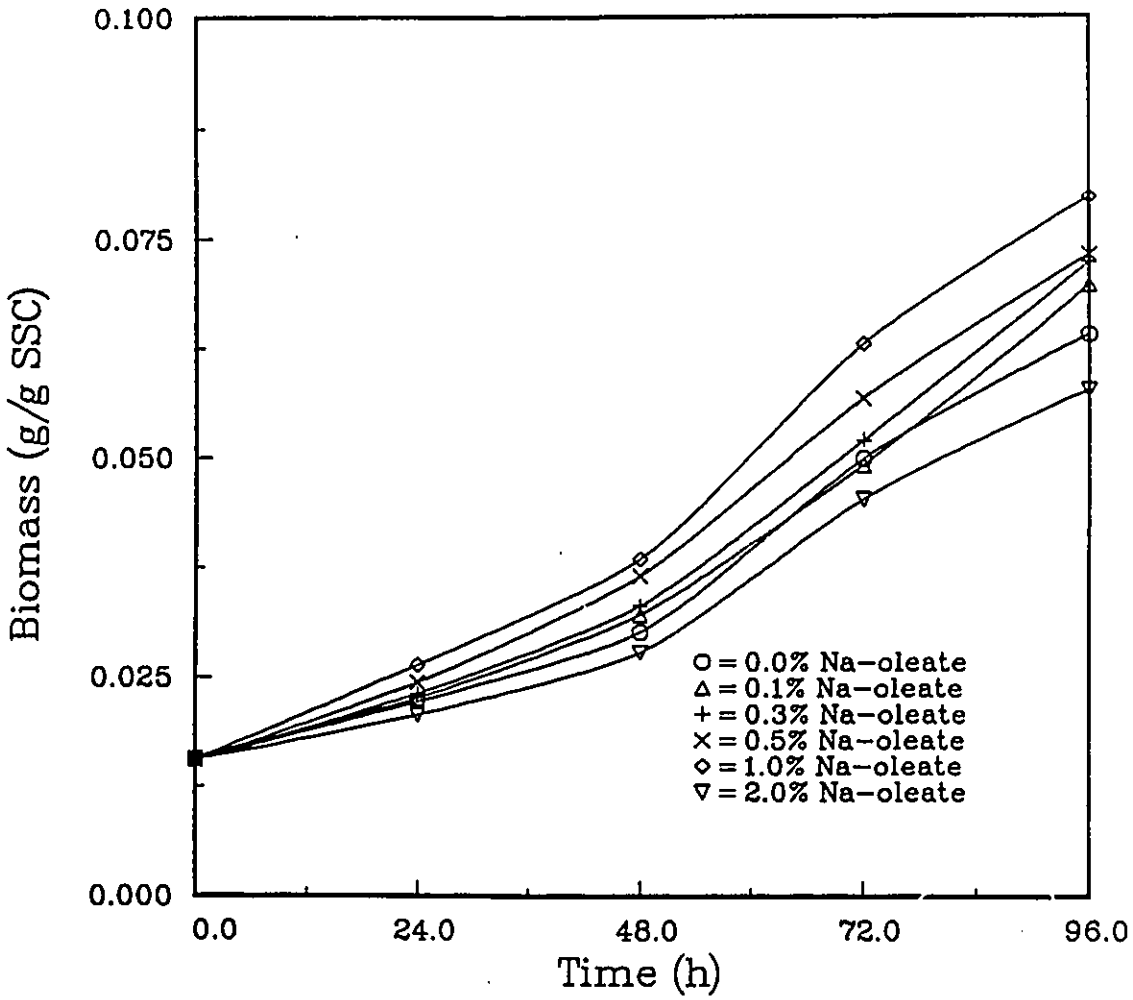


Figure 5.44: Effect of Na-oleate content on the biomass growth during the SSF process

in the surfactant concentration. But when its concentration was 2% the rate of biomass production and the amount of biomass produced were below the values obtained for the control.

The profile of enzyme activity (Fig. 5.45) was consistent with the result for biomass. In this case, 1% Na-oleate was also the optimum concentration at which the maximum enzyme activity was acheived. The medium with 2% Na-oleate produced about 30% less enzyme than that with 1% of the surfactant.

Phytic acid content reduction in the media supplemented with various amounts of Na-oleate is shown in Fig 5.46 Phytic acid had been completely eliminated after 36 h of fermentation in the systems containing 0.5% and 1% Na-oleate, while the control and the systems containing 0.1% and 0.3% Na-oleate required 48 h for it. It is seen in Fig. 5.46 that the optimum Na-oleate concentration (1%) exhibited the highest rate of phytic acid content reduction, while the slowest rate is observed in the system containing 2% Na-oleate; it required 72 h to reduce phytic acid content to zero.

To model the productions of biomass and phytase, and the reduction of phytic acid content in canola meal supplemented with Na-oleate during solid state fermentation, the values of the parameters X_m and μ_m for different concentrations of Na-oleate were calculated from Eq. 3.5 using the method of least squares (Marquardt algorithms) by fitting the equation to a set of data for each Na-oleate concentration (Table 5.12).

The results from Table 5.12 show that maximum rate of growth, corresponds

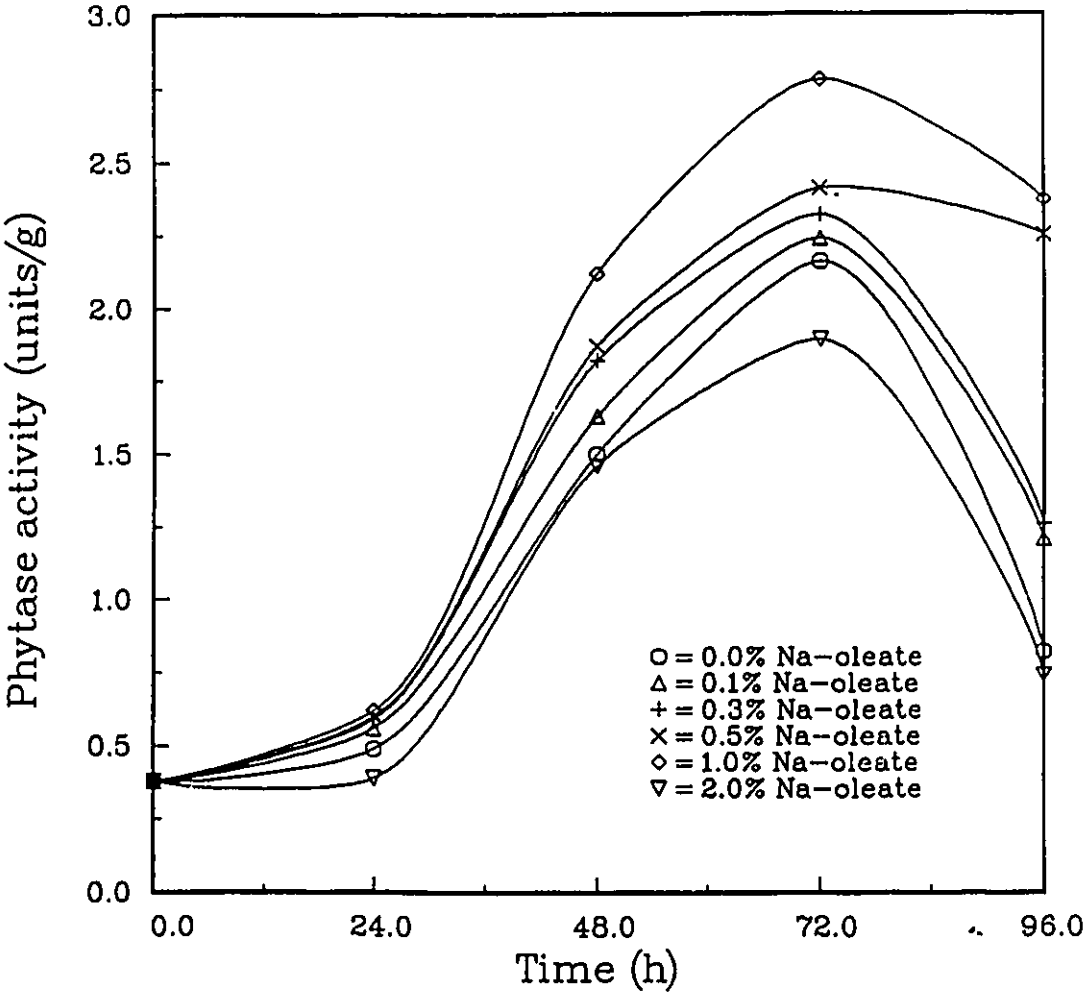


Figure 5.45: Effect of Na-oleate content on the phytase production during the SSF process

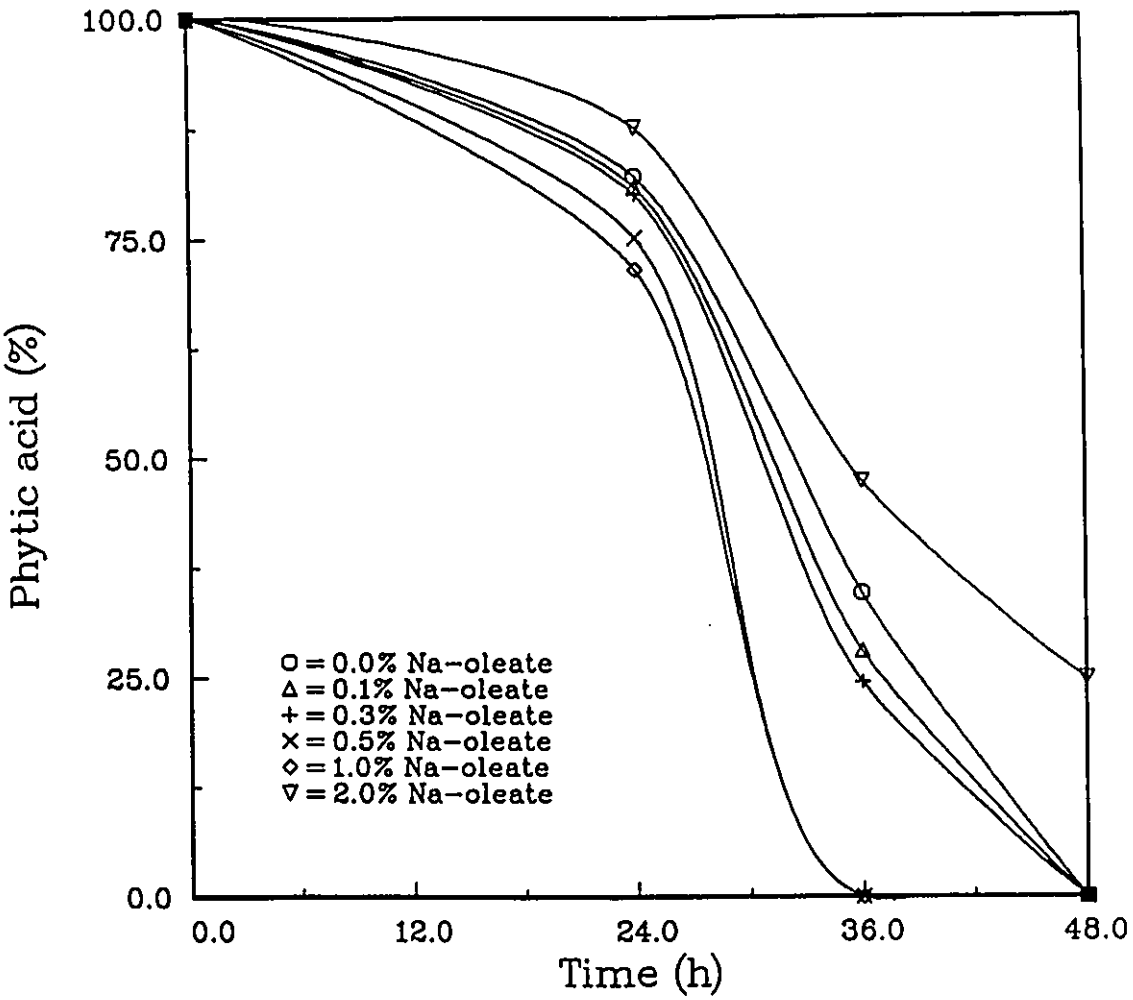


Figure 5.46: Effect of Na-oleate content on phytic acid content reduction during the SSF process

Table 5.12: Maximum biomass concentrations and maximum specific growth rate for different Na-oleate concentration

Sodium oleate concentration, (%)	Maximum biomass concentration, X_m (g/g SSC)	Maximum biomass specific growth rate, μ_m (h^{-1})
0.0	0.1633	0.0198
0.1	0.3072	0.0185
0.3	0.2310	0.0199
0.5	0.1122	0.0251
1.0	0.1122	0.0276
2.0	0.1207	0.0171

to a Na-oleate concentration of 1% which is consistent with the experimental results. The comparison of the biomass concentrations predicted by Eq. 3.5 and the experimental biomass results for different Na-oleate concentrations is shown in Fig. 5.47; the presented results show good agreement between the experimental results and the predicted data.

To use Eq. 3.10 to predict the enzyme concentration in the increasing phase of its production during solid state fermentation, the values of the parameter k_v for different Na-oleate concentrations had to be obtained. They were calculated by applying the previously obtained X_m and μ_m values for each Na-oleate concentration in Eq. 3.10 and fitting this equation to the experimental enzyme concentration data.

Generally, the maximum rate of increase in enzyme activity was acheived at the concentration of Na-oleate of 1% (Tables 5.13).

Comparison of the experimental and predicted values by Eq. 3.10 is shown in

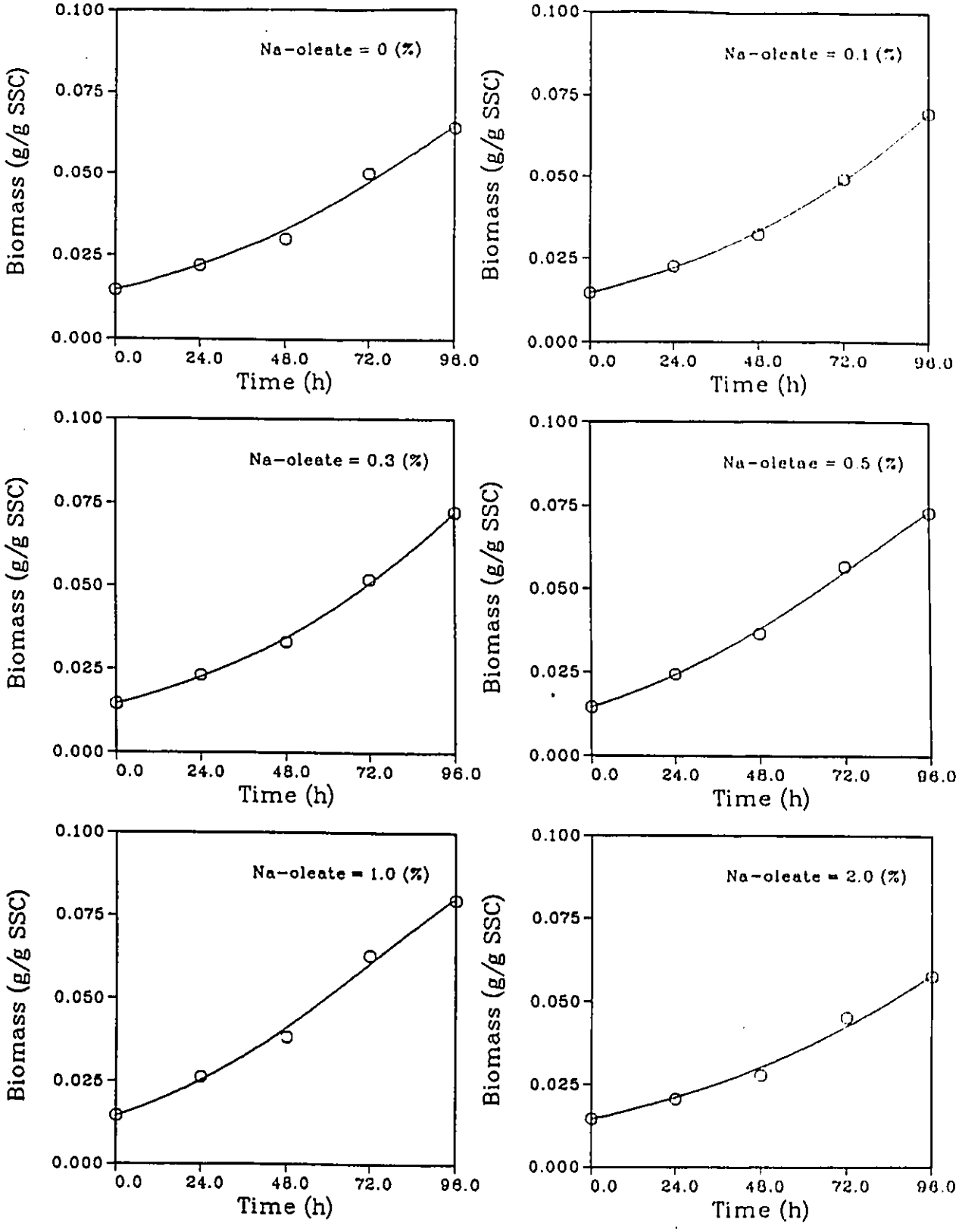


Figure 5.47: Comparison between predicted values of biomass (Eq.3.5) and the biomass produced in the SSF process for different Na-oleate concentrations

Table 5.13: Rate constant k_v, for various Na-oleate concentration for the model (Eq. 3.10) representing the increase phase of phytase production

Sodium oleate concentration, (%)	Rate constant, k_v (units/g.h)
0.0	0.888
0.1	0.943
0.3	0.977
0.5	0.940
1.0	1.037
2.0	0.819

Fig. 5.48 for different concentrations of Na-oleate. The shown results reveal that Eq. 3.10 predicts better the experimental enzyme data which were obtained with the media containing phosphate than with those supplemented with Na-oleate. Inspecting the presented data, it can be seen that Eq. 3.10 gives only a rough estimate of the experimental data for various Na-oleate concentrations.

It was shown that the phytic acid content reduction in canola meal was also influenced by Na-oleate. For modelling of this effect, it was necessary to calculate the rate constants of these processes, k_p, from Eq. 3.19 for each Na-oleate (Table 5.14) concentration.

In the evaluation of these constants, the X_m and μ_m values previously obtained were used as well.

Using Eq. 3.19 the phytic acid concentration in canola meal was calculated for different times of fermentation and shown in Fig. 5.49 together with the experimental results. The results from this figure show that Eq. 3.19 predicts well

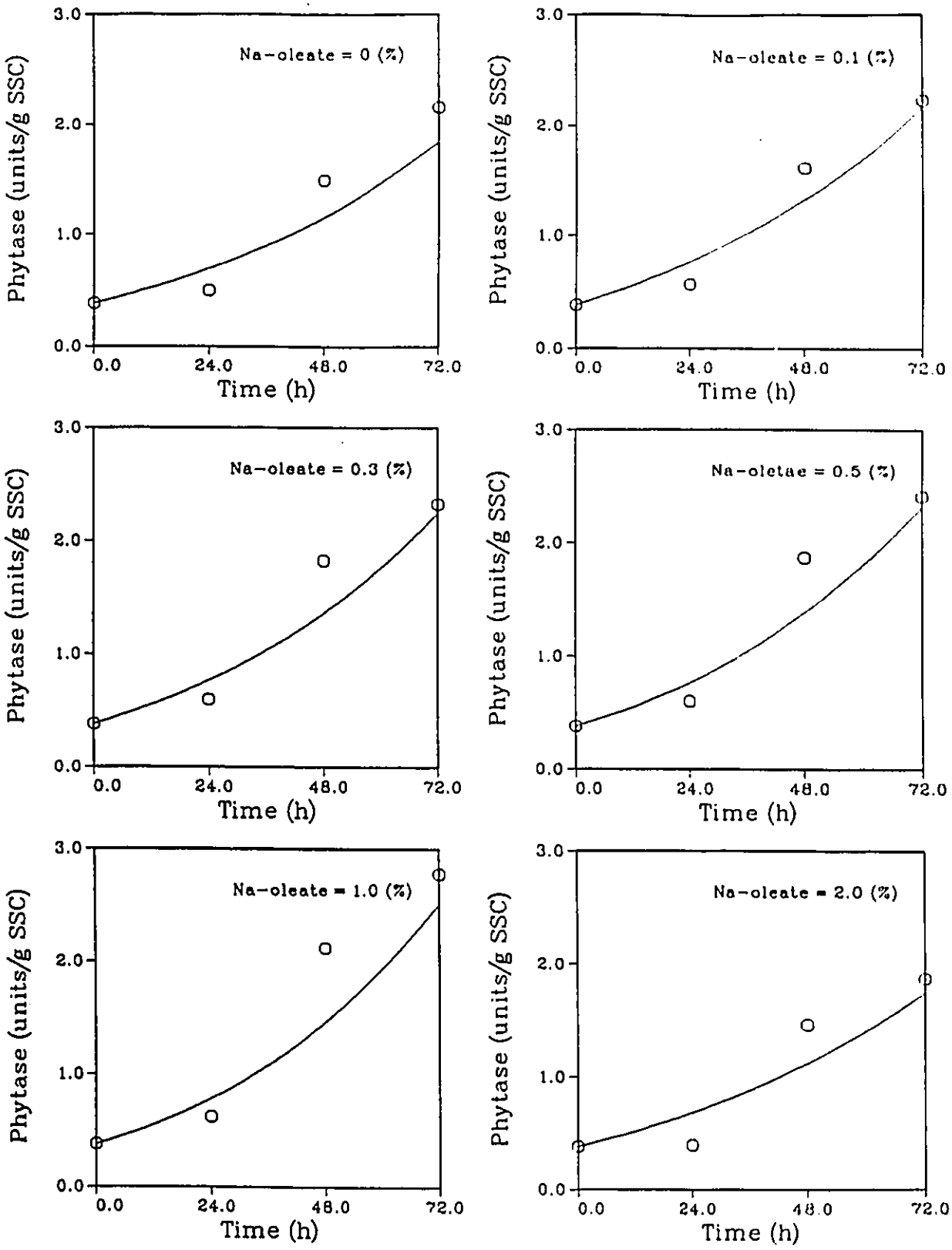


Figure 5.48: Comparison between predicted values of phytase activity (Eq.3.10 and 3.11) and the phytase activity produced in the SSF process for different Na-oleate concentrations

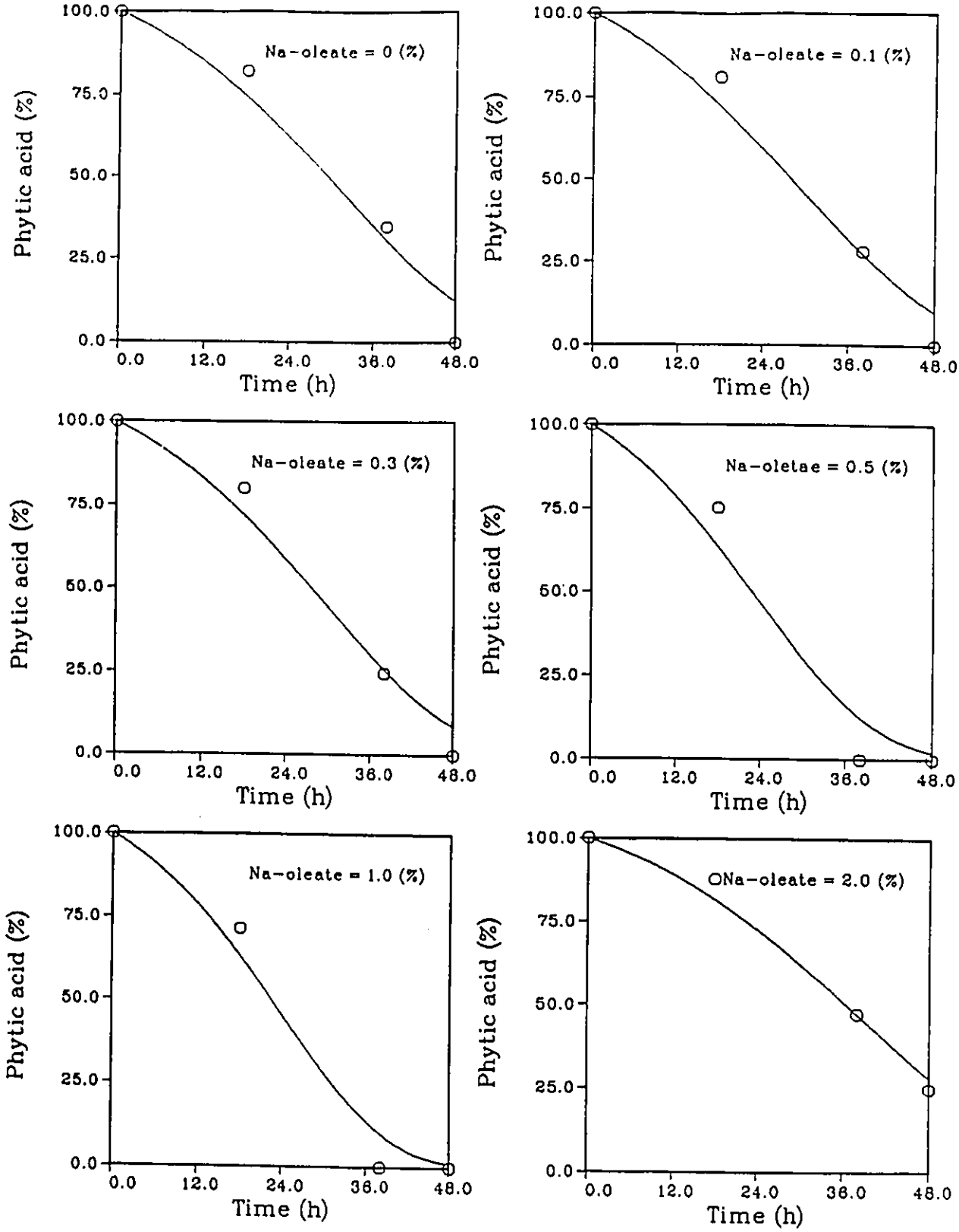


Figure 5.49: Comparison between predicted values of phytic acid content (Eq.3.19) and the phytic acid content determined experimentally in the SSF process for different Na-oleate concentrations

Table 5.14: Rate constant k_p, for various Na-oleate concentrations for the model (Eq. 3.19) representing the reduction of phytic acid content

Na-oleate concentration, (%)	Rate constant, k_p (h^{-1})
0.0	3160
0.1	3495
0.3	3432
0.5	4273
1.0	4046
2.0	2297

the data obtained in experimental work.

5.5.4 Effect of Combined Phosphate and Sodium Oleate

Realizing that phosphate and Na-oleate added separately to canola meal media for solid state fermentation influenced the biomass and phytase productions and the reduction of phytic acid content, it was of interest to examine their effects on the above processes if both of these compounds were together in the same medium.

Canola meal media were supplemented either by 1 mg phosphate, or 1% Na-oleate or by 1 mg phosphate and 1% Na-oleate. Neither phosphate nor Na-oleate were added to the control. The results shown in Figs. 5.50, 5.51 and 5.52 indicate that phosphate and Na-oleate exerted a synergistic effect on the productions of biomass and phytase and the reduction of phytic acid content in canola meal supplemented by both of these compounds. It is also noticable that the systems supplemented by phosphate, or by Na-oleate or by both of them gave better

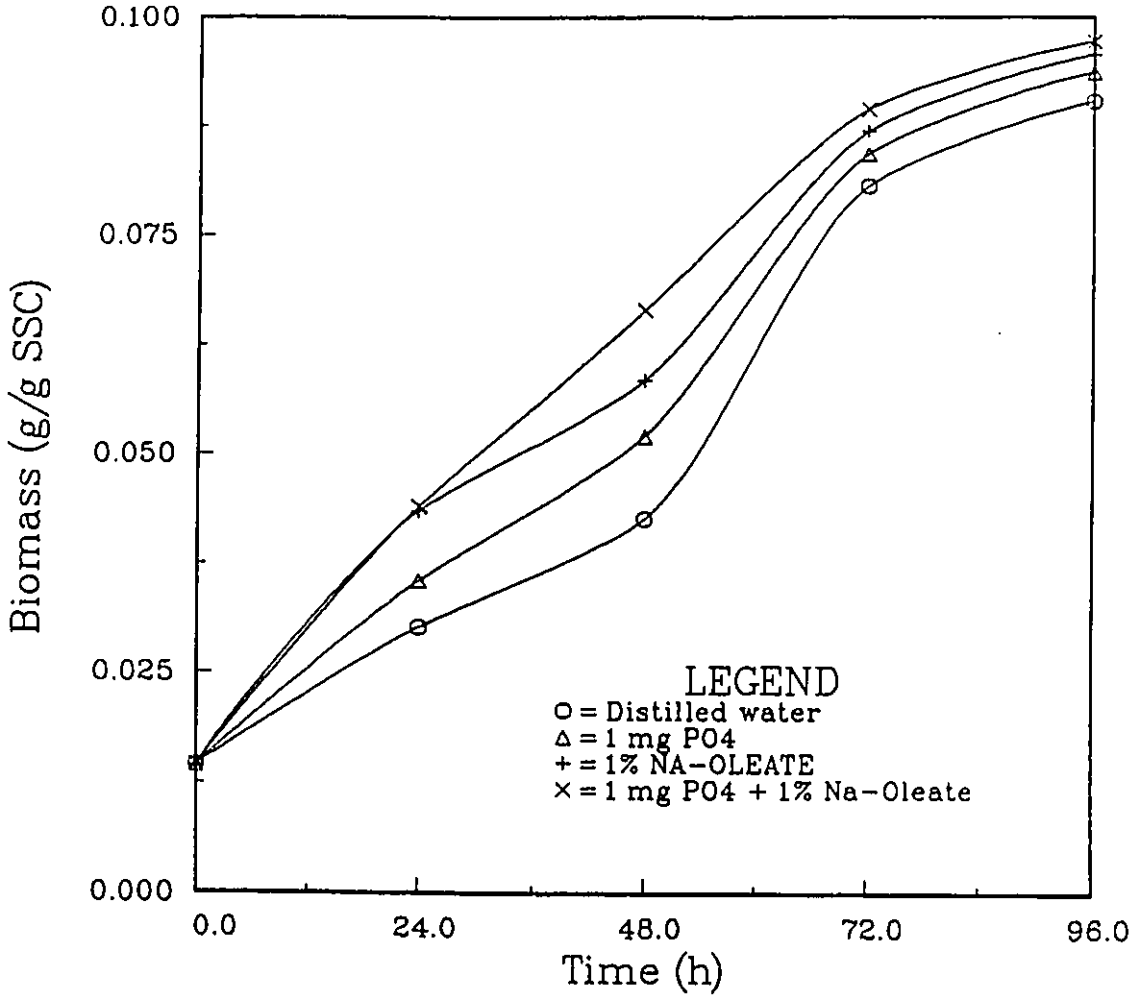


Figure 5.50: Effect of combined phosphate and sodium oleate on biomass production during the SSF process

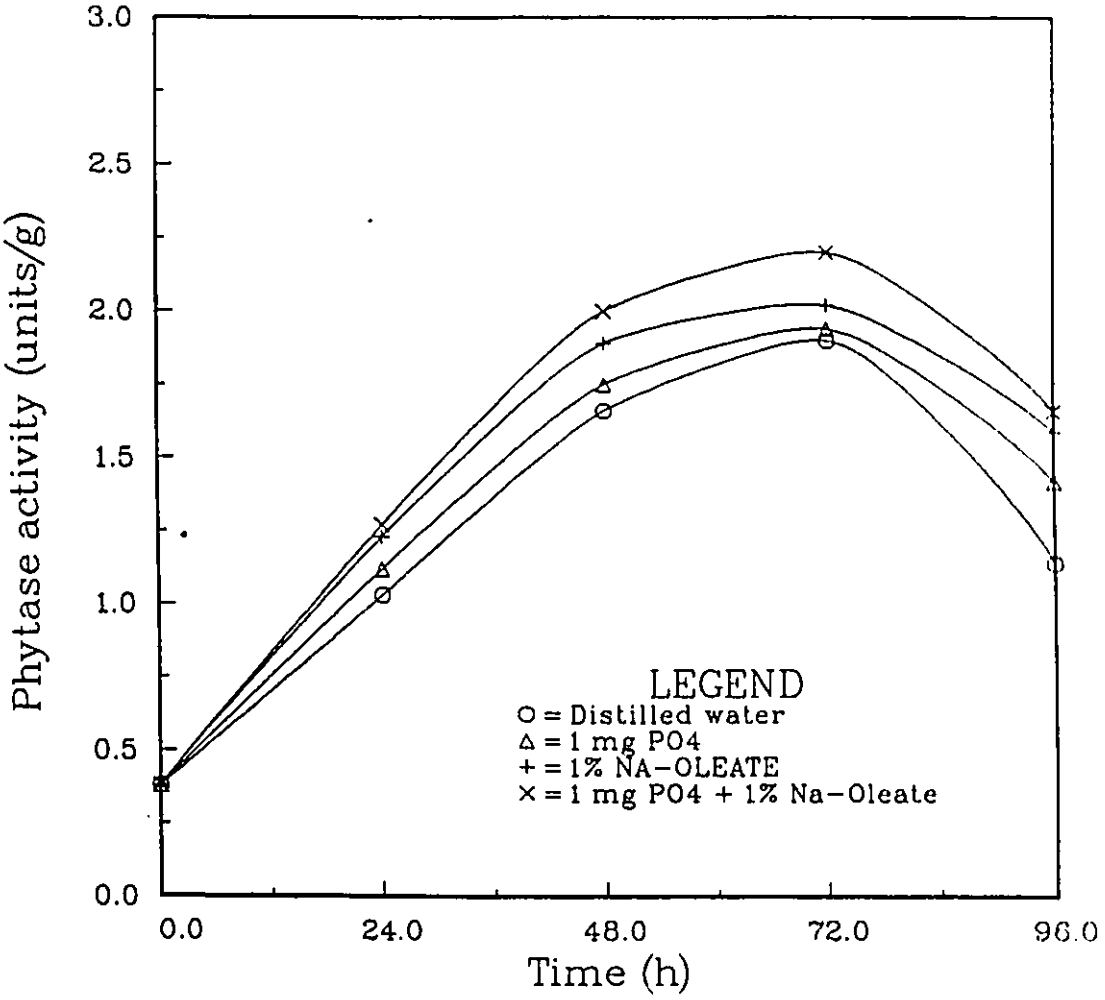


Figure 5.51: Effect of combined phosphate and sodium oleate on the phytase production during the SSF process

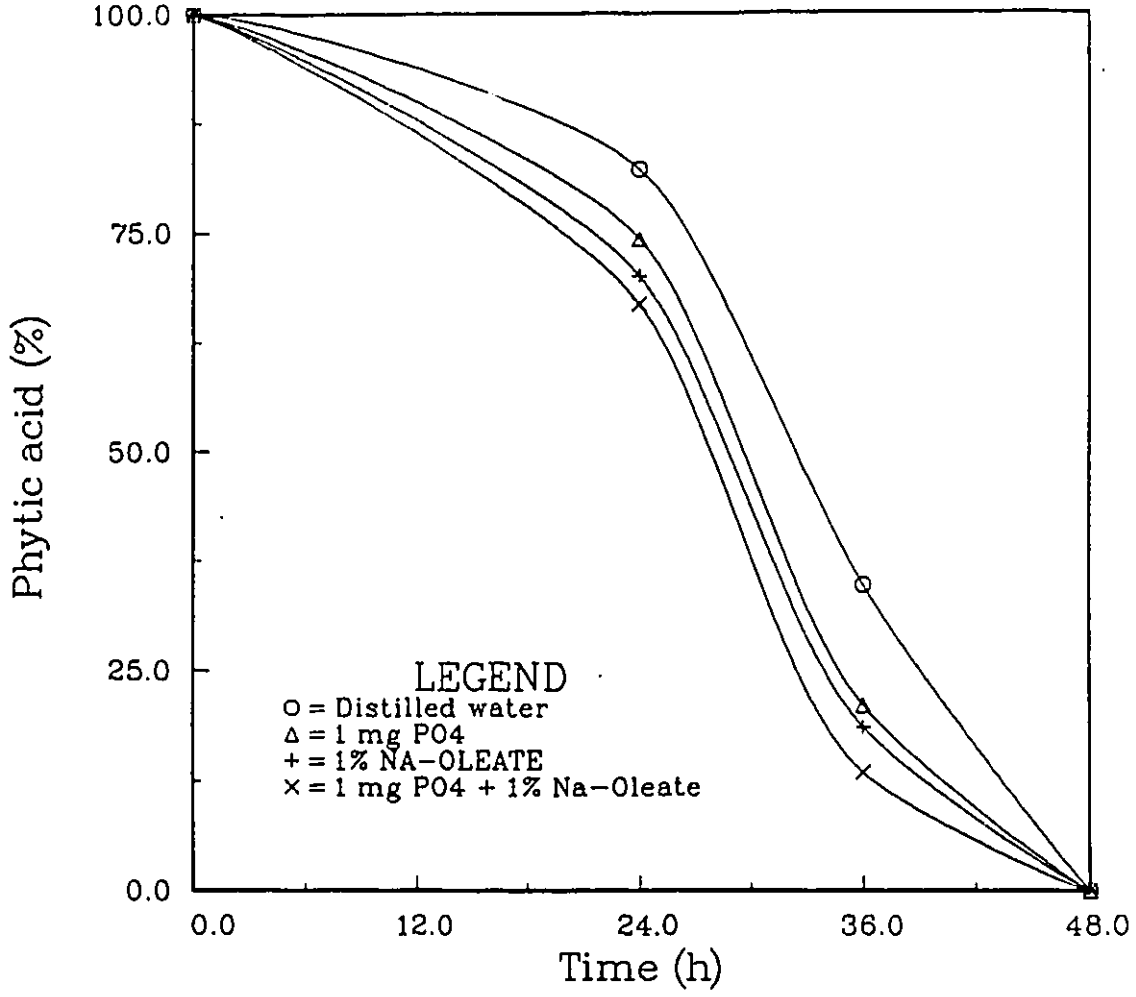


Figure 5.52: Effect of combined phosphate and sodium oleate on phytic acid content reduction during the SSF process

results than the control.

Chapter 6

Conclusions and Recommendations

6.1 Conclusions

Characteristics of the phytase of *A. carbonarius*, produced during a solid state fermentation process using canola meal as a substate, were studied. It was found that the K_m and v_{max} values for this enzyme using sodium phytate as the substrate are 0.345 mM and 0.807 units respectively. The optimum temperature and pH were found to be 53°C and 4.7 respectively. It was demonstrated that an activation of the studied enzyme was noticed when it was preincubated at higher temperatures for a period of time. The energy of activation, entropy change, and enthaly change of this phytase were estimated to be 7800 cal/mole, 73.95 cal/(mole.K), and 24000 cal/mole respectively.

Effects of the extractant:solid state culture ratio, and the time for single step extraction of phytase were evaluated.

It was found that *Aspergillus carbonarius* was a good producer of phytase in a solid state fermentation process. During this process it reduced the phytic acid content in canola meal.

The rate of phytic acid content reduction increased with an increase in the inoculum size.

The biomass and phytase production, and the rate of the phytic acid content reduction were affected by the inoculum homogenization. The optimum time of homogenization of the inoculum was found to be 120 seconds. The specific enzyme productivity was not affected very much by this treatment of the inoculum.

The optimum moisture content of the solid medium used in this study was found to be 53%.

Although both sterilized and unsterilized meal inoculated with this microorganism can be used for the reduction of the phytic acid content, better results were obtained with the former one.

Particle size of canola meal influenced the process of solid state fermentation. The optimum size was determined to be 1-1.4 mm.

The optimum initial pH for growth and enzyme production was found to be 5.6 when acetate or phosphate buffers were used. With citrate buffer the optimum pH was found to be 6.5.

The effect of glucose concentrations on the productions of biomass and phytase, and the reduction of phytic acid content in canola meal by a solid state fermentation process has been studied. The results showed that the glucose in

the amounts that did not exceed 6 g per solid state culture system had a positive effect on the rates of the biomass growth, enzyme production and the phytic acid content reduction, while glucose amounts above 6 g per solid state culture system reduced the rates below the one measured in the control which was not supplemented with glucose.

The systems with more than 6 g of glucose had extended growth phases which resulted in higher maximum enzyme activities compared to those attained in the systems with lower glucose concentrations.

The models for the biomass and phytase productions, and the phytic acid content reduction during a solid state fermentation process are given in this work. They fit the experimental data produced in this work reasonably well.

When the effect of phosphate was investigated it was found that 1-5 mg of phosphate per solid state culture system increased biomass growth, phytase production and phytic acid content reduction. The systems containing more than 10 mg of added phosphate showed slower biomass growth, and lower rate of phytase production, and phytic acid content reduction than the control system which was not supplemented with phosphate.

The effect of surfactants on this process was also studied. Sodium oleate was found to be the best surfactant for this process. Higher biomass and phytase concentrations and faster reduction of phytic acid content were observed in the presence of this surfactant than in the control or in the presence of other surfactants. The optimum concentrations of sodium oleate in the solid culture medium

was found to be 1%.

A synergistic positive effect on the biomass growth, phytase production and phytic acid content reduction was noticed when both phosphate and sodium oleate were added together to the same medium.

6.2 Recommendations

The thickness of the mass of the substrate was about 5 cm, and the continuous mixing of this solid culture destroys the mycelia formed during this process. Subsequently this reduces the biomass gowth, the enzyme production and the phytic acid content reduction. This problem should de addressed in a further study.

Utilization of a previous solid state culture, grown for 2 or 3 days, for inoculation of a new batch of solid medium rather than preparing the inoculum in the liquid medium is recommended to save time and materials used for the liquid culture medium.

It would be intersting to develope a continuous solid state fermentation process for the phytase production and reduction of the phytic acid content in canola meal since, in general, continuous processes are more attractive for industrial applications than the batch ones.

Solid state fermentation can be used for the reduction of some other undesirable materials in canola meal other than phytic acid, such as phenolic compounds. This can be done by using other microorganisms and producing enzymes which

are able to eliminate these materials.

Bibliography

[1] Aidoo K.E., Hendry R., Wood B.J.B. (1982) Solid substrate fermentations. *Advances in Appl. Microbiol.*, **28**, 201-237

[2] Al-Asheh S., Duvnjak Z. (1993) Production of phytase and reduction of phytic acid content in canola meal by *Aspergillus carbonarius* during a solid state fermentation process. *Fifteenth Symposium on Biotechnology for Fuels and Chemicals*, Colorado Spring, Colorado, May 10-14, 1993

[3] Allen R.J..L. (1940) The estimation of phosphorus. *Biochem. J.*, **34B**, 858-865

[4] Alli I., Houde R. (1987) Characterization of phytate in canola meal, 8^{th} progress report. *Canola Council of Canada*, 159-165

[5] Amtual J. S., Akhtar M. W., Malik N. N., Naz B. A. (1988) Production of free and substrate-bound cellulases of *Cellulomonas flavigena*. *Enzyme Microb. Technol.*, **10**, 626-631

[6] AOAC (1975) Official Methods of Analysis, 12^{th} ed., Association of Official Analytical Chemists, Washington D.C.

[7] Baily E., Ollis D. F. (1986) Biochemical Engineering Fundamentals, 2^{nd} Ed., *McGraw-Hill, Inc.*

[8] Bieth H., Jost P., Spiess B., Wehrer C. (1981) Effect of the alkali metal cations on the protonation constants of myi-inositol hexakis(phosphate). *Analytical letters*, **22**(3), 703-717

[9] Bitar, K., Reinhold J. G. (1972) Phytase and alkaline phosphatase activities in the intestinal mucosa of rats, chicken, calf, and man. *Biochim. Biophys.*, **268**, 442-452

[10] Blair R., Misir, R., Bell J. M., Clandinin D. R. (1987) The chemical composition and nutritional value of meal for chickens from triazine-tolerent canola. 8^{th} Progress Report, Canola Council of Canada, 51-57

[11] Blair R., Scougall R. K. (1975) Chemical composition, nutritive values of rapeseed meals. *Agricultural Research Council's Poultry Research Center*, 25-27

[12] Blix G. (1948) The determination of hexosamines according to Elson and Morgan. *Acta Chemica Scandinavica*, **2**, 467-473

[13] Boland A. R., Garner G. B., O'Dell B. L. (1975) Identification and properties of phytate in cereal grains and oilseed products. *J. Agric. Food Chem.*, **23**, 1186-1189

[14] Brown E. C., Heit M. L., Ryan D. E. (1961) Phytic acid: an analytical investigation. *Can. J. Chem.*, **39**, 1290-1297

[15] Butler W. H., (1975) In "The Filamentous Fungi", J. E. Smith and D. R. Berry, eds. *Vol. 1. Arnold*, London.

[16] Cannel E., Moo-Young M. (1980) Solid state fermentation systems. *Process Biochem.*, **6**, 2-7

[17] Chang C. W. (1967) Study of phytase and flouride effects in germinating corn seeds. *Cereal Chem.*, **44**, 129-142

[18] Chang R., Schwimmer S., Burr H. K. (1977) Phytate: removal from whole dry beans by enzymatic hydrolysis and diffusion. *J. Food Sci.*, **42**(5), 1098-1101

[19] Chauhan B. M., Suneja N., Bhat C. M. (1986) Nutritional value and fatty acid composition of some high yielding varieties of bajra. *Bull. Grail Technol.*, **21**, 41

[20] Chen B. H. Y., Morr C. V. (1985) Solubility and foaming properties of phytate-reduced soy protein isolate. *J. Food Sci.*, **50**, 1142-1985

[21] Cheryan M. (1980) The formation of protein-phytate complexes. *CRC Crit. Food Sci. Nutri.*, **13**, 297-300

[22] Clandinin D. R. (1986) Canola meal for livestock and poultry. Canola Council of Canada, 1-19

[23] Cosgrove D. J. (1966) The Chemistry and biochemistry of inositol polyphosphates. *Rev. Pure and Appl. Chem.*, **16**, 209-212

[24] Cosgrove D. J. (1980) Inositol phosphates. *Elsevier Scientific Publishing Co.*, 86-117.

[25] Courtois J., Joseph G. (1947) Recherches sur la phytase 6. Action de diverses preparations phosphatiques sur quelques esters phophoriques de l'inositol. *Bull. Soc. Chem. Biol.*, **29**, 951

[26] Courtois J., Perez C. (1948) Recherches sur la phytase 8. teneur en inositophosphate et activite phytasiques de diverse grains. *Bull. Soc. Chem. Biol.*, **30**, 195-198

[27] Czajkowska D., Ilnicka-Olejniczak O. (1988) Biosynthesis of protein by microscopic fungi in solid state fermentation-I. *Acta Biotechnol.*, **8**(5), 407-413

[28] DeRham, Jost T. (1979) Phytate-protein interactions in soybean extracts and low-phytate soy protein products. *J. Food Sci.*, 44(1), 496-600

[29] Desgrandes C., Vergoignan M., Georges M., Durand A. (1991) Biomass estimation in solid state fermentation. *Appl. Microbiol. Biotechnol.*, **35**, 200-205

[30] Detry R. W., Lillehoj E. B., Cigler A. (1972) Microbial Toxins, Vol. 6. Acadimic Press, New York

[31] Durand A., Chereau D. (1988) A new pilot reactor for solid state fermentation: application to the protein enrichment of sugar beet pulp. *Biotechnol. Bioeng.*, **31**, 476-486

[32] Erdman J. W. (1979) Oil seed phytates: Nutritional implications. *J. Am. Oil Chem. Soc.*, **56**, 736-741

[33] Fardiaz D., Markakis P. (1981) Degradation of phytic in oncom. *J. Food Sci.*, **46**, 523-525

[34] Ford J. R., Mustakas G. C., Schmutz R. D. (1978) Phytic acid removal from soybeans by a lipid protein concentrate process. *J. Am. Oil Cnem. Soc.*, **55**, 371-374

[35] Ghareub M. (1990) Biosynthesis, purification and some properties of extracellular phytase from *Aspergillus carneus*. *J. Acta MicCnem. Soc.*, **55**, 371-374

[36] Gibbins L. N., Norris F. W. (1963) Phytase and acid phosphatase in *Phaseolus vulgaris*. *Biochem. J.*, **86**, 86-71

[37] Gillberg L., Tornell B. (1976) Preparation of rapeseed protein isolates. *J. Food Sci.*, **41**, 1063-1069

[38] Graf E. (1986) Phytic acid-Chemistry and applications. The Pillsbury Co., Pilatus Press, Minneapolis.

[39] Graf E. (1983) The physico-chemical properties of phytic acid. *J. Agric. Food Chem.*, **31**, 851-854

[40] Graf E., Eaton J. W. (1985) Dietary phytate and calcium bioavailability. In: nutrutional bioavailability of calcium. ACS symposium series (Kies C. ed.). *American Chemistry Society*, **264**, 24,79

[41] Graf E., Empson K. L., Eaton J. W. (1987) Industrial implications of phytic acid. *J. Food Chem.*, **36**(6), 1183-1188

[42] Han Y. W., Anderson A. W. (1975) Semi-solid fermentation of ryegrass straw. *Appl. Microbiol.*, **30**, 930-934

[43] Han Y. W., Gallagher D. J. (1986) Phytase production by *Aspergillus ficuum*. *J. Ind. Microbiol.*, **1**, 295-301

[44] Han Y. W., Gallagher D. J. (1987) Phytase production by *Aspergillus ficuum* on solid substrate. *J. Ind. Microbiol.*, **2**, 195-200

[45] Han Y. W., Wilfred A. G. (1988) Phytase hydrolysis in soybean and cottonseed meals by *Aspergillus ficuum* phytase. *J. Agric. Food Chem.*, **36**, 259-262

[46] Han Y. W. (1988) Removal of phytic acid from soybean and cottonseed. *J. Food Chem.*, **36**(6), 1181-1183

[47] Harland B. F., Harland J. (1980) Fermentative reduction of phytate in rye, white and whole wheat breads. *Cereal Chem.*, **57**(3), 226-229

[48] Harland B. F., Morris E. R. (1985) Fibre and mineral absorption. In: Dietary Fibre Prespectives Review and Bioliography I (Leads, A. R. and Avenell, A., ed.), P. 72. John Libbey Co. Ltd., London

[49] Harris D. (1988) Canola Digest. Canola Council of Canada **22**(10), 1-12

[50] Hartman G. H. Jr. (1979) Removal of phytate from soy protein. *J. Am. Oil Chem. Soc.*, **56**, 731-735

[51] Haug W., Lantzsch H. J. (1983) Sensitive method for the rapid determination of phytate in cereals. *J. Sci. Food Agric.*, **34**, 1423-1426

[52] Hesseltine C. W. (1972) Solid state fermentation. *Biotechnol. Bioeng.*, **14**, 517-532

[53] Howson S. J., Davis R. P. (1983) Production of phytate-hydrolysing enzyme by some fungi. *Enzyme Microb. Technol.*, **5**, 277-382

[54] Hussain H., Khan S., Ismail M., Satter A. (1989) Effect of roasting and autoclaving on phytic acid content of chickpea. *Die Nahrung*, **33**(4), 345-348

[55] Irving G. C. J., Cosgrove D. J. (1972) Inositol phosphate phosphatase of microbiological origin: the inositol pentaphosphate products of *Aspergillus ficuum* phytases. *J. Bacteriol.*, **112**, 434-438

[56] Irving G. C. J., Cosgrove D. J. (1974) Inositol phosphate phosphatases of microbiological origin: Some properties of partially purified phosphatases of *A. ficuum* NRRL 3135. *Aust. J. Biol. Sci.*, **27**, 361-368

[57] Jager A., Coran S., Kirk T. K. (1985) Production of ligninases and degradation of lignin in agitated submerged culture of *Phanerochaete chrysosporium. Applied and Environmental Microbiology* **50**, 1274-1278

[58] Khan N., Zaman R., Elahi M. (1988) Effect of phytic acid content of Bengal Grams Products. *J. Agric. Food Chem.*, **Vol. 36**, 1274-1276

[59] Laskin A. I. (1982) Applied Microbiology, Vol. 28. *J. Acadamic Press*, 201-236

[60] Lolas M., Markakis P. (1975) Phytic acid and other phosphorus compounds of beans. *J. Agric. Food Chem.*, **23**, 13-17

[61] Lolas M., Markakis P. (1977) Phytase of navy beans. *J. Food Sci.*, **42**(4), 1094-1097

[62] Lowry O. H., Rosebrough N. J., Farr A. L., Randall R. J. (1951) Protein measurement with Folin Phenol reagent. *J. Biol. Chem.*, 265-275

[63] Mahjan S., Chauhan B. M. (1987) Phytic acid and extractable phosphorus of pearl millet as affected by natural lactic acid fermentation. *J. Sci. Food Agric.*, **41**, 381

[64] Mandal N. C., Burman S., Biswas B. B. (1972) Isolation, purification and characterization of phytase from germinating mung beans. *Phytochemistry*, **11**, 495-502

[65] Markakis S., Diamantoglous S. (1990) Enrichment of deseeded pod with fungal protein. *Cryptogamie, Micol.*, **11**(3), 189-201

[66] Markakis S., Diamantoglous S. (1990) Fungal isolation from leaves of some Mediterranean evergreen Sclerophyllous shrubs. Enzymatic activity of the isolated fungi. *Gryptogamie, Mycol.*, **11**(4), 243-254

[67] Mitchell D. A., Greenfield P. F., Doelle H. W. (1991) An emperical model of growth of *Rhizporus* in solid-state fermentation. *J. Ferment. Bioeng.*, **72**(3), 224-226

[68] Mitchell D. A., Greenfield P. F., Doelle H. W. (1990) Mode of growth of *Rhizopous oligosporus* on a model substrate in solid-state fermentation. *World J. of Microb. and Biotechnol.*, **60**, 201-208

[69] Moo-Young M., Dougulis A. J., Chahal D. S., MacDonald D. G. (1979) The Waterloo Process for SCP production from waste biomass. *Process Biochem.*, **14**(10), 38-40

[70] Nair V. (1990) Reduction of phytic acid content in canola meal by solid state fermentation. M. A. Sc. thesis. *Dept. of Chemical Engineering, University of Ottawa.*

[71] Nair V., Duvnjak Z. (1990) Reduction of phytic acid content in canola meal by *Aspergillus ficuum* in solid state fermentation process. *Appl. Microbiol. and Biotechnol.* **34**(2), 183-188

[72] Nair V., Duvnjak Z. (1991) Phytic acid content reduction in canola meal by various microorganisms in solid state fermentation process. *Acta Biotechnologica* **16**(3), 211-218

[73] Okazaki N., Sugama S., Tanaka T. (1980) Mathematical model for surface culture of koji mold. *J. Ferment. Technol.*, **58**, 471-476

[74] Pandey A. (1991) Effect of particle size of substrate on enzyme production in solid state fermentation. *Bioresource Technology*, **37**, 169-172

[75] Peers F. G. (1953) Phytase of wheat. *Biochem. J.*, **53**, 102-110

[76] Rackis J. J. (1974) Mineral availability in soy protein products. *J. Am. Oil Chem. Soc.*, **51**, 161-164

[77] Raimbault M., Alazard D. (1980) Culture methods to study fungal growth in solid state fermentation. *E. J. Appl. Microbiol. Biotechnol.*, **9**, 199-209

[78] Raimbault M., Revah S., Pina F., Villalobos P. (1985) Protein enrichment of cassava by solid state fermentation using molds isolated from traditional foods. *J. Fermen. Technol.*, **63**(4), 395-399

[79] Raimbault M., Deschamps F., Mayer F., Senez J. C. Direct protein enrichment of starchy products by fungal solid fermentation. *Vth International on glopal impact of Appl. Microbiol.*, Bangkok, 1977

[80] Ranhotra G. S., Loewe R. J., Puyat L. V. (1974) Phytic acid in soy and its hydrolysis during bread making. *J. Food Sci.*, **39**, 1023-1025

[81] Reddy N. R., Pierson M. D., Sathe S. K., Salunkhe D. K. Phytase in cereals and legumes. CRC Press, Florida, 1989

[82] Rees E. T., Maguire A. (1969) Surfactants for enzyme production by microorganisms. *Appl. Microbiol.*, **17**, 242-245

[83] Rivera-Munoz G., Tinoco-Vaencia J. R., Sanchez S., Farres A. (1991) Production of Microbial lipases in a Solid state fermentation. *Biotechnology Letters*, **13**, 277-280

[84] Sakurai Y., Lee T. H., Shiota (1976) On conventional method for glucosamine estimation in koji. *Agric. Biol. Chem.*, **41**, 619-624

[85] Senez J. C. (1978) Solid state fermentation of starchy products *S. vulgaris*. U, N. U., Intern. Conf. Guatemala City, 1978

[86] Sharma C. B., Goel M., Irshad M. (1978) Myoinositol hexaphosphate as a potential inhibitor of α-amylase of different origin. *Phytochemistry*, **17**, 201-204

[87] Shieh T. R., Ware J. H. (1968) Survey of microorganisms for the production of extracellular phytase. *Appl. Microbiol.*, **16**(9), 1348-1351

[88] Statistics Canada (1993) Cereals and Oilseeds Review. *Catalogue 22-007*, **16**, 1

[89] Stone F. E., Hardy R. W., Spinelli J. (1984) Autolysis of phytic acid and protein in canola meal, wheat bran and and fish silage blends. *J. Sci. Food Agric.*, **35**, 513-519

[90] Sudarmadji S., Markakis P. (1977) Phytate and phytase of soybean tempeh.. *J. Sci. Food Agr.*, **28**, 381-517

[91] Takahashi J., Abekawa G., Yamada K. (1960) Effect of non-ionic surface active agent on mycelial form and amylase production of *A. niger*. *Nippon Nozei Kogaku Korishi*, **34**, 1043-1045

[92] Ullah A. H. J. (1988) Production, rapid purification and catalytic characterization of extracellular phytase from *Aspergillus ficuum*. *Preparative Biochemistry*, **18**, 443-458

[93] Vaisey-Genser M., Harris D. F. (1987) Current markets for canola oil. In: canola oil properties and performance. *Canola Council of Canada.*

[94] Weiner J., (1978) A method for phytic acid determination in wheat and weat fractions. *Cereal Chem.*, **48**, 312-320

[95] Zuk B. Blake J., Craig W., Coxworth E., Strayer R. (1981) Canola oil as a fuel for farm diesel engines- final report, File no. 34SZ.01706-0-0100, *Agriculture Canada*, Ottawa.

Appendix A

Experimental Results

Table A.1: Effect of phytic acid concentration on phytase activity at 53°C and pH 4.7

s (mM)	Phytase (units/h)
0	0
0.10	0.097
0.15	0.272
0.40	0.454
0.50	0.514
0.60	0.521
1.00	0.563

Table A.2: Effect of temperature of incubation on phytase activity at pH 4.7

T (°C)	Phytase (units/h)
30	0.538
40	0.684
45	0.799
50	0.835
53	0.859
55	0.811
70	0.523

Table A.3: Effect of pH on phytase activity at 53°C

pH	Phytase (units/h)
4.4	0.602
4.6	0.708
4.7	0.715
4.8	0.702
5.2	0.642
5.6	0.593

Table A.4: Effect of incubation time on phytase activity at 53°C and pH 4.7

Time (min)	Phytase (units/g)
0	0
10	0.077
15	0.085
20	0.098
30	0.108
40	0.113
50	0.117
60	0.112

Table A.5: Effect of enzyme concentration on phytase activity at 53 °C and pH 4.7

Stock enzyme (%)	phytase (units/g)
100	2.125
50	1.062
33	0.604
25	0.416
20	0.395
10	0.230
5	0.167

Table A.6: Effect of time of extraction on phytase activity

Time (min)	Phytase (units/g)
0	0
5	1.67
10	1.75
15	1.89
30	1.97
60	2.05
120	2.13

Table A.7: Effect of volume of extraction on phytase activity

Volume (ml/g)	Phytase (units/g)
0	0
1	5.49
2	7.35
3	8.12
5	8.22
10	8.69

Table A.8: Effect of preincubation temperature on phytase activity

	Preincubation temperature (°C)			
Time (min)	50	60	70	90
	Phytase activity (%)			
0	100	100	100	100
15	107	103	116	120
30	116	113	130	130
45	121	126	140	95
60	124	136	132	67
90	126	129	120	55
120	127	118	106	50
180	115	95	87	44
240	92	79	72	43
300	81	67	64	42

Table A.9: Biomass, phytase activity, glucose concentration and pH in liquid medium

Time (h)	Biomass (mg/mL)	Glucose (mg/100 mL)	Phytase (units/ml)	pH
0	0	0.850	0	6.95
24	0.145	0.793	0.470	6.17
48	0.340	0.030	0.610	4.82
60	0.448	0	0.663	5.65
72	0.521	0	0.587	6.17
96	0.484	0	0.554	6.63
100	0.412	0	0.532	6.74

Table A.10: Effect of initial glucose concentration on the growth rate in liquid medium

Initial glucose (g/L)	Growth rate (mg/h.mL)
0	0
2.5	0.0093
5.0	0.0200
7.5	0.0260
15.0	0.0353
25	0.0413

Table A.11: Effect of aeration on glucose concentration in liquid medium

	Volume of the flask (mL)	
Time (h)	50	100
	Glucose (mg/100 mL)	
0	0.850	0.850
24	0.490	0.804
36	0.021	0.054
48	0	0
60	0	0
72	0	0

Table A.12: Effect of aeration on biomass production in liquid medium

	Volume of the flask (mL)	
Time (h)	50	100
	Biomass (mg/mL)	
0	0	0
24	0.213	0.160
36	0.506	0.253
48	0.667	0.453
60	0.560	0.533
72	0.540	0.500

Table A.13: Changes in biomass production, phytase activity, phytic acid content reduction, protein content and carbohydrates utilization during SSF process

Time (h)	Biomass (g/g SSC)	Phytase (units/g)	Phytic acid (%)	Protein (%)	Carbohydrates (%)
0	0.0349	0.62	100.0	100.0	100.0
24	0.0489	1.13	66.3	110.1	89.9
40	0.0823	2.20	0	127.3	83.1
48	0.0922	3.35	0	117.7	74.9
72	0.1010	4.70	0	110.5	73.0

Table A.14: Effect of moisture content on phytic acid content reduction

	Moisture content (%)			
Time (h)	29	53	60	65
	Phytic acid (%)			
0	100.0	100.0	100.0	100.0
22	84.5	78.9	89.9	96.3
32	75.0	64.3	66.7	89.7
46	67.5	22.6	27.0	59.4
56	59.5	0	0	26.6
72	59.0	0	0	0

Table A.15: Effect of inoculum concentration on phytic acid content reduction

	Inoculum (mg)					
Time (h)	60	80	150	170	220	240
	Phytic acid (%)					
0	100.0	100.0	100.0	100.0	100.0	100.0
28	94.0	84.6	83.0	73.8	70.0	52.4
44	36.2	8.6	0	0	0	0
53	20.6	0	0	0	0	0

Table A.16: Effect of sterilization, inoculation and buffer media on phytic acid content reduction

Time (h)	Sterilized inoculated	Sterilized uninoculated	Unsterilized inoculated	Unsterilized uninoculated	Buffer inoculated
	Phytic acid (%)				
0	100.0	100.0	100.0	100.0	100.0
20	92.3	95.0	97.4	97.5	95.7
29	67.1	99.1	76.2	81.0	83.4
43	29.4	97.8	43.0	59.1	70.0
53	0	100.0	38.5	52.2	31.1

Table A.17: Effect of time of homogenization on biomass production

	Homogenization time (sec)				
Time (h)	10	30	60	120	240
	Biomass (g/g SSC)				
0	0.0146	0.0146	0.0146	0.0146	0.0146
24	0.0334	0.0374	0.0390	0.0405	0.0348
48	0.0563	0.0583	0.0625	0.0710	0.0555
72	0.0573	0.0662	0.0700	0.0828	0.0630
96	0.0740	0.0790	0.0885	0.0937	0.0817

Table A.18: Effect of time of homogenization on phytase production

	Homogenization time (sec)				
Time (h)	10	30	60	120	240
	Phytase activity (units/g)				
0	0.303	0.302	0.302	0.302	0.302
24	0.344	0.520	0.620	0.733	0.647
48	1.380	1.460	1.540	1.680	1.450
72	1.780	1.870	1.930	2.040	1.950
96	1.570	1.450	1.090	1.010	1.820

Table A.19: Effect of time of homogenization on phytic acid content reduction

	Homogenization time (sec)				
Time (h)	10	30	60	120	240
	Phytic acid (%)				
0	100.0	100.0	100.0	100.0	100.0
24	84.3	78.6	72.6	70.6	79.9
48	43.4	30.7	22.3	12.0	36.5
72	0	0	0	0	0

Table A.20: Effect of particle size of canola meal on biomass production

	Particle size (mm)					
Time (h)	S_1	S_2	S_3	S_4	S_5	S_6
	Biomass (g/g SSC)					
0	0.0125	0.0125	0.0125	0.0125	0.0125	0.0125
21	0.0241	0.0272	0.0288	0.0292	0.0314	0.0293
33	0.0272	0.0314	0.0334	0.0240	0.0350	0.0334
48	0.0350	0.0371	0.0402	0.0429	0.0439	0.0397
72	0.0423	0.0506	0.0532	0.0574	0.0600	0.0501
96	0.0444	0.0527	0.0553	0.0610	0.0616	0.0522

Table A.21: Effect of particle size of canola meal on phytase production

	Particle size (mm)					
Time (h)	S_1	S_2	S_3	S_4	S_5	S_6
	Phytase (units/g)					
0	0.38	0.38	0.38	0.38	0.38	0.38
21	0.57	0.59	0.65	0.67	0.70	0.65
33	0.64	0.70	0.72	1.02	1.09	0.92
48	1.30	1.45	1.52	1.75	1.89	1.73
72	1.67	0.53	0.59	0.95	1.36	0.72
96	1.44	0.07	0.13	0.24	0.27	0.12

Table A.22: Effect of particle size of canola meal on phytic acid content reduction

	Particle size (mm)					
Time (h)	S_1	S_2	S_3	S_4	S_5	S_6
	Phytic acid (%)					
0	100.0	100.0	100.0	100.0	100.0	100.0
21	97.4	93.4	92.0	90.8	83.6	91.0
33	95.8	84.4	75.0	53.0	45.9	61.7
48	67.0	40.8	37.3	28.1	0	32.2
72	0	0	0	0	0	0

Table A.23: Effect of initial pH of acetate buffer medium on phytase production

	pH				
Time (h)	3.6	4.7	5.2	5.6	Water
	Phytase (units/g)				
0	0.55	0.55	0.55	0.55	0.55
24	0.53	0.94	1.12	1.07	1.91
42	0.72	1.05	1.82	1.93	2.50
60	0.88	1.16	2.26	2.56	3.20
72	1.01	1.68	2.68	3.33	3.86
96	0.85	2.40	3.80	4.38	3.57
120	1.45	3.40	4.63	5.73	2.05
148	2.80	1.88	5.51	6.03	0.68
172	2.99	1.01	4.75	5.47	0.39

Table A.24: Effect of initial pH of acetate buffer medium on phytic acid content reduction

	pH				
Time (h)	3.6	4.7	5.2	5.6	Water
	Phytic acid (%)				
0	100.0	100.0	100.0	100.0	100.0
24	89.7	81.8	80.1	79.4	71.2
42	85.1	79.1	75.2	67.4	32.6
60	84.4	77.7	34.3	21.5	0
72	82.9	71.9	6.6	0	0
96	80.8	0	0	0	0

Table A.25: Effect of initial pH of phosphate buffer medium on phytase production

	pH		
Time (h)	5.6	6.0	5.6
	Phytase (units/g)		
0	0.42	0.42	0.42
24	1.45	1.43	1.36
48	2.45	2.20	2.14
72	3.38	2.86	2.72
96	1.85	3.02	2.94
120	0.61	2.70	1.55

Table A.26: Effect of initial pH of phosphate buffer medium on phytic acid content reduction

	pH		
Time (h)	5.6	6.0	5.6
	Phytic acid (%)		
0	100.0	100.0	100.0
24	94.0	96.6	98.3
48	23.9	62.7	79.0
60	0	24.1	37.5
72	0	0	3.09

Table A.27: Effect of initial pH of citrate buffer medium on phytase production

	pH		
Time (h)	5.6	6.0	5.6
	Phytase (units/g)		
0	0.40	0.40	0.40
24	1.05	1.07	1.12
48	2.10	2.25	2.28
72	2.45	2.62	2.80
96	2.52	2.60	2.96
120	0.86	0.88	0.98

Table A.28: Effect of initial pH of citrate buffer medium on phytic acid content reduction

	pH		
Time (h)	5.6	6.0	5.6
	Phytic acid (%)		
0	100.0	100.0	100.0
24	96.0	95.9	91.4
48	46.5	41.7	37.4
60	30.4	11.5	0
72	0	0	0

Table A.29: Effect of glucose content on biomass production

	Glucose (g)					
Time (h)	0	2	4	6	12	12
	Biomass (g/g SSC)					
0	0.0145	0.0145	0.0145	0.0145	0.0145	0.0145
24	0.0323	0.0339	0.0359	0.0370	0.0281	0.0250
48	0.0635	0.0688	0.0708	0.0734	0.0495	0.0453
72	0.0724	0.0750	0.0786	0.0813	0.0641	0.0526
96	0.0745	0.0802	0.0838	0.0865	0.0682	0.0583
120	0.0776	0.0854	0.0870	0.0896	0.0776	0.0693
144	0.0568	0.0583	0.0615	0.0650	0.0849	0.0818
168	0.0146	0.0167	0.0177	0.0198	0.0885	0.0948

Table A.30: Effect of glucose content on phytase production

	Glucose (g)					
Time (h)	0	2	4	6	12	12
	Phytase (units/g)					
0	0.38	0.38	0.38	0.38	0.38	0.38
24	0.84	0.91	0.93	0.99	0.57	0.52
48	1.43	1.53	1.62	1.72	1.07	0.79
72	2.10	2.24	2.37	2.54	1.70	1.03
96	0.75	0.86	1.05	1.63	2.45	1.74
120	0.12	0.35	0.34	0.46	3.25	2.93
144	0.02	0.06	0.27	0.29	3.03	3.66
168	0	0	0.04	0.07	1.68	3.15

Table A.31: Effect of glucose content on phytic acid content reduction

	Glucose (g)					
Time (h)	0	2	4	6	12	12
	Phytic acid (%)					
0	100.0	100.0	100.0	100.0	100.0	100.0
24	78.2	71.1	65.0	62.2	91.1	97.4
48	13.8	11.3	8.2	0	48.3	53.0
72	0	0	0	0	5.6	35.9

Table A.32: Effect of added phosphate on biomass production

	Phosphate (mg)					
Time (h)	0	1	5	10	50	100
	Biomass (g/g SSC)					
0	0.0145	0.0145	0.0145	0.0145	0.0145	0.0145
18	0.0292	0.0370	0.0318	0.0255	0.0250	0.0198
38	0.0427	0.0479	0.0432	0.0422	0.0375	0.0349
48	0.0542	0.0594	0.0578	0.0563	0.0531	0.0406
60	0.0651	0.0729	0.0682	0.0630	0.0588	0.0458

Table A.33: Effect of added phosphate on phytase production

	Phosphate (mg)					
Time (h)	0	1	5	10	50	100
	Phytase (units/g)					
0	0.32	0.32	0.32	0.32	0.32	0.32
18	0.50	0.83	0.67	0.50	0.45	0.34
38	1.30	1.52	1.47	1.40	1.37	1.27
48	1.76	2.07	1.91	1.82	1.78	1.69
60	1.95	2.29	2.18	1.97	1.90	1.76
84	1.63	2.13	1.87	1.68	1.49	1.34

Table A.34: Effect of added phosphate on phytic acid content reduction

	Phosphate (mg)					
Time (h)	0	1	5	10	50	100
	Phytic acid (%)					
0	100.0	100.0	100.0	100.0	100.0	100.0
18	82.3	66.2	76.2	80.4	81.7	88.1
38	34.0	0	22.5	30.4	33.2	42.5
48	6.2	0	0	4.5	6.8	19.4
60	0	0	0	0	0	0

Table A.35: Effect of surfactants on biomass production

	Surfactant			
Time (h)	Water	Na-oleate	Tween-80	Triton X-100
	Biomass (g/g SSC)			
0	0.0145	0.0145	0.0145	0.0145
24	0.0290	0.0375	0.0361	0.0277
48	0.0515	0.0690	0.0575	0.0320
72	0.0630	0.0765	0.0700	0.0595
96	0.0716	0.0899	0.0750	0.0640

Table A.36: Effect of surfactants on phytase production

	Surfactant			
Time (h)	Water	Na-oleate	Tween-80	Triton X-100
	Phytase (units/g)			
0	0.38	0.38	0.38	0.38
24	0.89	1.01	0.91	0.83
48	1.84	2.36	2.05	1.60
72	2.20	2.66	2.46	1.31
96	0.57	1.27	0.65	0.37

Table A.37: Effect of surfactants on phytic acid content reduction

	Surfactant			
Time (h)	Water	Na-oleate	Tween-80	Triton X-100
	Phytic acid (%)			
0	100.0	100.0	100.0	100.0
24	77.2	65.0	71.8	85.2
36	27.8	0	22.8	38.0
48	0	0	0	0

Table A.38: Effect of Na-oleate concentration on biomass production

	Na-oleate (%)					
Time (h)	0	0.1	0.3	0.5	1.0	2.0
	Biomass (g/g SSC)					
0	0.0156	0.0156	0.0156	0.0156	0.0156	0.0156
24	0.0222	0.0226	0.0231	0.0244	0.0264	0.0207
48	0.0302	0.0322	0.0332	0.0366	0.0385	0.0279
72	0.0499	0.0492	0.0520	0.0568	0.0630	0.0453
96	0.0641	0.0698	0.0724	0.0732	0.0797	0.0578

Table A.39: Effect of Na-oleate concentration on phytase production

	Na-oleate (%)					
Time (h)	0	0.1	0.3	0.5	1.0	2.0
	Phytase (units/g)					
0	0.38	0.38	0.38	0.38	0.38	0.38
24	0.49	0.56	0.59	0.60	0.62	0.39
48	1.50	1.63	1.82	1.87	2.12	1.46
72	2.16	2.24	2.32	2.41	2.78	1.89
96	0.82	1.21	1.26	2.25	2.37	0.74

Table A.40: Effect of Na-oleate concentration on phytic acid content reduction

	Na-oleate (%)					
Time (h)	0	0.1	0.3	0.5	1.0	2.0
	Phytic acid (%)					
0	100.0	100.0	100.0	100.0	100.0	100.0
24	82.2	81.0	80.1	75.2	71.5	87.7
36	34.7	28.1	24.5	0	0	47.4
48	0	0	0	0	0	24.9

Table A.41: Effect of combined phosphate and 1.0% Na-oleate on biomass production

Time (h)	Water	phosphate 1 mg	Na-oleate %	Phosphate + Na-oleate
	Biomass (g/g SSC)			
0	0.0145	0.0145	0.0145	0.0145
24	0.0302	0.0355	0.0435	0.0440
48	0.0427	0.0522	0.0586	0.0666
72	0.0807	0.0844	0.0870	0.0895
96	0.0904	0.0937	0.0957	0.0972

Table A.42: Effect of combined phosphate and 1.0% Na-oleate on phytase production

Time (h)	Water	phosphate 1 mg	Na-oleate %	Phosphate + Na-oleate
	Phytase (units/g)			
0	0.38	0.38	0.38	0.38
24	1.03	1.12	1.23	1.27
48	1.66	1.75	1.89	2.00
72	1.90	1.94	2.02	2.20
96	1.14	1.42	1.59	1.66

Table A.43: Effect of combined phosphate and 1.0% Na-oleate on phytic acid content reduction

Time (h)	Water	phosphate 1 mg	Na-oleate %	Phosphate + Na-oleate
	Phytic acid (%)			
0	100.0	100.0	100.0	100.0
24	82.2	74.2	70.0	66.8
36	34.7	21.0	18.5	13.4
48	0	0	0	0

Appendix B

Standard Curves

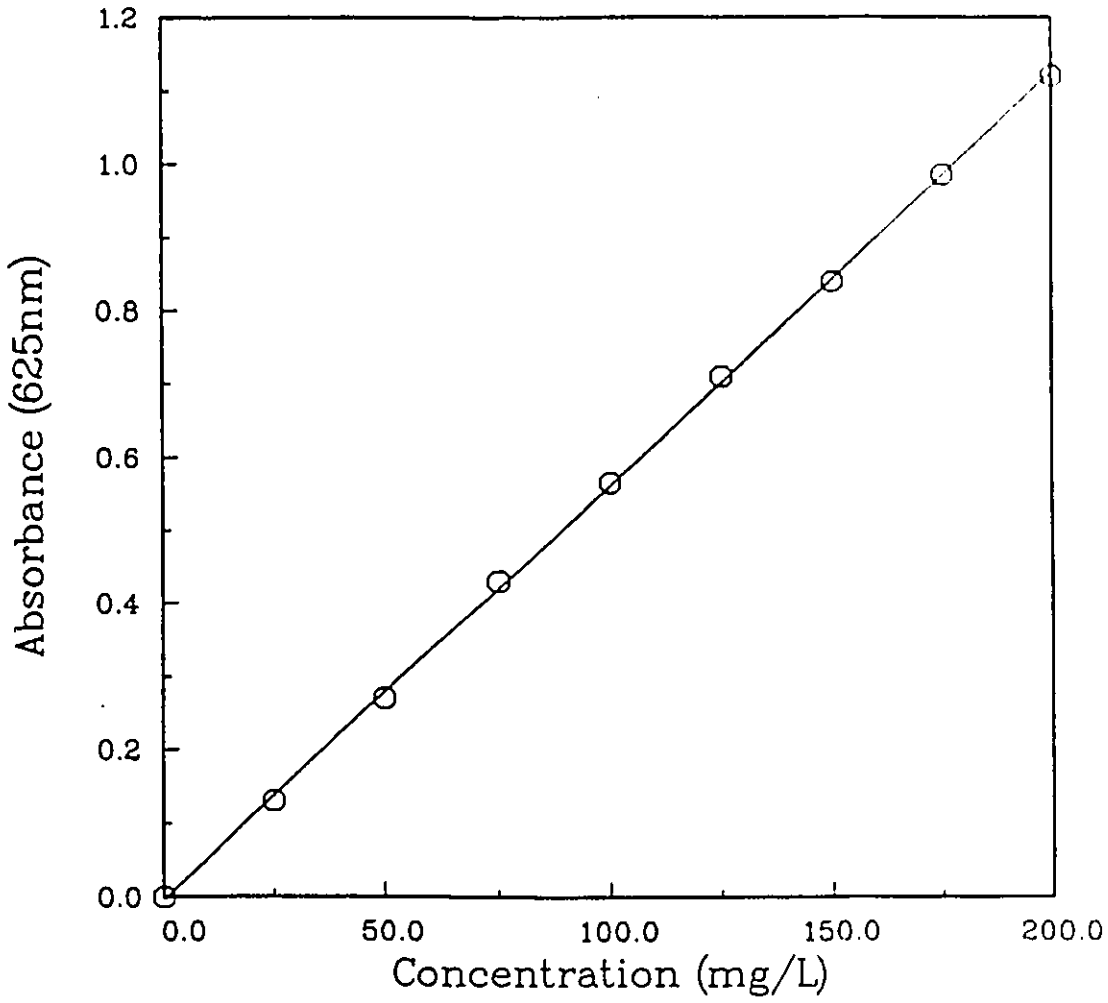


Figure B.1: Standard curve for measurement of total available carbohydrates (AOAC, 1975), using sucrose as the standard

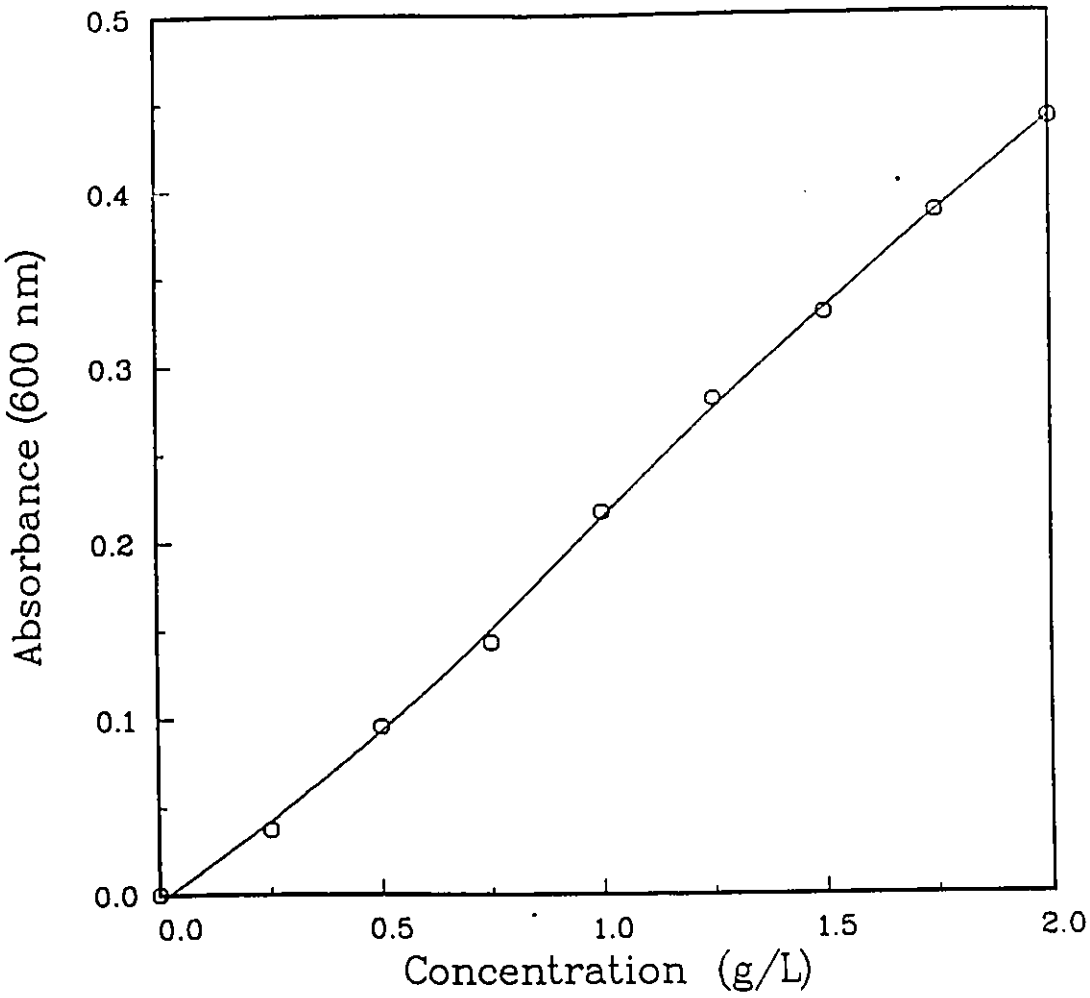


Figure B.2: Reducing sugar determination by the method described by Weiner (1978)

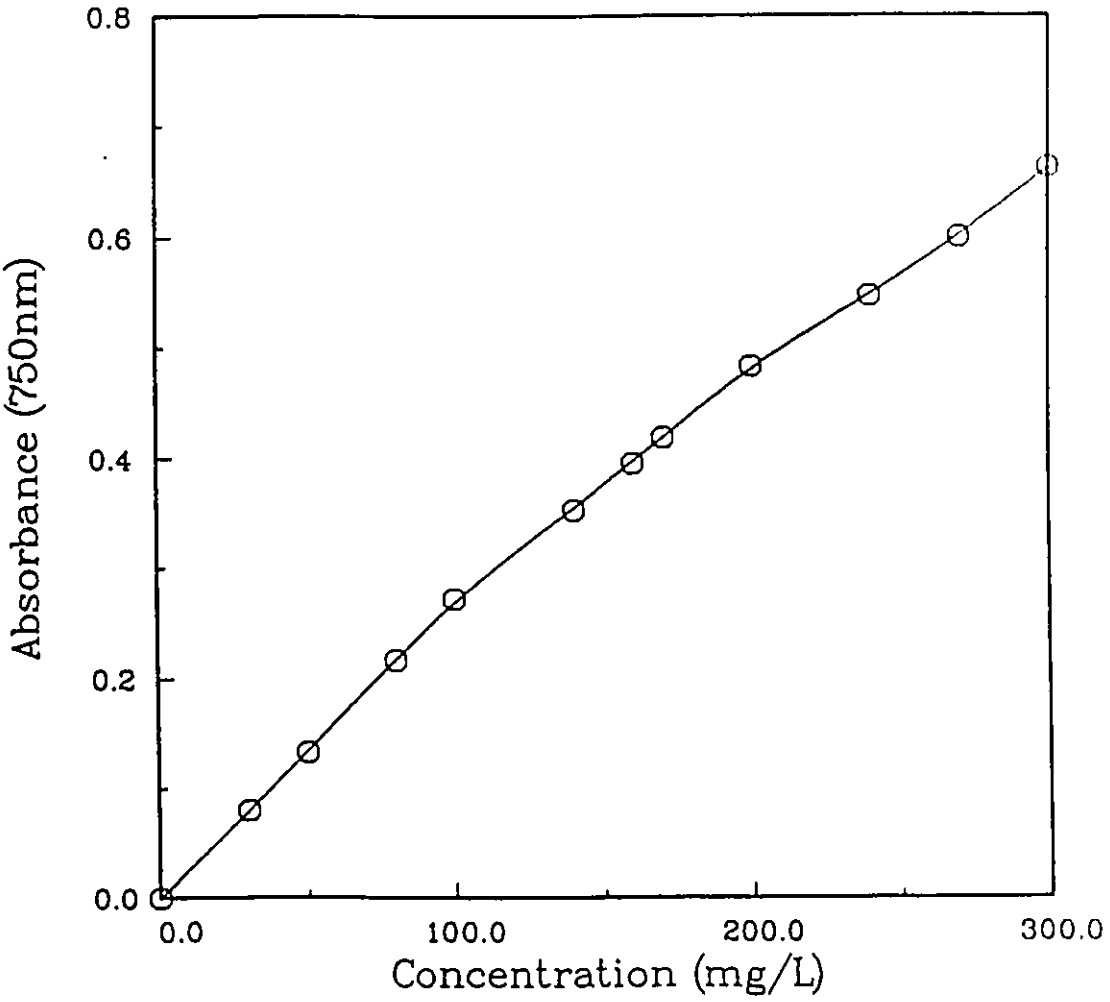


Figure B.3: Standard curve for measurement of protein by the method described by Lowry et al. (1975)

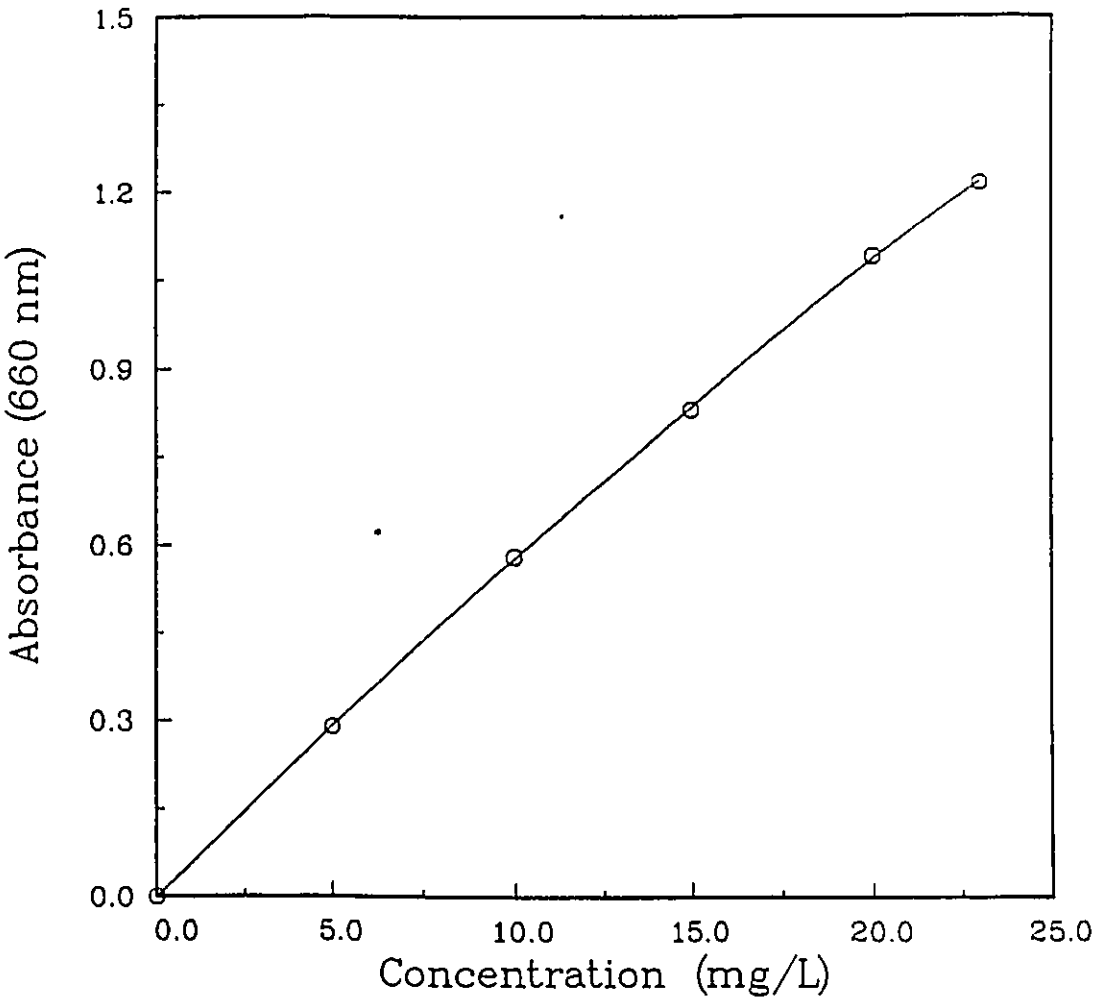


Figure B.4: Standard curve for measurement of inorganic phosphorus by the method described by Harland and Harland (1980)

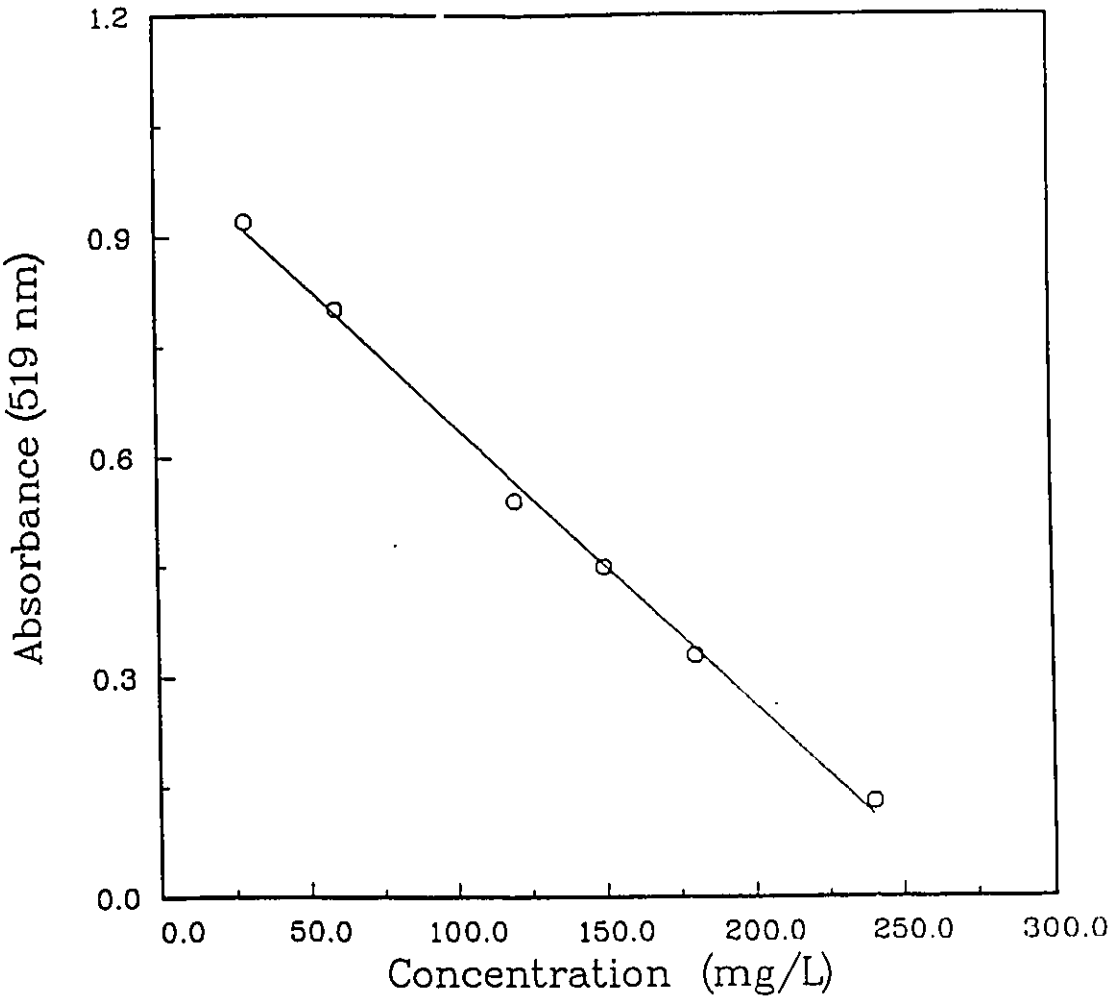


Figure B.5: Standard curve for measurement of phytic acid concentration by the method described by Haug and Lantzch (1983)

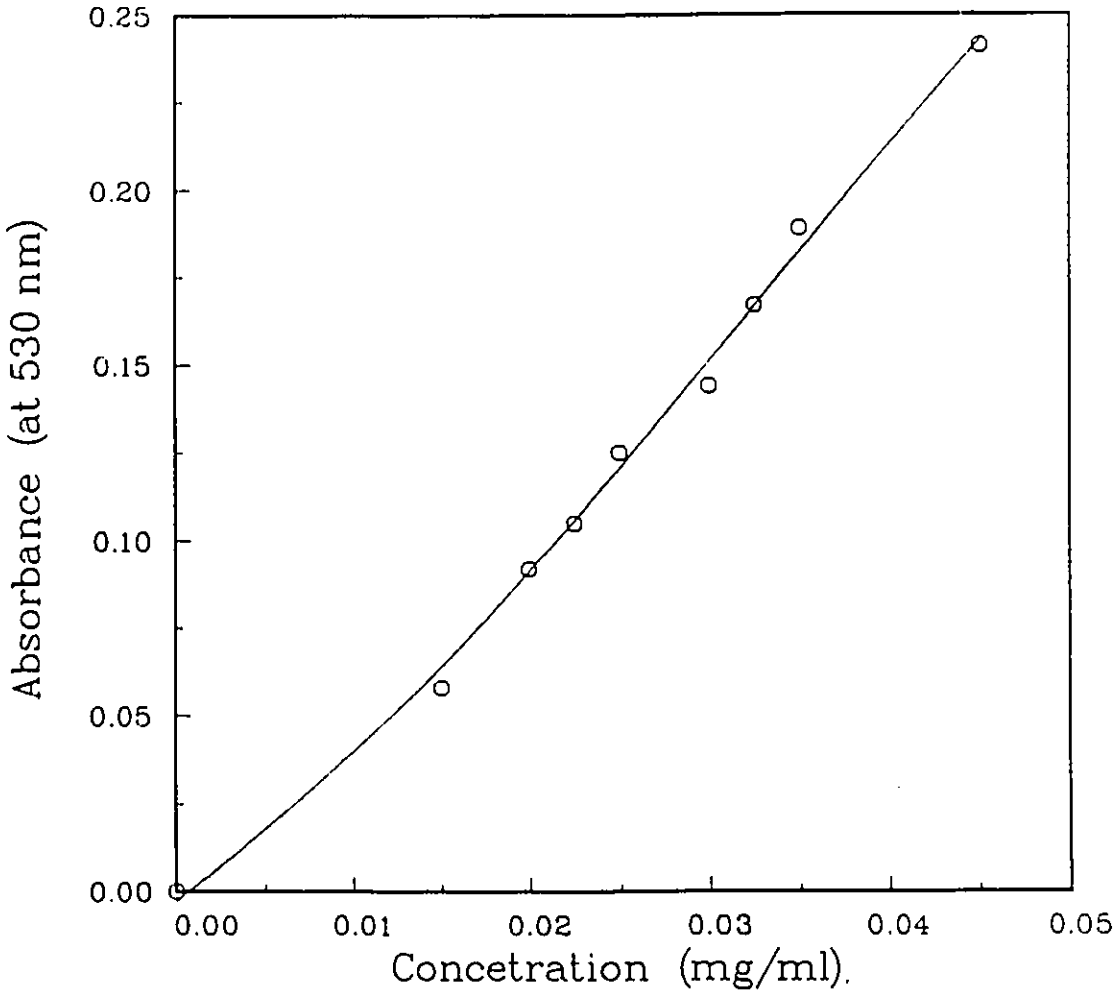


Figure B.6: Standard curve for measurement of glucosamine concentration by the method described by Blix (1948)

Appendix C

Relatioship Between Biomass and Glucosamine Content

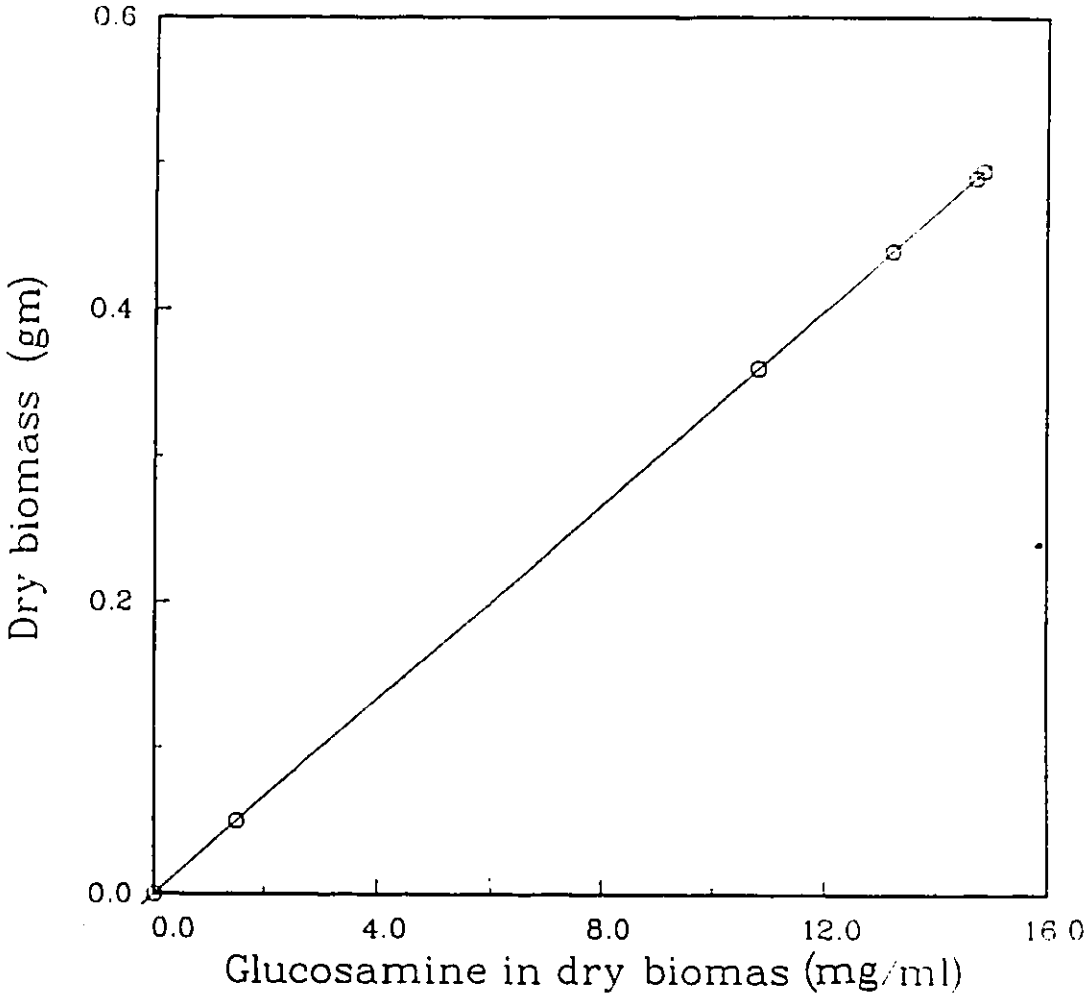


Figure C.1: Changes in dry weight biomass and glucosamine content in liquid medium