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REDUCTION OF PHYTIC ACID CONTENT IN CANOLA MEAL BY SOLID STATE FERMENTATION

A Thesis submitted to the University of Ottawa in partial fulfillment of the requirement for the degree of Master of Applied Science in Chemical Engineering

By

VIPINACHANDRAN NAIR

OTTAWA, ONTARIO

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UNIVERSITÉ D'OTTAWA
UNIVERSITY OF OTTAWA

Abstract

Solid state fermentation of canola meal has been carried out for the reduction of its phytic acid content. Several microorganisms were surveyed for this purpose. The phytic acid content of canola meal was reduced by 26%, 60% and 66% using *Saccharomyces cerevisiae*, *Rhizopus oligosporus* NRRL 2990 and *Aspergillus niger* NRC 401121 respectively after 120 hours of fermentation.

Aspergillus ficuum NRRL 3135 was found to be the best for the reduction of phytic acid content. For this reason, this microorganism was studied more extensively.

In the study of the phytase (EC 3.1.3.8) from *Aspergillus ficuum* NRRL 3135, it was found that the production of the enzyme in a submerged batch process was inhibited by high concentrations of glucose. The inhibition was overcome by applying a fed batch technique in the production of the enzyme.

Tests carried out at different oxygen concentrations revealed that aeration had a beneficial effect on the production of the enzyme.

The study of some characteristics of the enzyme showed that the optimum pH and temperature for the activity of this phytase are 5.0 and 60°C respectively. Preincubation of the enzyme preparation at 60°C resulted in relatively fast denaturation of the enzyme. Upon storage at 4°C, it lost only 15% of the activity in five weeks.

Aspergillus ficuum NRRL 3135 also produced phytase when grown on canola meal by a solid state technique. The enzyme catalyzed the degradation of the phytic acid present in the meal and completely eliminated it, rendering the commodity more suitable for animal feed. As a result of the growth of the microorganism, a 15% increase in the total amount of protein in the meal was noticed.

In certain batches, using solid state fermentation of canola meal by *Aspergillus ficuum* NRRL 3135, a complete reduction of the phytic acid content in canola meal was achieved in 48 hours.

The larger amount of biomass in the inoculum and the older inoculum increased the rate of phytic acid hydrolysis.

The optimum moisture content of the medium was found to be 67% for the hydrol-

ysis of phytic acid in a SSF process.

Both homogenization of the inoculum and the substitution of water in the semi-solid medium with acetate buffer resulted in a faster reduction of the phytic acid content.

The enzyme preparation extracted from canola meal after it was treated in a SSF process was also used for the reduction of the phytic acid content in new batches of canola meal both in a semi-solid medium and in a liquid medium. In the semi-solid medium, 58% of the phytic acid was hydrolyzed at 45°C in 20 hours, while 100% hydrolysis was noticed at 50°C in 12 hours in the liquid medium.

The SSF process seems to be beneficial for the upgrading of the canola meal by both reducing its phytic acid content and increasing the amount of protein.

Nomenclature

CHO	carbohydrate
DEAE	diethyl amino ethyl
EDTA	ethylene diamine tetraacetic acid
g	gravity force (as a measure of rotational speed)
K_i	inhibition constant
K_m	Michaelis-Menten constant
M	multivalent cation
NO_x	nitrogen oxides
PA	phytic acid
PD	potato-dextrose
Phy-P	phytate phosphorus
PRO	protein
rpm	revolutions per minute
SSF	solid state fermentation
TCA	trichloro acetic acid
v	volume
v/v	volume/volume (mL/100 mL)
V_{max}	maximum rate of enzyme reaction
w	weight
w/v	weight/volume (g/100 mL)

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Chapter 1

Introduction

Rapeseed was brought into Canada for cultivation in 1942. At that time, rapeseed oil was used only as a lubricant due to its adhesive property to wet metal surfaces. In Canada, edible oil was first extracted from rapeseed in 1956. However, the oil was not nutritionally viable due to the presence of high levels of cholesterol and saturated fatty acids. In the 1970's, Canadian scientists developed a new cultivar of rapeseed which has very low levels of cholesterol and saturated fatty acids. The industry has estblished the name "Canola seed" to characterize this particular cultivar. Since then, the market value of this seed and oil has increased considerably. During the past decade, canola seed has become the most important oil seed in the world economy. Canada has become the world's largest producer of the seeds and the largest exporter of canola oil and seeds (Harris, 1988).

Canola seed is very rich in oil (40-45%) and protein (20-25%). Canola oil is extracted from the seed and is used for the production of margarine, shortening and salad oil. Due to low levels of cholesterol and saturated fatty acids, canola oil is more popular than soybean oil, corn oil, palm oil, and peanut oil (Harris, 1988).

Canola meal is a by-product of canola oil production and is widely used in Canada in rations for poultry, pigs and cattle because of its high nutritional value and availability at a competitive price with other protein supplements. The protein content of canola meal ranges from 35 to 40%. The other major protein supplement

usually used in animal feed is soybean meal which contains 40-45% protein. While canola meal is rich in some of the amino acids, soybean meal is rich in some others. Thus, it has been found that both canola meal and soybean meal complement each other when used in rations (Clandinin, 1986). The presence of larger amounts of total phosphorus in canola meal than in soybean meal is also a definite advantage. Canola meal is also a better source of selenium and choline. Detailed comparison of canola meal and soybean meal is given in Appendix A. However, the presence of phytic acid in large amounts reduces the nutritional value of the canola meal. Phytic acid (*myo*-inositol hexaphosphoric acid) is the major storage form of phosphorus in many seeds and cereals; up to 80% of the phosphorus has been reported to be in the form of phytate (Lolas and Markakis, 1977). Phytic acid forms complexes with multivalent cations such as Ca^{2+}, Fe^{3+}, and Zn^{2+} and thus reduces the bioavailability of these minerals. Phytic acid also forms complexes with protein and thereby obstructs their enzymatic degradation. It is also known to inhibit many enzymes such as α-amylases (Sharma et al., 1978), trypsin, tyrosinase and pepsin (Graf, 1986).

Harland and Harland (1980) reported that no phytate is present in banana, celery, citrus fruits, lettuce, mushroom, onion and prune. Very low amounts of phytate were found in apple, carrot, broccoli, and green bean. Moderate amounts of phytate were found in potatoes, figs, artichokes and strawberries. The greatest amounts of phytate have been reported in legumes, cereals and nuts. The hydrolysis of phytic acid will liberate inorganic phosphorus which can be easily utilized in metabolic processes.

It is necessary to hydrolyze phytic acid content of food and feed because of the nutritional implications stated above. Although chemical methods can be adopted for the removal of phytic acid, due to the expense of the process as well as the loss of some nutritional value of the material, other methods have been developed. Solid state fermentation (SSF) has been found to be an effective way to hydrolyze phytic acid. A fermentation process in which the medium is not a free liquid is

termed solid state fermentation. These processes have been in use for hundreds of years, and are still used on a commercial scale for the production of various types of fermented foods. Examples of SSF are bread dough formation, cultivation of mushrooms, production of cheese, production of oriental foods such as soy sauce, tempeh, and oncom, and production of citric acid, vineger and aflatoxin.

The objectives of this study are:

1. to study the reduction of the phytic acid content in canola meal: a) by solid state fermentation using various microorganisms under different conditions; b) using the enzyme preparation extracted from the canola meal treated in a solid state fermentation process; and

2. to study the production and the characteristics of the enzyme.

Chapter 2

Literature Review

2.1 Canola oil

Canada produced more than 4.3×10^6 tons of canola oil in 1988/89 (Statistics Canada). The oil is used mainly for the production of margarine, salad oil and shortening. Detailed comparison of different types of oil production in Canada is given in Appendix A. Recently, canola oil has been investigated for its feasibility in diesel engines (Zuk et al., 1981). They concluded that canola oil could be substituted for diesel fuel without a major change in the engine performance and that there were only low levels of particulates, NO_x and aldehydes in exhaust emissions when canola oil was used. However, they pointed out that the higher viscosity of canola oil could be a problem in cold weather but the addition of alcohol to the oil in different proportions would reduce the viscosity and hence would make the mixture more feasible. It was also speculated that due to the expense of processing, canola oil can compete with crude oil only when the price of crude oil exceeds $40.00 per barrel.

2.2 Canola meal

Canola meal is a by-product of canola oil production. It is composed of 35-40% proteins, 28-32% carbohydrates, 11-15% crude fiber, 3-7% phytic acid and some minerals. Since large quantities of canola oil are produced every year, canola meal is also available in large amounts. Due to the presence of large amounts of nitrogen and phosphorus, canola meal was used mainly as a fertilizer. Recent developments have shown that canola meal can be used as a protein supplement in animal feed due to its richness in protein (Clandinin, 1986).

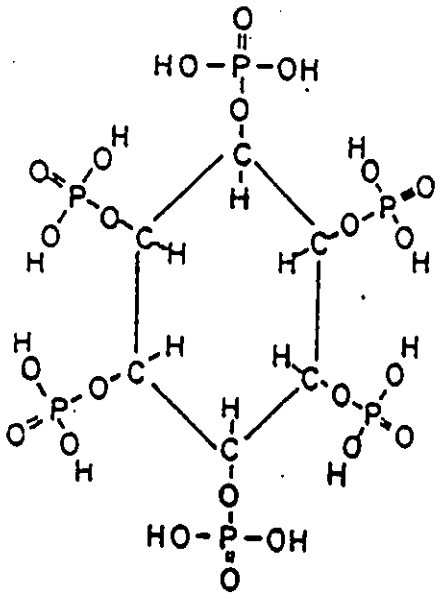
2.3 Phytic acid

2.3.1 Structure and Occurrence

Phytic acid (*myo*-inositol hexaphosphoric acid) occurs primarily as a salt of mono- and divalent cations. The major accumulation sites of phytate in cereals and legumes are the electron-dense aleurone layers called globoids (Reddy et al., 1989). Phytate is the major storage form of phosphorus in many seeds and cereals. Up to 80% of their phosphorus content has been reported to be in the form of phytate (Lolas and Markakis, 1975;1977). Phytate contents of many cereals and seeds are given in Appendix A.

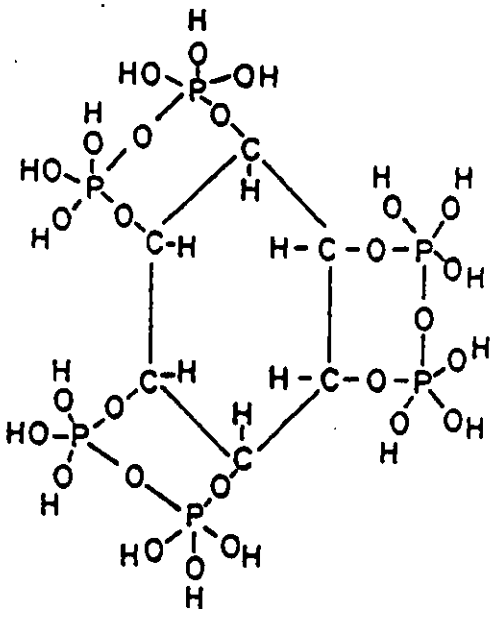
Analytical investigation on phytic acid by Brown et al. (1961) verified that phytic acid exists in two different forms. These structures are given in Fig. 2.1 a,b,c,d.

(a)



ANDERSON

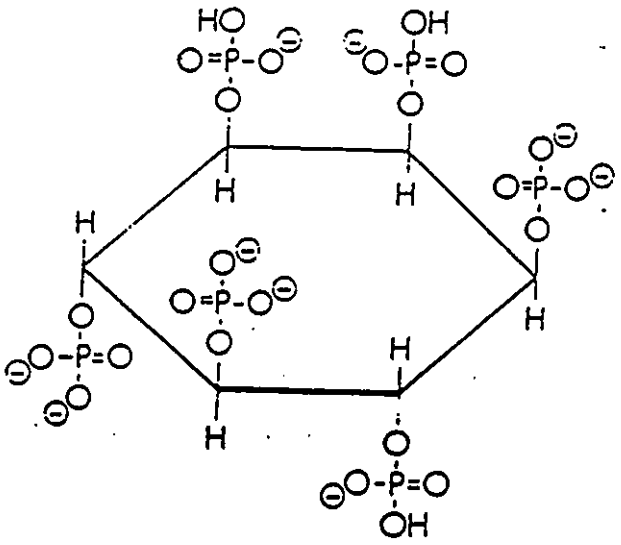
(b)



NEUBERG

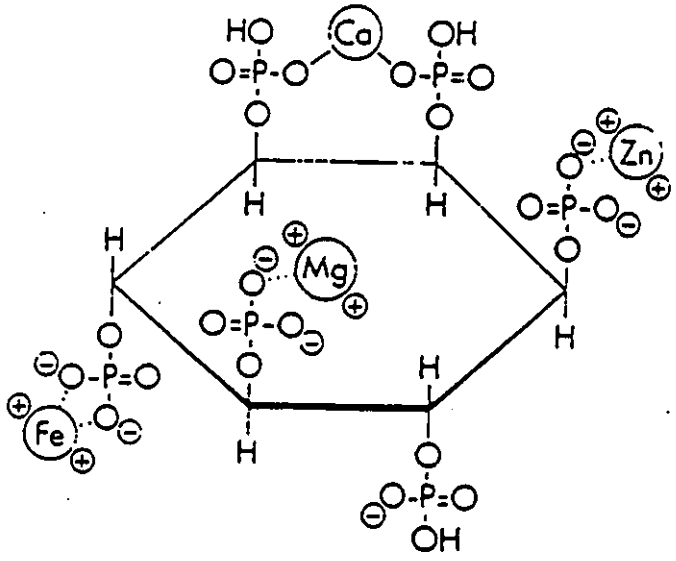
Figure 2.1: Structures of phytic acid; (a) - Anderson model, (b) - Neuberg model

(c)



PHYTIC ACID

(d)



PHYTIC ACID CHELATE

Figure 2.1: Structures of phytic acid (cont'd); (c) - Ionic form in solution, (d) - Phytic acid chelate

2.3.2 Applications

There are many applications for phytic acid. It is used in many countries as a food preservative in order to prevent product discolouration and to improve the quality and the shelf-life of the food materials (Reddy et al., 1989). It has been reported that phytic acid inhibits the oxidation of ascorbic acid (vitamin C), stabilizes sorbic acid and prevents the peroxidation of fats and oils (Graf, 1986). Due to its chelating property, phytic acid can be used to remove metals from liquid products such as wines and other beverages.

Phytic acid is also known to promote commercial fermentation processes for the production of antibiotics, yeasts, lactic acid and enzymes (Graf, 1986). The iron-chelating property of phytic acid leads to its industrial use as an anti-corrosion agent (especially in cooling water and antifreeze), a water additive for preventing scale formation, an iron-stabilizing agent in the paper industry and an antistatic agent in the polymerization of ethylene to produce polymers with good transparency (Reddy et al., 1989).

Numerous medical applications have also been derived from phytic acid. It binds to hydroxyapatite (a chief structural form of vertebrate bone) to form a uniform monomolecular surface layer that inhibits several physiological processes such as bone desorption, plaque formation and enamel dissolution. Phytate may also provide some protection against toxic mineral absorption in humans (Reddy et al., 1989).

2.3.3 Nutritional Implications

Phytic acid complexes with proteins at various pH values. At low pH, phytic acid has a strong negative charge while many proteins may have positive charges. In such cases, $-NH_3^+$ groups on protein will bind to the phosphate groups of phytic acid (Fig. 2.2 a). Multivalent metal 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 the 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.

The major concern over phytate-mineral interaction arises due to the ability of phytate to form insoluble complexes with minerals at physiological pH values. The bioavailability of minerals such as calcium, magnesium, iron and zinc from the food materials derived from cereals, seeds and legumes is considerably reduced due to the presence of phytic acid (Reddy et al., 1989).

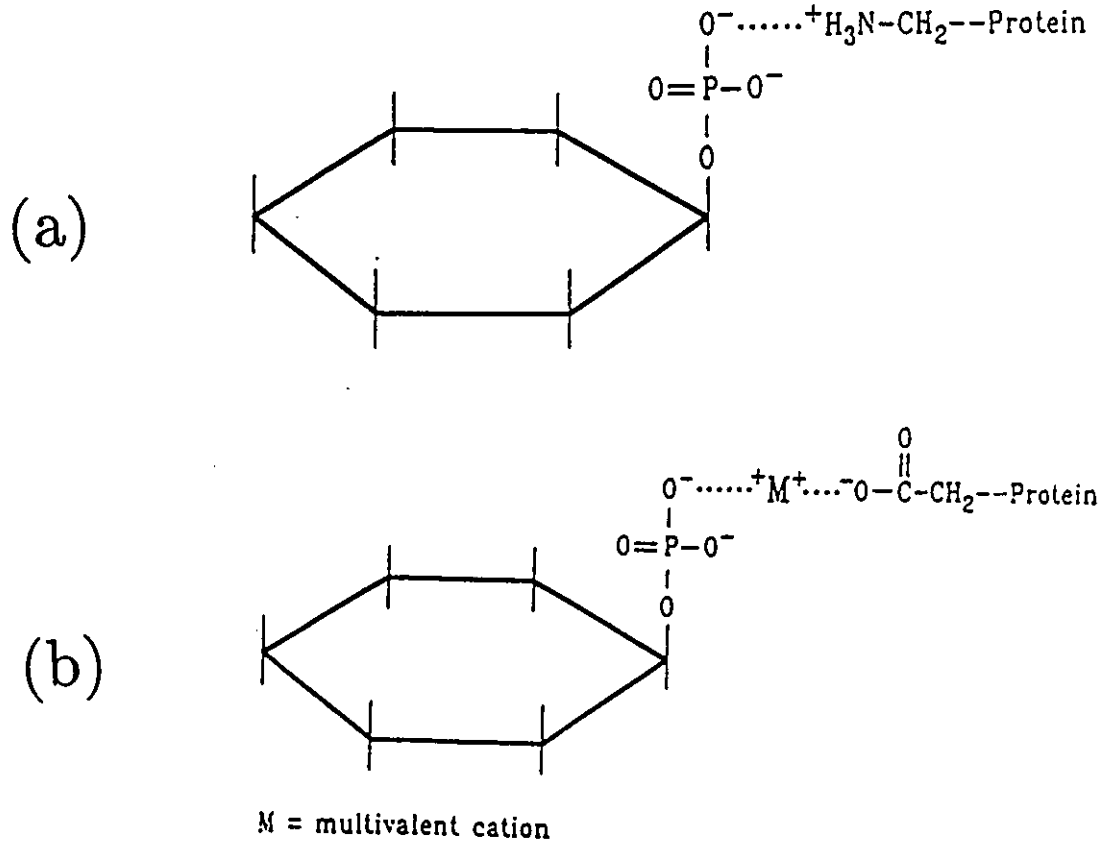


Figure 2.2: Complexation of phytic acid with protein; (a) - low pH, (b) - intermediate pH

2.3.4 Phytate Removal

In cereals and seeds, phytate is associated with specific components and can be selectively removed by mechanical processes (Appendix A). About 89% of the phytate in corn can be removed by milling followed by germ separation. Polishing of rice has also been shown to remove significant amounts of phytate. However, dehulling (removal of seed coat) apparently increased the phytic acid content of beans (Deshpande et al., 1982).

Gillberg and Tornell (1976) studied the dissolution of nitrogen and phosphorus containing substances from defatted rapeseed meal. Results from these experiments showed that the removal of phytic acid also caused the removal of some minerals such as calcium, zinc and sodium. They also observed that the extraction of rapeseed meal at pH higher than 8.0 removes a large amount of protein. The solubility of phytic acid and its salts decreases with increase in pH.

Ford et al. (1978) developed a process for the removal of phytate from soy protein. They used a combination of high pH and low calcium concentration or a low pH and high calcium concentration and succeeded to remove up to 90% of phytate from soybean extracts. However, this process also removed most of the protein content and some minerals.

Erdman Jr. (1979) investigated many possible ways to reduce phytic acid content. Heat treatment of several oil seeds proved rather inefficient, since only 10% of the phytic acid content was reduced by autoclaving for 30 minutes. Soaking oil seeds was also found to be inappropriate due to the low endogenous phytase of oil seeds. Some oil seeds can be fractionated and processed directly by extracting the specific components of the seeds, but this process is very selective and depends on pH, chelating agents, etc., and therefore, a common method could not be used for all oil seeds.

Phytate removal from soy protein was reported by Hartman Jr. (1979). The process involved the precipitation of phytate at high pH followed by the centrifugation or filtration. Sequential control of pH and temperature followed by purification

of soy extract proved satisfactory for the removal of 90% of the phytic acid. Temperature and pH control were very crucial not only for the effective removal of phytic acid but also for the prevention of protein degradation.

The extraction of canola meal with water at pH 7.0 resulted in the solubilization of only 8-12% of the phytic acid (Alli and Houde, 1987). This process also solubilized 24-31% of protein. The extraction at higher pH (11.0) solubilized only 6-9% of the phytic acid. However, the extraction of the meal at pH 4.5 led to the solubilization of 56-62% of phytic acid, as well as 40-50% of protein. Sequential extractions of the meal at pH 1.5 followed by the one at pH 4.5 resulted in the solubilization of 75-80% of phytic acid. The conclusions drawn from this study were: canola phytate is relatively insoluble in water, phytate has relatively high solubility at low pH, and protein has relatively high solubility at high pH. This study also points out that although most of the phytate can be removed by extraction at acidic pH, some protein associated with phytic acid will also be removed. This will result in the loss of some nutritional value of the meal.

Smith and Rackis (1957) reported that 72% of the phytate in soybean could be removed by dialysis at pH 6.5 without an appreciable loss of proteins. Okubo et al. (1975) investigated the removal of phytic acid from defatted soybean meal to produce low-phytate protein concentrates. The process involved two steps; the dissociation of phytate from protein at different pH, and the removal of dissociated phytate by ultrafiltration using a membrane. It was found that the amount of phosphorus removed by dialysis is a function of pH. More than 95% of phosphorus was removed by dialysis at pH 5.5. Ultrafiltration has been reported to be economically and nutritionally more feasible in the food processing industry than other methods such as acid leaching for the removal of phytate.

All these methods were effective for extracting the phytic acid, but the extraction process also resulted in the loss of some protein and minerals. Therefore, other methods have to be investigated for the removal of phytic acid without the loss of the nutritional value of the substrate.

Several researchers have reported the use of enzymes produced by microorganisms or plants for the hydrolysis of phytic acid. Solid state fermentation has been found to be an effective way for the reduction of phytic acid content of oncom (Fardiaz and Markakis, 1981).

2.4 Solid State Fermentation (SSF)

Solid state fermentation (SSF) has been known for hundreds of years. Food fermentations such as cheesemaking, oriental fungus-processed foods, the preservation of fish and animal products and the production of vineger are different types of solid state fermentation processes (Hesseltine, 1967, 1972; Aidoo et al., 1982).

The production of enzymes using mold was reported as early as 1894 (Hesseltine, 1972; Han and Anderson, 1975). Since then, SSF has been widely used for the production of enzymes using *Aspergillus, Penicillium* and *Rhizopus* species. *Aspergillus oryzae* has been found to be best suited for industrial purposes because of its ease of handling.

The koji process for the production of enzymes by soy bean fermentation uses wooden trays in which soy beans, rice and wheat are inoculated with the koji mold (usually *A. oryzae*) and incubated for three days (Hesseltine, 1967; Aidoo et al., 1982). The problem with the tray method is the difficulty in controlling the operating conditions. Although rotary drums have been used to replace trays, low enzyme production has been reported due to the damage of the mycelia caused by rotation of the drum.

Although citric acid had been produced by submerged-culture techniques, tremendous improvement in its production in the SSF process of cane or beet pulp impregnated with sucrose or molasses has been reported (Hesseltine, 1972). The substrate was inoculated with *A. niger* and 45% yield in citric acid was obtained. Further improvement in yield by adding methanol to the medium has been reported by Lakshminarayana et al. (1975).

Conversion of agricultural wastes to more useful products has received 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) developed a fermentation process for the bioconversion of agricultural wastes into proteinaceous food products using *Chaetomium cellulolyticum.*

Composting is another SSF process which consists of two phases. During the first phase, readily available sugars are fermented leading to the production of heat which in the second phase favours the thermophilic microbes in attacking less readily available sugar sources such as polysaccharides (Hesseltine, 1972).

Raimbault and Alazard (1980) and Raimbault et al. (1985) investigated the growth of *A. niger* in a solid state fermentation process for the protein enrichment of starchy materials such as cassava. They studied the growth kinetics and the changes in pH, protein, and carbohydrate. In this study, an incubation device (Fig. 2.3) that provides the substrate with humidified air at a controlled temperature was used. The optimum conditions for the growth of *A. niger* were 50-55% moisture, 35°C, and $2x10^7$ spores per gram of substrate. The aeration rate was 4 to 6 L/h per 20 g of the substrate.

Czajkowska and Ilnicka-Olejniczak (1988, 1989) studied the protein biosynthesis by microscopic fungi in a SSF process. They used *A. oryzae* to study the effects of humidity of the medium, the dose of inoculum, and aeration on protein biosynthesis from starchy raw materials. They observed that the inoculum should contain at least 10^6 spores per gram of the medium and the pH of the medium should be near 6.5. They concluded that the moisture level and the aeration should be 60% and 4 L/h per 100 g of the medium respectively for the maximum yield of protein. Under these optimum conditions, the microorganism consumed 25 g of carbohydrates and produced 6.0-6.3 g of protein per 100 g of the medium.

All these investigators reported advantages and disadvantages of solid state fer-

mentation. These are summarized in the next section.

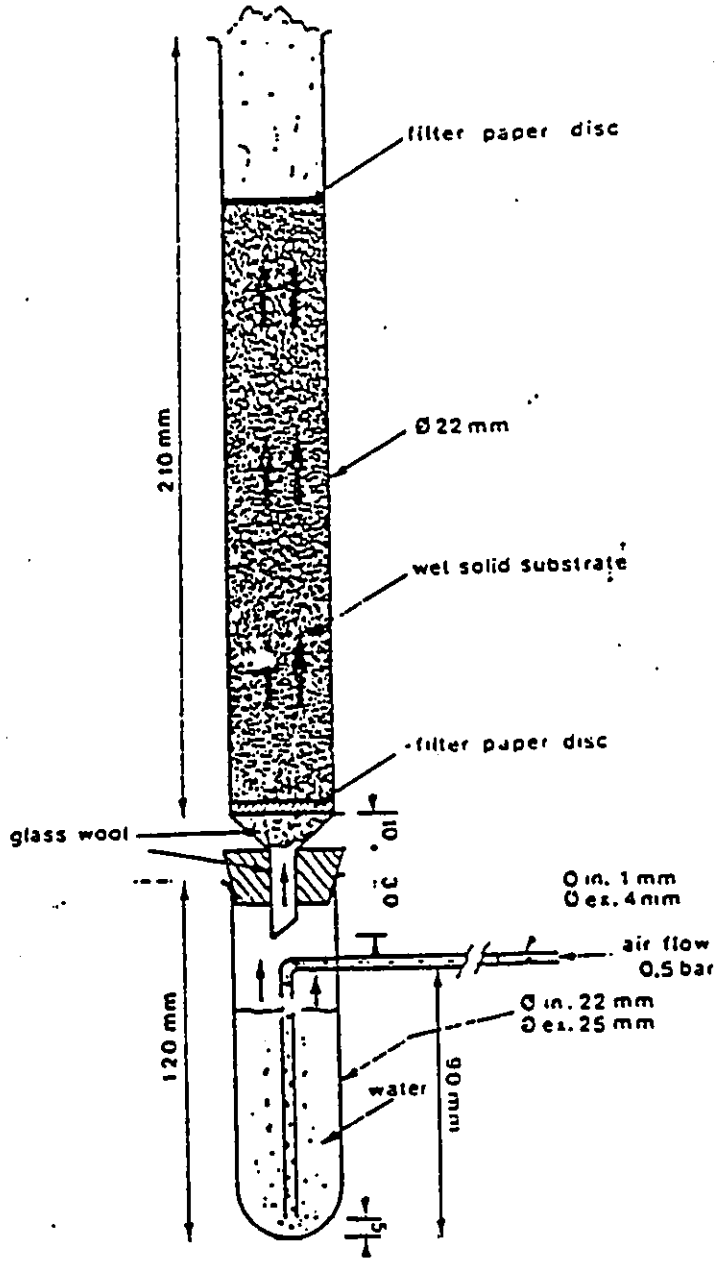


Figure 2.3: Incubation device designed by Raimbault and Alazard (1980) - Single unit

2.4.1 Advantages and Disadvantages of SSF

There are many advantages of SSF, among them are the following:

1. In many cases, since the substrate is itself rich in nutrients, the addition of extra nutrients is not necessary. Substrate supplemented with water will suffice for the microbial growth.
2. Usually, the pretreatment of substrate is not necessary.
3. The space requirement for the reaction vessel is minimal, since the substrate is very concentrated.
4. Due to a low moisture content, most bacterial contamination are avoided.
5. The product yield is much higher than in liquid media.
6. The product may be extracted directly from the reaction vessel, and as a result, less volume of extractant is required and a more concentrated product is formed than in a submerged culture.

Some of the disadvantages of SSF are:

1. The power requirements may be high for continuous agitation of solid material.
2. A large amount of inoculum is required.
3. The chances of contamination are high if water is to be added during the course of fermentation.

2.4.2 Phytic Acid Reduction by SSF

Solid state fermentation has been reported to be an effective way for the hydrolysis of phytic acid. It enhances the hydrolysis of phytic acid, releasing inorganic phosphorus into the medium, and at the same time improves the nutritional value of the substrate.

Ranhotra et al. (1974) investigated the hydrolysis of phytic acid in soy products during the bread making. They observed that there was no phytase activity in soy flour. Complete hydrolysis of phytate was observed when the bread was made from

wheat flour. During bread making using soy-fortified wheat flour (10% soy flour and 90% wheat flour), the amount of phytic acid was reduced by 46% to 88% depending on the amount of yeast used. They found that without adding any yeast, there was approximately 46% reduction in phytic acid, which was due to the phytase activity of wheat. When they added 9 g of yeast per loaf of bread (1 lb), 88% of the phytic acid was hydrolyzed. There was no further reduction in phytic acid even after the addition of 15 g of yeast. They concluded that the phytase activity in wheat and yeast was quite high. Increased hydrolysis of phytate was accompanied by an increase in the level of inorganic phosphorus which may have inhibited further hydrolysis.

Chang et al. (1977) investigated enzymatic hydrolysis of phytate in whole dry beans. Phytic acid was extracted from bean tissues as follows: five grams of whole beans were cleaned with 0.007 M NaCl and distilled water, and rehydrated for 5 hours at room temperature. The rehydrated beans were blended with 100 mL of 3% trichloro-acetic acid (TCA) at full speed for two minutes. The slurry was then extracted at 60°C for 30 minutes. Phytate phosphorus was determined colorimetrically as described by Makower (1970). Inorganic phosphorus was also determined colorimetrically by Allen's method (1940). The effects of temperature, pH, and freezing on phytate hydrolysis were studied. They found that 3% TCA was a better extractant for phytic acid than 0.5 N HCl. Mild heating (near 60°C) also improved the removal of phytate. Incubation of beans at different temperatures revealed that 49% of phytic acid could be hydrolyzed at 60°C, which was found to be the optimum temperature. Soaking for 24 hours in acetate buffer (pH 4.0 to 5.8) caused an apparent decrease in phytate content of beans. The greatest decrease was noticed between pH 5.0 and 5.5. Fifty percent decrease in phytic acid content was observed by exposing rehydrated beans to water-saturated air at 60°C.

Sudarmadji and Markakis (1977) performed the hydrolysis of phytic acid in soybeans by fermenting with *Rhizopus oligosporus* NRRL 2710. They found that overnight soaking did not reduce the phytic acid content of soybeans; boiling the

seeds for 30 minutes reduced it by 14% and the fermentation of tempeh at 30°C reduced 33% of the phytic acid content. They concluded that there was no phytase activity in soybeans and the reduction of phytate was caused by the phytase produced by *Rhizopus oligosporus*. In order to verify the presence of phytase in *R. oligosporus*, the mold was grown on different media and the enzyme was extracted from the ground mycelia using a 2% aqueous solution of $CaCl_2$. They found that the potato-dextrose agar medium supplemented with peptone was luxuriant for the growth of the microorganism and it yielded the highest phytase activity. The optimum pH for phytase activity was reported to be 5.6 and the K_m value was 0.28 mM using sodium phytate (10^{-3} M) as the substrate.

Harland and Harland (1980) obtained 65% reduction of phytic acid content in rye bread and 33% in both white and whole wheat breads using yeast. It was observed that an increase in yeast cells and fermentation time increased the reduction of phytic acid. The major reduction of phytic acid content in all three breads occurred during the first two hours of rising period.

Reddy and Salunkhe (1980) investigated quantitative changes in phosphorus compounds and mineral contents of black gram, rice and their mixture during fermentation. Equal parts of rice and black gram cotyledons were washed, soaked for 2 hours and then ground to a fine paste, containing 60% moisture. One percent salt was added to the medium and incubated at 30°C. Similar experiments were also performed with rice and black gram individually. The fermentation of rice alone for 8 hours resulted in complete hydrolysis of phytate. After 45 hours of fermentation, only 13.3% and 48.8% of phytate were hydrolyzed in black gram, and black gram and rice blends respectively. During this fermentation, no apparent changes were observed in mineral contents.

Phytic acid reduction in oncom (fermented peanut press cake) was investigated by Fardiaz and Markakis (1981). They used several molds but the best one for the reduction of phytic acid was *Rhizopus oligosporus* ATCC 22959. The cake was pre-treated with citric acid to remove the oil and at the same time to keep the

pH of the cake at the desired level where the phytase activity was reported to be high. Addition of some starch to the cake promoted the mold growth. For the inoculation, spores were harvested with sterilized distilled water after one week of mold growth on potato-dextrose agar. Approximately $2x10^9$ spores were used to inoculate 1500 g of the substrate. The incubation was performed in Petri dishes at 30°C. Phytic acid was determined using the method described by Wheeler and Ferrel (1971). Inorganic phosphorus was determined colorimetrically according to Allen's method (1940). They observed an excellent mold growth after 36 hours of fermentation. After 72 hours of fermentation, *Rhizopus oligosporus* and *Neurospora sitophila* reduced the phytic acid content of oncom by 95% and 58% respectively.

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.

All these research works imply that a SSF process would be appropriate for the effective removal of phytic acid from the canola meal. However, a survey of various microorganisms is necessary before proceeding with the fermentation process.

2.4.3 Phytase production and study of its characteristics

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. The optimum pH was found to be 5.15 and the optimum temperature was 55°C. 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.

Gibbins and Norris (1963) extracted phytase from dwarf beans (*Phaseolus vulgaris*). The cotyledons were homogenized with 5 volumes of 0.01 M maleate 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 chromatography and ultrafiltration. The crude enzyme had an activity of 0.025 units[1] and the purified enzyme had a maximum activity of 0.1 units. The optimum pH was 5.2 and the K_m value was 0.15 mM. The phytase was inhibited by higher substrate concentrations (4 mM sodium phytate). Calcium and magnesium ions were found to have a stimulatory effect, while iodoacetamide and fluoride ions had an inhibitory effect on the phytase activity.

Chang (1967) extracted phytase from germinating corn seeds, and studied the properties of the enzyme. The germination of corn seeds was conducted in darkness 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). These 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. The optimum pH was 5.6 and the optimum temperature was 50°C. It was observed that heating the enzyme at 50°C for ten minutes did not affect the activity, while at 60°C, 40% of the activity was lost. Ten minutes of heating at 80, 90 and 100°C completely inhibited the phytase activity. Calcium chloride (0.01 M) activated the enzyme and sodium fluoride (0.05 to 10 mM) inhibited it.

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 10,000 x g. The supernatant was designated as the crude

[1]One unit of enzyme was defined as the amount of enzyme required to release one mg of phosphorus per hour at the given temperature and pH.

enzyme which was then subjected to further purification. The first step was the heat treatment at 60°C for 6 min. followed by the ammonium sulfate fractionation. The fraction precipitating between 50% and 70% saturation was collected and dialyzed against 0.001 M Tris-HCl buffer (pH 7.0). An equal volume of cold acetone was added to the dialyzed fraction. After centrifuging, the pellet obtained was washed with cold acetone and dissolved in a small volume of buffer. The last purification step involved the addition of 1 g bentonite to every 30 mg enzyme and suspending in cold water. The pellet was collected and dissolved in a minimum volume of buffer. The specific activity increased from 0.001 units/mg of total protein for the crude enzyme to 0.08 units/mg for the purified enzyme. The optimum temperature for the phytase activity was 57°C, and the optimum pH was 7.5. The K_m value was reported to be 0.61 mM and V_{max} was 0.13 units. A competitive inhibition was observed in the presence of inorganic phosphorus (K_i= 0.4 mM).

Lolas and Markakis (1975, 1977) extracted the phytase from navy beans (*Phaseolus vulgaris*). The crude enzyme was prepared by homogenizing beans with a 2% aqueous solution of $CaCl_2$ in a blender. The ratio of extractant to beans was 10:1. The filtrate was centrifuged and used as the crude enzyme. The crude enzyme was also partially purified by saturating with ammonium sulfate. The fraction between 35% and 70% saturation had the highest activity. The optimum pH for the phytase activity was found to be 5.3, and the optimum temperature was 50°C. This phytase was stable at 2°C for at least six weeks in maleate buffer (pH 6.5). The phytase was found inhibited by concentrations of phytate higher than 2 mM. The K_m value was 0.018 mM. Iodoacetamide and N-ethylmaleimide (0.1 M) were found to inhibit up to 70% of the phytase activity. NaF (0.01 M) and NaCl (0.5 M) inhibited 80% and 50% of the phytase activity respectively. The presence of metal ions at low concentration (10^{-5} M) increased phytase activity while at high concentration inhibited it.

Bitar and Reinhold (1972) investigated the phytase activities in intestinal mucosae of chicken, rat, calf and man. The optimum phytase activity of the partially

purified enzyme extended from pH 7.0 to pH 7.5 in rats, while a sharp maximum was located at pH 7.4 in homogenates of human mucosa. The pH optima for the phytase of chicken and calf were 8.2 and 8.6 respectively.

Dox and Golden (1911) investigated the phytase production by *Aspergillus* species. They observed that *A. niger* has the power of utilizing phytic acid as a source of phosphorus. *A. niger* was found to produce both intra- and extra-cellular phytases. It was also observed that the intracellular phytase activity increases with the age of the microbial culture.

Shieh and Ware (1968) surveyed many microorganisms for the production of extracellular phytase. A number of *Aspergillus niger* group were found to be good producers of extracellular phytase; *Aspergillus ficuum* NRRL 3135 was found to produce the most active phytase. They also reported that high concentrations of inorganic phosphorus in the medium repressed the production of phytase. They also tested the phytase activity when produced by the microorganisms on different media. Similar observations were also reported by Han and Gallagher (1987).

Shieh et al. (1969) studied the phytase of *A. ficuum* NRRL 3135, and reported that this microorganism produces two types of enzymes for the hydrolysis of inositol phosphates; a non-specific orthophosphoric monoester phosphohydrolase that had a pH optimum of 2.0, and *myo*-inositol hexaphosphate phosphohydrolase (phytase) that had an optimum pH of 5.5.

Irving and Cosgrove (1971a,b, 1972, 1974) verified the existence of these two types of enzymes in *A. ficuum* NRRL 3135. They purified the acid phosphatase and the phytase produced by *A. ficuum* NRRL 3135 and reported that the optimum pH and the K_m value for the phytase were 5.3 and 0.013 mM respectively. They also concluded that the enzyme was not inhibited by 1 mM concentrations of oxalate, citrate or ethylene diamine tetra acetic acid (EDTA); however, 69% of the enzyme activity was inhibited by 1 mM fluoride.

Han et al. (1987) studied the phytase production by *A. ficuum* in a SSF process. They found that the minimal amount of water required for the growth of the

microorganism and the enzyme production was 15%, while the optimum level of moisture for the production of the enzyme was between 25 and 35%. The optimum pH for the phytase production was between 4 and 6. They also found that wheat bran was better as a substrate for the phytase production than soy meal and corn meal.

Han and Wilfred (1988) have reported the use of *A. ficuum* NRRL 3135 phytase in the reduction of phytic acid content in soybean and cottonseed meals. Application of microbial phytase enhanced the hydrolysis of both soluble and insoluble phytate. The enzyme was extracted from wheat bran after it was treated with *A. ficuum* NRRL 3135 in a SSF process. When the enzyme level reached about 40 units[2] in about 1-2 weeks, the enzyme was extracted from the medium using water. Thirty mL of diluted enzyme preparation (1 unit/g of substrate) was added to 2 g of the meal at pH 5.4 and incubated for several hours at 37°C. They found that the removal of phytate in soybean meal was more effective than in cottonseed meal. After 25 hours of incubation, about 85% of the total phytate in soybean meal was hydrolyzed by the enzyme treatment, while only 67% of the phytate in cottonseed meal was hydrolyzed using the same amount of the enzyme.

Based on all these information, it is proposed that a SSF process be carried out for the reduction of the phytic acid content in canola meal. Many microorganisms will be surveyed for their capability to hydrolyze phytic acid, and the best one will be studied extensively for the production of the enzyme phytase.

[2]One unit was defined as the amount of enzyme required to liberate 1 μmol of inorganic phosphate per min. under the assay condition.

Chapter 3

Materials and Methods

3.1 Microorganisms

In this work, the following microorganisms were investigated for the phytase production and the reduction of phytic acid content in canola meal in a solid state fermentation process:
Rhizopus oligosporus NRRL 2990 (NRC 2831), *Aspergillus niger* NRC 401121, *Aspergillus niger* NRC 5765, *Aspergillus carbonarius* NRC 401124, *Saccharomyces cerevisiae*, and *Aspergillus ficuum* NRRL 3135.

All microorganisms were maintained on agar slants and the inoculum were prepared either by collecting the spores from the slants or by growing them in liquid medium (Table 3.1). All the ingredients given in the table were dissolved in 100 mL of distilled water.

Table 3.1: Growth media

Table 3.1: Nutrient media[1] for the growth of the microorganisms and the inoculum preparation

	A. niger *A. ficuum* *A. carbonarius*		*R. oligosporus*		*S. cerevisiae*	
Liquid	glucose	0.5 g	glucose	2.0 g	glucose	1.0
	yeast extract	0.5 g	bactopeptone	1.0 g	yeast extract	3.0
	nutrient broth	0.8 g	KH_2PO_4	0.1 g	bactopeptone	0.35
	water	100 mL	$NaNO_3$	0.1 g	KH_2PO_4	0.2
			$MgSO_4.7H_2O$	0.5 g	$MgSO_4.7H_2O$	0.1
			water	100 mL	$(NH_4)_2SO_4$	0.1
					water	100 m
Solid	malt extract	3.0 g				
	yeast extract	0.5 g	all of the above +		all of the above +	
	glucose	0.5 g	P.D. agar	3.0 g	agar	2.0
	agar	1.5 g	agar	1.5 g		
	water	100 mL	water	100 mL	water	100 m

[1]Obtained from NRC

3.1.1 *Rhizopus oligosporus* NRC 2831

Rhizopus oligosporus NRC 2831 was found to grow well on agar slants, but very poorly in a liquid medium. It was also observed that the mycelia grown on agar slants sporulate within a few days and therefore, spores were harvested with sterile water and used as the inoculum for the fermentation. The number of spores in the inoculum was determined as follows: the spore suspension was diluted in series, a known volume of the diluted suspension was spread on agar plates, and the number of colonies developed after 24 hours of incubation at 30°C was counted. The plate containing between 50 and 100 colonies was used to calculate the actual number of spores in the inoculum, because this sample would be statistically viable.

Initially, experiments were performed in Petri dishes. One hundred grams of canola meal were placed in a large beaker, mixed with 100 mL of water and sterilized at 121°C for 45 minutes. After cooling, an amount of inoculum containing between $2x10^8$ and $3x^9$ spores was added and mixed thoroughly under asceptic conditions. The inoculated meal was then distributed into several sterile Petri dishes (approximately 20 g in each), and incubated at 30°C for several days. Analyses were done in duplicate by taking two Petri dishes each time at intervals of 24 hours and the contents were separately analyzed for phytic acid, moisture, pH, carbohydrate and protein.

Experiments were also performed in aerated chambers (Fig. 3.1) in order to see the effect of aeration and humidification on the microbial growth and the reduction of phytic acid. One hundred grams of canola meal were treated as before and transferred into the sterilized chamber and incubated at 30°C. Sterile air was provided at a rate of 500 mL/min.

The effects of spore concentration in the inoculum, addition of buffer, and aeration on the reduction of phytic acid content were studied.

A certain degree of contamination was noticed in experiments that were carried out in aerated chambers. After 72 hours of fermentation, some other microorganisms were found growing in the medium. To avoid contamination, a few experiments were

carried out in 500 mL Erlenmeyer flasks. For a typical batch fermentation, 50 g canola meal was placed in 500 mL Erlenmeyer flask, 25 mL of 0.2 M acetate buffer (pH 4.7) was added and then, after mixing thoroughly, the flask and its contents were sterilized for 45 minutes at 121°C. After cooling, the meal was inoculated with the spores, mixed thoroughly, and then incubated at 30°C for several days.

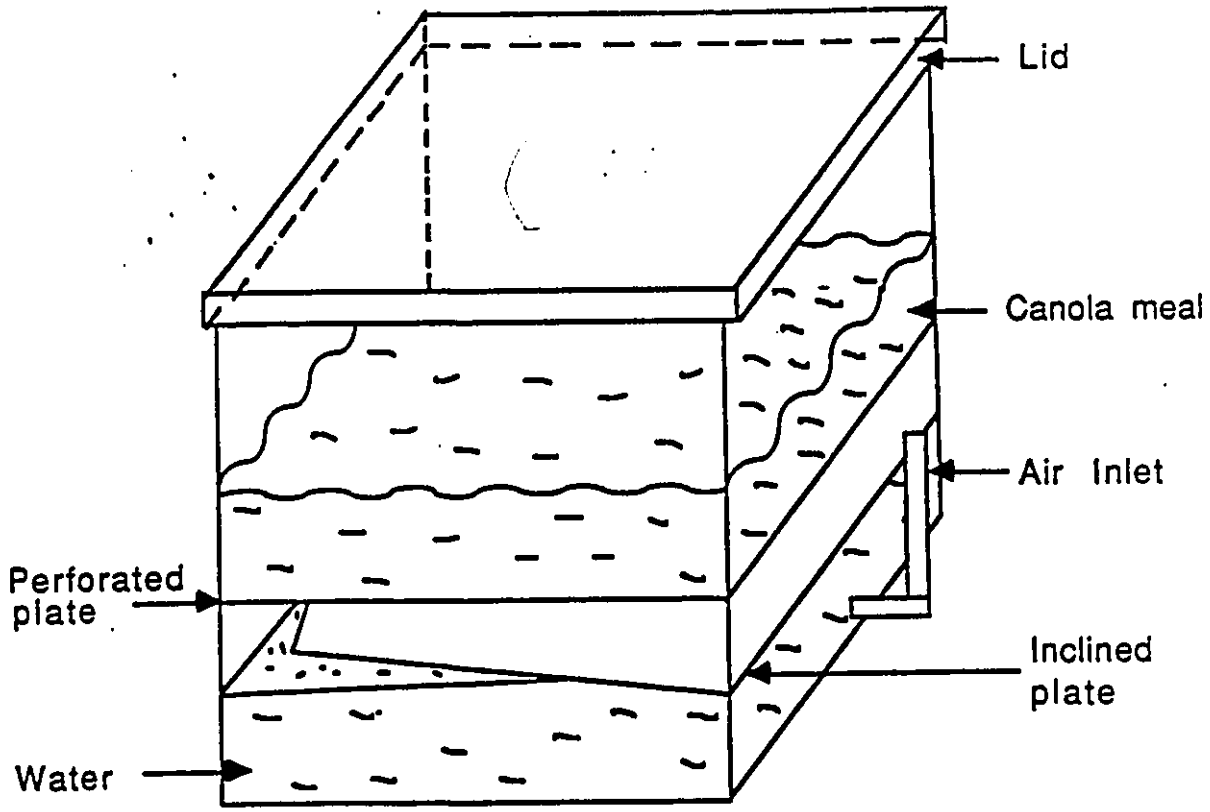


Figure 3.1: Aeration chamber designed in this work

3.1.2 *Aspergillus niger* and *Aspergillus carbonarius*

Aspergillus niger (NRC 401121 and NRC 5765) and *Aspergillus carbonarius* NRC 401124 were found to sporulate easily and therefore, a procedure similar to that for *R. oligosporus* was followed for the inoculum preparation.

The effects of spore concentration, the addition of buffer, and the effect of aeration on the reduction of phytic acid were studied using these microorganisms.

3.1.3 *Aspergillus ficuum* and *Saccharomyces cerevisiae*

Wild *S. cerevisiae* strain was grown in a liquid medium and the broth was used as the inoculum. The number of cells in the inoculum was determined by a series of dilutions and then spreading them on agar plates as in the case of *R. oligosporus*.

Aspergillus ficuum NRRL 3135 was found to be a better producer of phytase than the other microorganisms used. For this reason, *A. ficuum* NRRL 3135 was studied in more detail. This microorganism did not sporulate well on a solid agar surface, but grew well in pellet form in the liquid medium. In this case, the inoculum was prepared by growing the fungus in the liquid medium; the biomass was determined before inoculation. Experiments were carried out for the production of phytase and for the reduction of phytic acid content in canola meal. The effects of biomass concentration, the age of inoculum, the addition of buffer, the moisture content of the medium, the sterilization of the meal, and the effect of homogenization of the inoculum on the reduction of phytic acid content were studied.

Effect of glucose concentration: To study the biomass and phytase productions by *A. ficuum* NRRL 3135, several media containing 0.8% [2] nutrient broth, 0.5% yeast extract and different glucose concentrations ranging from 0.5% to 5.0% were used. The biomass and the enzyme production, and the pH were followed for several days.

In another set of experiments, the effect of fed batch technique of substrate

[2]All % are w/v (g/100 mL), unless otherwise specified

addition on enzyme and biomass production was studied. Initially, 150 mL of medium containing 0.5% glucose was prepared. After sterilization and inoculation, the medium was incubated at 30°C and after every 24 hours, 0.5% glucose was added to the medium.

Effect of aeration: To study the effect of aeration on the biomass and the enzyme production, several 500 mL Erlenmeyer flasks were filled with 50 mL, 100 mL, and 150 mL of the liquid medium. These flasks were shaken at 200 rpm for aeration. Since the volume of the broth in the flasks were different, the surface area of the broth in contact with the air were also different, and hence the aeration were also different in each flasks. After sterilization, the media were inoculated with 10% (v/v) of the inoculum. One flask from each group was taken for analysis at a time.

3.2 Analysis of samples

3.2.1 Biomass

A known volume of sample was centrifuged at 12000 x 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.

3.2.2 Reducing sugars

The supernatant after centrifuging the sample was used directly for reducing sugar determination using the dinitrosalicylic acid (DNS) method described by Weiner (1978) (see Appendix B for standard curve). The DNS reagent was prepared as follows: A) five grams 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 tartrate 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 the DNS reagent and boiled for exactly 5 min. in a water bath. After cooling, 20 mL of distilled water was added to the mixture and the absorbance was read at 600 nm.

3.2.3 Phytic acid

Many methods have been reported for phytic acid determination in cereals, flour, and protein concentrates (Makower, 1970; Wheeler and Ferrel, 1971; Latta and Eskin, 1980; Uppstrom and Svensson, 1980; Thompson and Erdman, 1982; Haug and Lantzsch, 1983). Wheeler and Ferrel (1971) have reported an indirect method for the determination of phytic acid in wheat. Phytate is extracted from the sample and reacted with iron for the iron-phytate complex formation. Assuming that Fe to P ratio is 4:6, the amount of phosphorus in phytic acid is calculated from the concentration of iron. The procedure is as follows: 2-3 g of meal was extracted with 50 mL of 3% TCA for 30 min. with mechanical shaking. The suspension was centrifuged and 10 mL of the supernatant was transferred into a centrifuge tube. Four mL of $FeCl_3$ solution (2 mg ferric iron per mL of 3% TCA) was added to the tube and the tube was heated in a boiling water bath for 45 min. After cooling and centrifuging, the supernatant was decanted. The precipitate was washed twice by dispersing well in 25 mL of 3% TCA, heating for 10 min. and centrifuging. After washing once with water, the precipitate was dissolved in 3 mL of 1.5 N NaOH and diluted to 30 mL with water. The solution was heated in a boiling water bath for 30 min. and filtered hot through Whatman no.2 filter paper. The precipitate was washed with 60 mL of hot water, dissolved in 40 mL of 3.2 N hot HNO_3, and diluted to 100 mL in a volumetric flask. Five mL aliquot was transferred into another flask and 20 mL of 1.5 M KSCN was added and diluted to 100 mL. The colour was read immediately at 480 nm. Iron content was calculated from a standard curve using $Fe(NO_3)_3$ (Appendix B). Phytate phosphorus was calculated from Fe:P ratio of 4:6.

Although this method gave good results and was used for the first part of the experiments, due to its lengthy procedure another method reported by Haug and Lantzsch (1983) for rapid determination of phytic acid by measuring the residual iron concentration was adopted for further study. The procedure is as follows: approximately 3 g samples were used for phytic acid analyses. Phytic acid was extracted from the samples using 50 mL of 3% TCA under continuous shaking for 1 hour (200 rpm). After centrifuging (6000 x g, 15 min.), the supernatant was collected. Five mL of the supernatant was mixed with 10 mL of 0.1% ferrous ammonium sulphate solution in 3% TCA, and boiled for half an hour. After cooling and centrifuging, 2 mL of the supernatant was treated with 3 mL of bipyridine- thioglycollic acid reagent and the absorbance was read at 519 nm. The concentration of phytic acid was calculated from a similarly prepared standard curve (Appendix B). The bipyridine-thioglycollic acid reagent was prepared by dissolving 10.0 g bipyridine in 10.0 mL thioglycollic acid and then making the solution up to 1.0 L.

3.2.4 Proteins

Approximately 3 g samples were taken in a 150 mL Erlenmeyer flask and boiled gently with 50 mL 1 N NaOH. After cooling and centrifuging (6000 x g, 15 min.), the supernatant was diluted appropriately for the analysis. The method described by Lowry et al. (1951) was adopted for protein determination. The following reagents were used for the analysis: A) 2% Na_2CO_3, B) 0.5% $CuSO_4.5H_2O$ in 1% sodium tartrate, C) 50 mL A + 1 mL B (daily prepared), and D) 1:1 Folin phenol reagent (1 N). Two mL of diluted sample was mixed with 10 mL of the reagent C and let stand for 15 minutes. Then 1 mL of the reagent D was added and mixed thoroughly. The absorbance was read at 750 nm after 45 minutes, using water as the reference. Protein concentration was calculated from the standard curve prepared similarly using albumin (Appendix B).

3.2.5 Carbohydrates

Total carbohydrates were determined by the method described by Weiner (1978). The extraction procedure was adopted from the Official Methods of Analysis (AOAC, 1975). Approximately 3 g samples were boiled for 2 hours with 50 mL of water and 10 mL of conc. HCl. The supernatant was appropriately diluted and 3 mL was treated with 10 mL of the anthrone reagent and boiled for 20 minutes. The absorbance was read at 625 nm using water as the reference. The carbohydrate content was calculated from the standard curve prepared similarly using sucrose (Appendix B). The Anthrone reagent was prepared by dissolving 1.0 g anthrone and 10.0 g thiourea in 1.0 L of 85% H_2SO_4.

3.2.6 Crude fiber

The method for fiber determination was also adopted from the Official Methods of Analysis (AOAC, 1975). Approximately 3 g samples were taken and extracted for one hour with 50 mL of petroleum ether to remove oil. The residue was then dried at 105°C for 24 hours, and placed in a 500 mL Erlenmeyer flask. Approximately 2.0 g of asbestos fiber and 200 mL of 1.25% H_2SO_4 (boiling) were added to the sample. The flask was then fitted with a condenser and boiled gently on a hot plate for 30 min. The mixture was then filtered through Buchner funnel, washed four times with 50 mL of boiling water, and the residue was sucked dry using vacuum. The residue was then transferred to a 500 mL Erlenmeyer flask, 200 mL of 1.25% NaOH was added, and boiled for 30 min. on a hot plate. The mixture was then filtered and washed with 25 mL of 1.25% H_2SO_4, three portions of 50 mL of boiling water, and finally with 25 mL of ethyl alcohol. The suction-dried residue was then transferred into an ashing dish and dried for 2 hours at 130°C. After cooling and weighing, the residue was burnt at 600°C for 30 min. The weight was again measured after cooling.

Similar procedure was followed with a sample of asbestos fiber alone and the

weight loss of blank was determined.

% fiber was calculated as follows:

$$\%fiber = \frac{(wt.lossofsample - wt.lossofblank)X100}{drywt.ofsample}$$

3.2.7 Phosphorus

Phosphorus was determined according to the method described by Harland and Harland (1980). The following chemicals were used for this analysis: Ammonium molybdate tetrahydrate ($(NH_4)_6Mo_7O_{24}.4H_2O$), 10 N Sulphuric acid (H_2SO_4), Ferrous sulfate heptahydrate ($FeSO_4.7H_2O$) and Potassium dihydrogen phosphate (KH_2PO_4).

Taussky-Schoor reagent

10.0 g ammonium molybdate was placed in a 100 mL flask and made up to the volume with 10 N H_2SO_4. Five g ferrous sulfate was placed in another 100 mL flask, 10.0 mL of the ammonium molybdate solution was added, and diluted to the volume with distilled water. This reagent (Taussky-Schoor) was made fresh daily.

Determination of phosphorus

Five mL of the supernatant was mixed with 5.0 mL of Taussky-Schoor reagent the absorbance was read at 660 nm immediately, using water treated similarly, as the reference. Phosphorus concentratio was determined from the standard curve prepared using KH_2PO_4 (Appendix B).

3.2.8 pH measurement

Approximately 2 g samples were taken and stirred thoroughly in 20 mL distilled water and then the pH was measured using Fisher Accumet pH meter (model 805 MP).

3.3 Enzyme Preparation and Assay Procedures

The enzyme activity was determined in the supernatant of the liquid medium and also in the crude enzyme extracted from the canola meal after the mold was grown for several days. The crude enzyme was extracted from the meal using a 2% aqueous solution of $CaCl_2.2H_2O$. The meal to extractant ratio was 1:2 (unless otherwise specified). The meal with good growth of the mold was stirred thoroughly in $CaCl_2$ solution for one hour (200 rpm). The liquid was squeezed out and filtered through a double layer cheese cloth and centrifuged (5000 x g, 15 min., 4°C). The clear supernatant was designated as the crude enzyme.

The enzyme activity was assayed by measuring the amount of phosphorus released by using sodium phytate as the substrate. Spectrophotometric determination of phosphorus was done using Taussky-Schoor reagent as described by Harland and Harland (1980). The reactions were carried out at 50 or 60°C in a water bath. A typical reaction mixture contained 5 mL of 0.2 M acetate buffer (pH 4.7), 1 mL of 15 mM phytic acid solution and 1 mL of the crude enzyme. The reaction was stopped by adding 5 mL of 10% TCA.

The effect of temperature, pH, substrate concentration, enzyme concentration, incubation time, preincubation of enzyme at 50°C and 60°C and also the effect of storage at 4°C on phytase acivity have been determined.

It should be noted that all the experiments were duplicated and the average value is shown in the figure. The standard deviation of the observed values were within 5%.

One unit of enzyme activity is defined as the amount of enzyme required to release one mg of phosphorus from 1 mL of 15 mM phytic acid solution per hour at the given temperature and pH.

Chapter 4

Results and Discussion

Solid state fermentation was carried out for the reduction of the phytic acid content in canola meal. Commercially available canola meal was used in this study. Initially, complete analysis of canola meal was performed and the composition is given in Table 4.1, where the analytical results are compared with the reported values. The results are in good agreement with the reported values.

Table 4.1: Composition of canola meal

Components	This work (%)	Literature value (%)	References
Moisture	7.5	8.0 - 11.0	[3], [8], [20]
Crude protein	34.0	34.0 - 38.0	[3], [6], [8], [45]
Carbohydrate	26.0	28.0 - 32.0	[3], [6], [8],[45]
Crude fiber	15.0	11.0 - 15.0	[3], [6], [8]
Ether extract	5.0	3.0 - 6.0	[3], [6]
Phytic acid	6.0	3.0 - 7.0	[3], [6], [8], [15], [20], [26], [42], [59]
Ash	6.5	6.0 - 8.0	[3], [26]

4.1 *Rhizopus oligosporus* NRRL 2990

Preliminary experiments were carried out in Petri dishes using *Rhizopus oligosporus* NRRL 2990. Phytic acid content reduction in canola meal using this microorganism is shown in Fig. 4.1; this shows that there was 48% reduction of phytic acid content in canola meal in 139 hours of fermentation in Petri dishes. The inoculum concentration used in this experiment was 3.1×10^8 spores/100 g meal. Further experiments were carried out using the same inoculum to see whether the addition of nutrients[1] to the medium would promote the growth of the microbe and hence a better reduction in phytic acid content. It was observed that there was no significant difference in mycelial growth between the two cases. Excellent growth of mycelia was noticed after 24 hours of fermentation. The addition of nutrients did improve the reduction of phytic acid (Fig. 4.1). Without adding the nutrients, there was only 48% reduction in 139 hours, while there was 55% reduction with the addition of nutrients. The addition of nutrients also caused the reduction of 68% of total carbohydrates. Only 52% of total carbohydrates were used up when no nutrients were added to the medium. The difference in the amount corresponded to the carbohydrate added (as glucose) to the medium. Results from this experiment also show (Fig. 4.2) that there were significant decreases in moisture content and dry weight. The reduction in dry weight during the incubation implied that some of the materials in the canola meal medium were probably being converted into gaseous products.

These preliminary results showed that much improvement was necessary in order to achieve complete hydrolysis of phytic acid. *Rhizopus oligosporus* is an aerobic microbe and therefore, aeration of the medium was looked upon as a possible solution.

[1]All the nutrients required for the growth of the microorganism in the liquid medium (Table 3.1) were added as weight percent per 100 g meal.

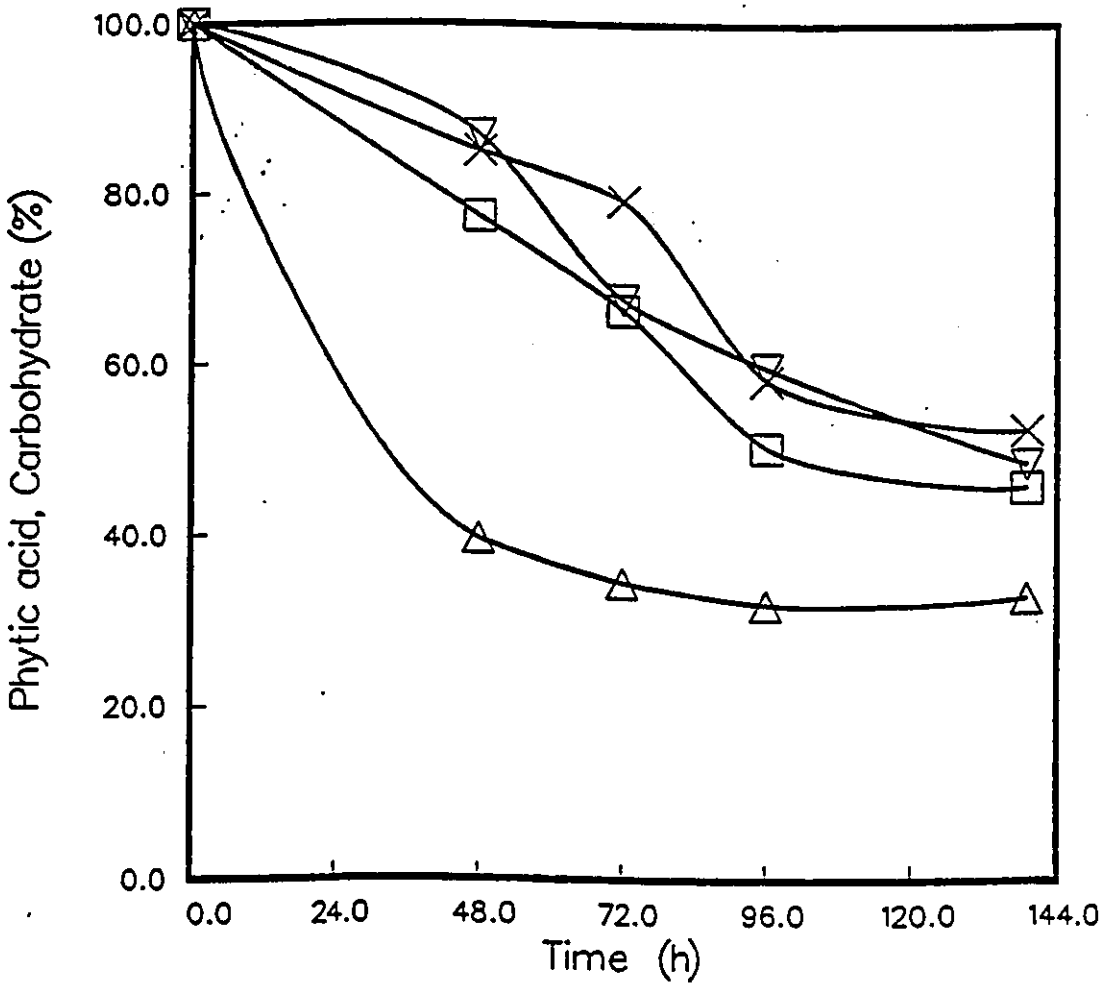


Figure 4.1: Phytic acid reduction and carbohydrate consumption in canola meal using *R. oligosporus* NRRL 2990. (With nutrients : □ - phytic acid, △ - total CHO; Without nutrients : X - phytic acid, ▽ - total CHO)

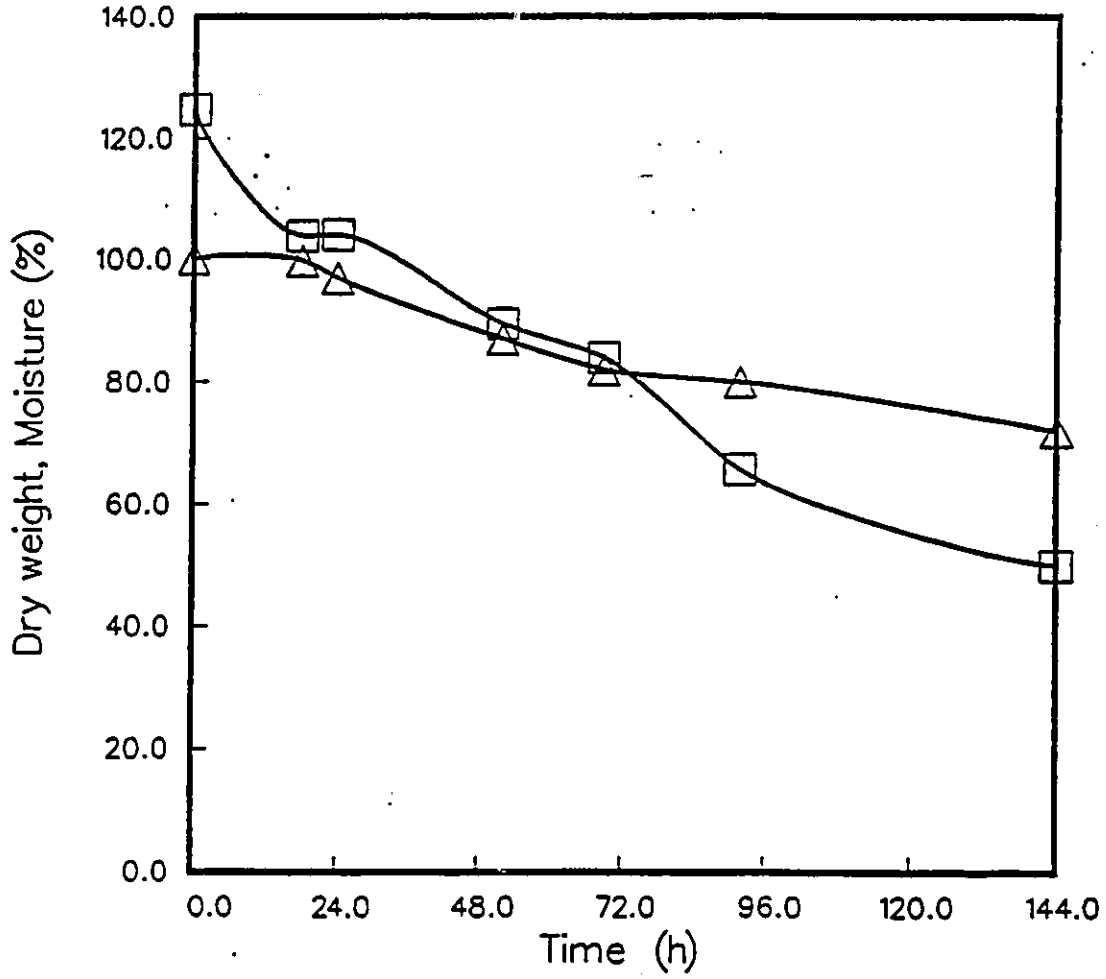


Figure 4.2: Moisture change and dry weight reduction in canola meal medium using *R. oligosporus* NRRL 2990. (□ - moisture, △ - dry weight)

Experiments were carried out in the chamber to see whether aeration would promote both the growth of the microbe and the reduction of the phytic acid content. Visual inspection indicated that the growth of the microbe was much more vigorous in the aerated chamber than in Petri dishes. However, preliminary experiments in the aerated chamber showed (Fig. 4.3) that only 38% of the total phytic acid content was hydrolyzed in 120 hours of fermentation. The amount of spores used in this experiment was 3.1×10^8 spores/100 g of meal. It should be noted that the minimum recommended inoculum concentration for the solid state fermentation of oncom (Fardiaz and Markakis, 1981) and for the protein biosynthesis from starchy materials (Czajkowska and Ilnicka-Olejniczak (1988; 1989)) is 2.0×10^8 spores/100 g.

To improve the reduction of phytic acid, the number of spores was double in another set of experiments. In this experiment, two separate chambers were used to study the effect of aeration. The results (Fig. 4.3) show that aeration caused a faster phytic acid content reduction. Fifty five percent of phytic acid was hydrolyzed without aeration in 120 hours of fermentation, while 51% reduction was achieved with aeration in 72 hours of fermentation. Further increase in spore concentration to 7.5×10^8 sp./100 g reduced almost 60% of the phytic acid content. Comparing these results with the previous batch where a low concentration of spores was used, it can be concluded that increase in spore concentration did have a significant effect on the reduction of phytic acid. When the medium was aerated, the maximum reduction in phytic acid was attained after 72 hours of fermentation, while in the non-aerated system, the reduction in phytic acid was linear and continued decreasing during the course of fermentation. Thus aeration seemed beneficial in terms of reducing the fermentation time.

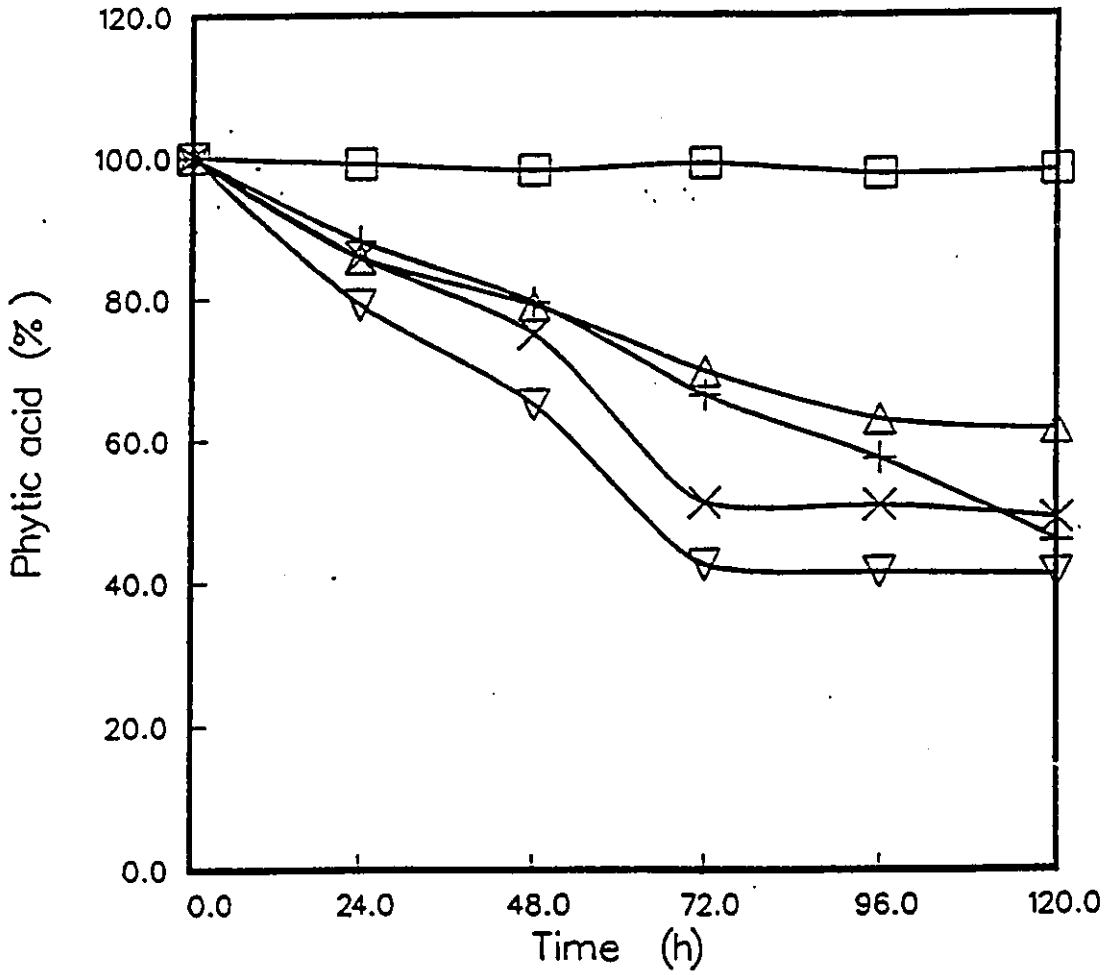


Figure 4.3: Effect of aeration and spore concentration in inoculum on the reduction of phytic acid content in canola meal using *R. oligosporus* NRRL 2990. (□ - Control (no inoculum), △ - 3.1×10^8 spores, X - 6.2×10^8 sp., ▽ - 7.5×10^8 sp.; (all with aeration); + - 6.2×10^8 sp., no aeration)

The summary of protein change in the systems inoculated with various spore amounts with and without aeration is shown in Fig. 4.4. In all four cases, there was a significant amount of protein loss after 96 hours of fermentation. Petri dish experiments suffered the greatest loss (22%), while the experiments in non-aerated chamber had the slightest loss (12%). An increase in spore concentration under aeration resulted in a lower amount of net protein loss. It was observed that during the first 24 hours of fermentation, there was a net gain of protein content in canola meal (Fig. 4.4). Excellent growth of the microorganism was observed during this period of time and therefore, an increase in total amount of protein was expected. When the spore concentration of inoculum was increased, there was an increase in the biomass production and hence an increase in the amount of total protein. However, since there was only about 5-10% difference in the amount of protein among all the cases discussed, the results are not conclusive.

The pH of the medium was measured for the duration of fermentation. The results (Fig. 4.5) show that there was a sharp increase in pH during the first 48 hours; in all cases, the pH value reached a plateau within 72 hours. Researchers have reported that the phytase has an optimum pH range of 4.8 to 5.6 (Graf, 1986). Noticing that the pH of the system increased beyond this range, it was speculated that the addition of buffer to the system may be helpful in achieving a better reduction of phytic acid content in canola meal.

It was observed that incubation of non-sterile meal at 30°C after the inoculation with *R. oligosporus* NRRL 2990 resulted in a lower amount of reduction in phytic acid than sterile meal (Fig. 4.6). Forty two percent of phytic acid was hydrolyzed when sterile meal was used, while only 25% reduction was achieved in the non-sterile meal. The addition of buffer was found to stabilize the pH of the system for a longer period of time and therefore, better reduction in phytic acid content was achieved. In 120 hours, 69% of phytic acid content was reduced by *R. oligosporus* NRRL 2990 in the aerated chamber. In all cases the inoculum concentration was 3.2×10^8 spores per 100 g of the meal.

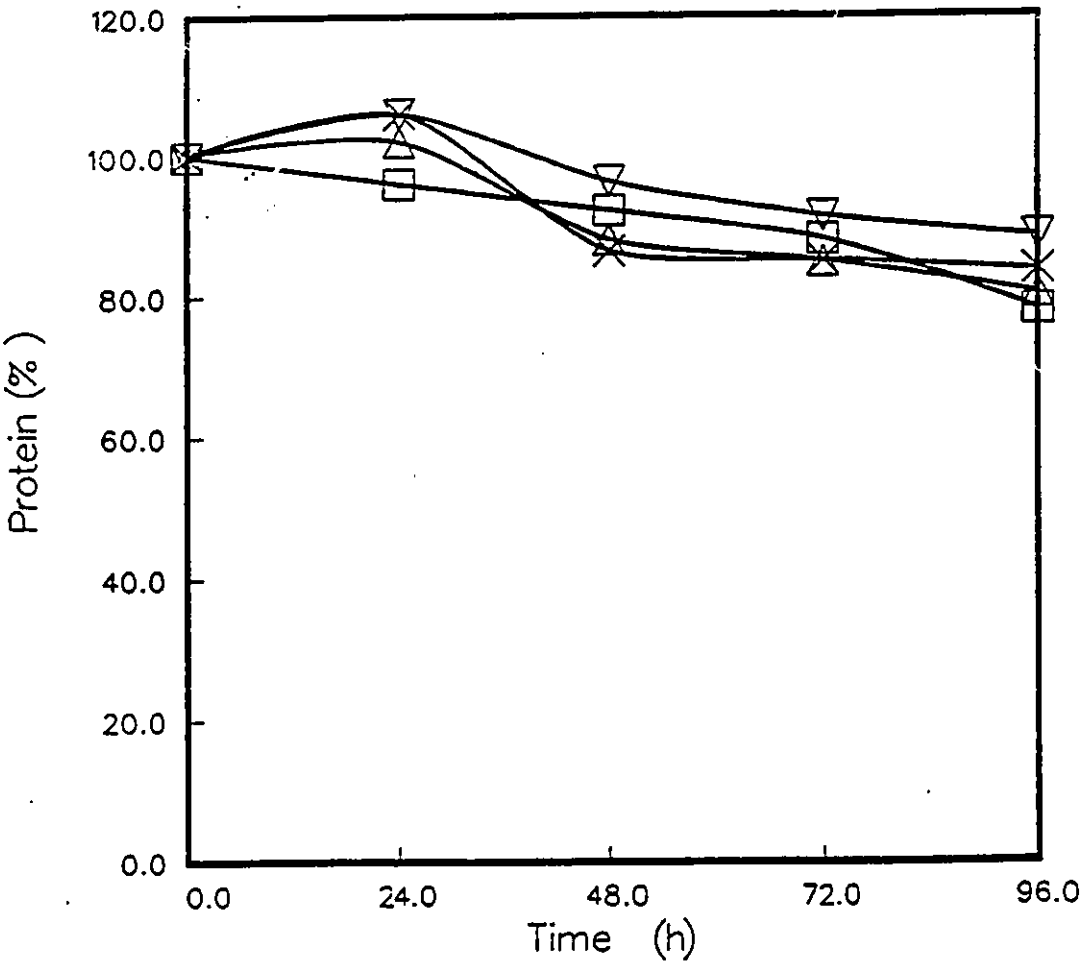


Figure 4.4: Protein change in canola meal medium treated with *R. oligosporus* NRRL 2990. (□ - Petri dish ($3.1x10^8$ sp.); Chamber : Δ - $3.1x10^8$ sp., with aeration, X - $6.2x10^8$ sp., with aeration, ∇ - $6.2x10^8$ sp., without aeration)

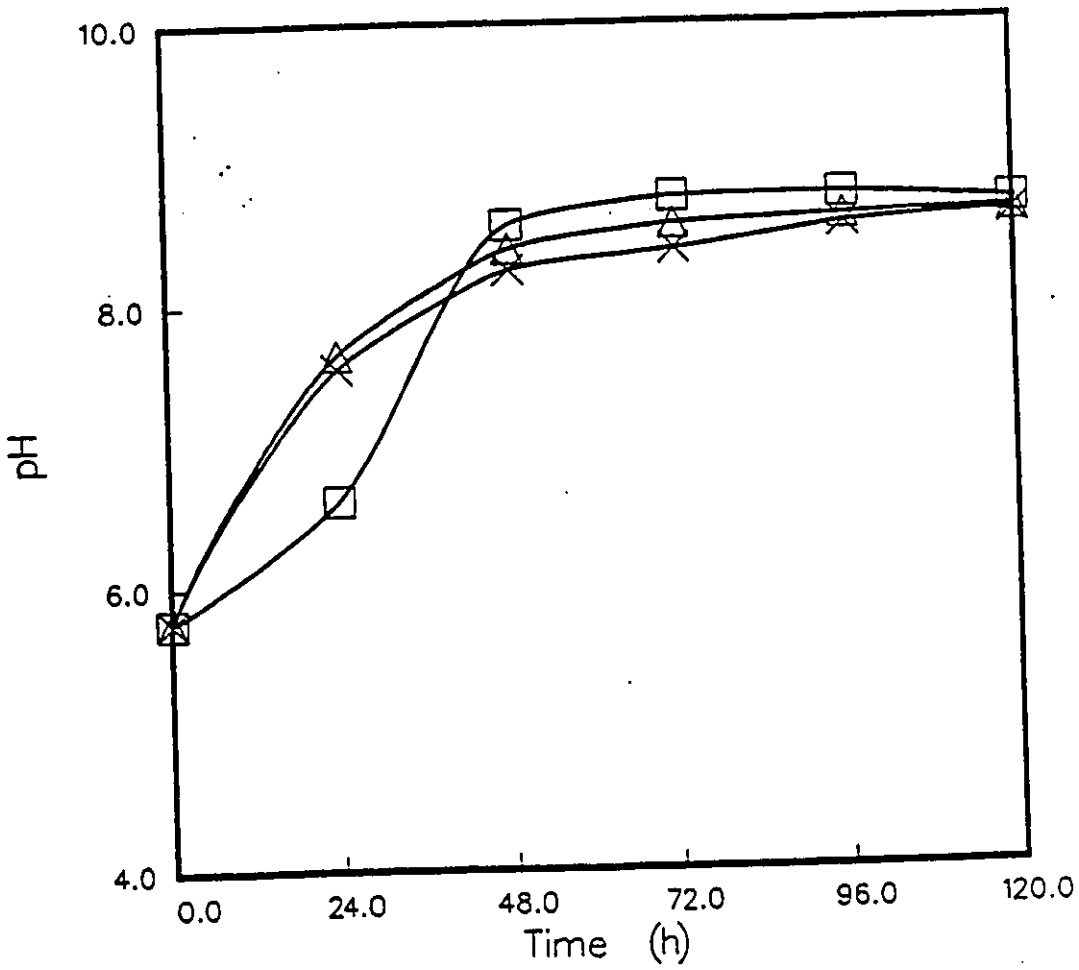


Figure 4.5: The change in pH during the incubation of canola meal with *R. oligosporus* NRRL 2990. (□ - Petri dish, Chamber : △ - with aeration, X - without aeration)

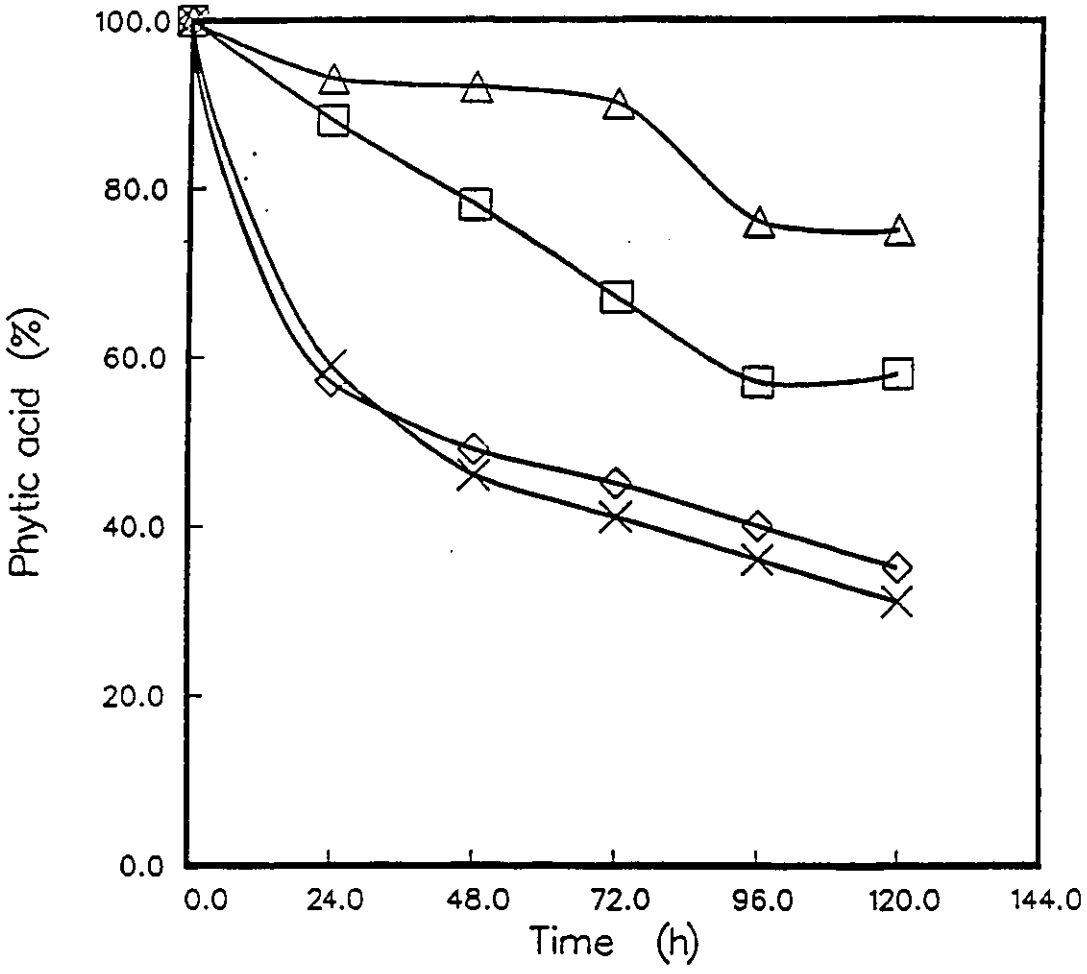


Figure 4.6: Phytic acid content reduction in canola meal using *R. oligosporus* NRRL 2990. (Chamber : □ - sterile meal, △ - non-sterile meal, X - sterile meal with buffer; ◇ - sterile meal with buffer in flask)

A certain degree of contamination was noticed in the experiments carried out in aerated chambers. To avoid contamination, further experiments were performed in Erlenmeyer flasks. These results were similar to the ones obtained using aerated chambers; however, only 65% of phytic acid was hydrolyzed in 120 hours (Fig. 4.6).

Different sterilization times were tested for their effects on the reduction of phytic acid. The results (Fig. 4.7) show that the sterilization time did not have any significant effect on phytic acid reduction during the first 48 hours; however, after 120 hours of fermentation, 10% more reduction was noticed when 120 minutes of sterilization time was used. Since the cost of sterilization increases with the time of sterilization, it is recommended that the minimum sterilization time be used in order to achieve a reasonable reduction in phytic acid content. For this reason, 45 minutes of sterilization time was used here. Canola meal may contain spores of several microorganisms. Even after sterilization for 45 minutes, some of these spores may survive. Under these circumstances these spores will be competing with the *Rhizopus* spores for nutrients. Thus the effective reduction of the phytic acid content in canola meal by *Rhizopus* may be affected by the presence of other microorganisms. Sterilization for a longer period of time will get rid of most of the microorganisms and hence a better reduction in phytic acid can be achieved.

These experiments using *Rhizopus oligosporus* NRRL 2990 showed that this microorganism is capable of phytic acid content reduction. However, the results were not satisfactory since the purpose was to reduce the phytic acid content completely. At this point, another microorganism was tested for its capability to reduce the phytic acid content in canola meal.

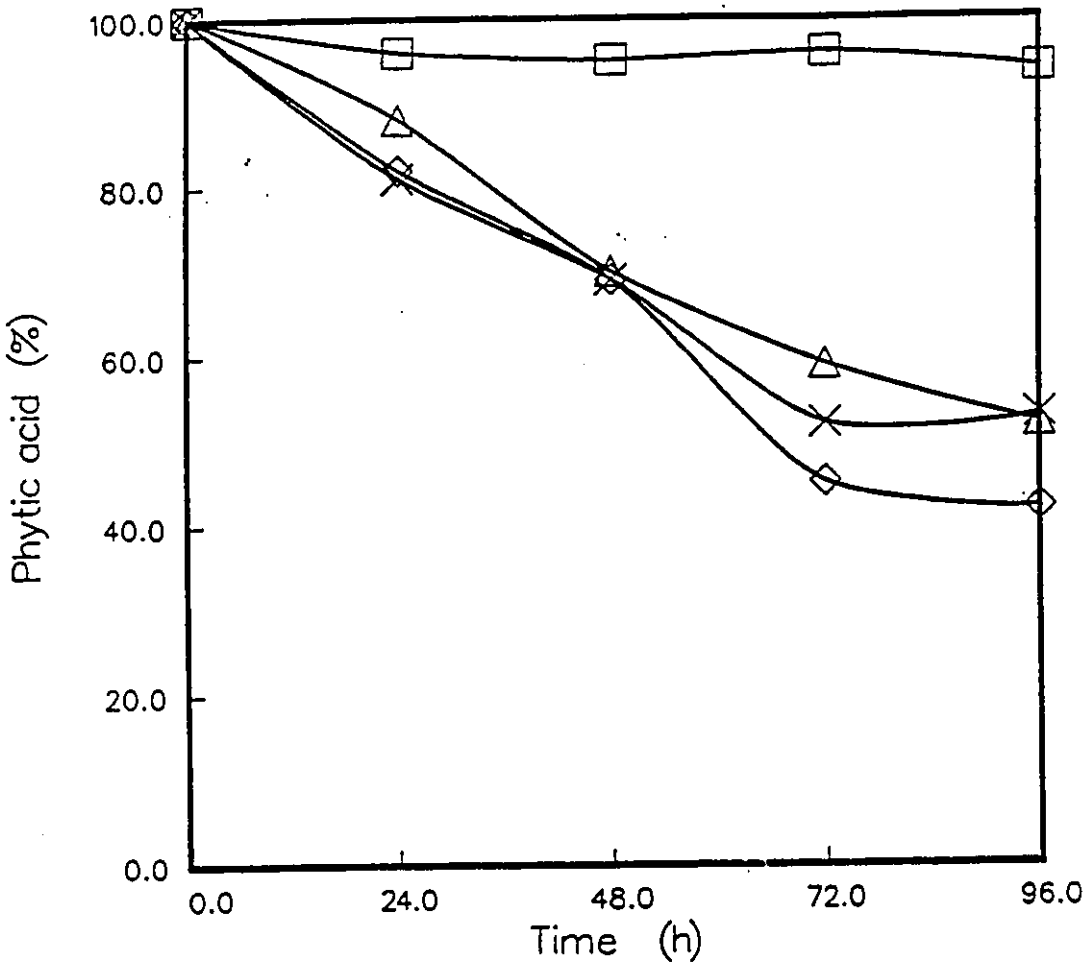


Figure 4.7: Effect of sterilization time on the reduction of phytic acid content in canola meal using *R. oligosporus* NRRL 2990. (Time : □ - 45 min., control (no inoculum), △ - 25 min., X - 45 min., ◇ - 120 min.)

4.2 *Aspergillus niger*

Aspergillus niger has been reported to be one of the better producers of the enzyme phytase (Shieh and Ware, 1968). For this reason, experiments were performed using this microorganism. Two strains of *Aspergillus niger* (NRC 401121 and NRC 5765) were tested for their ability to produce phytase and hence to reduce the phytic acid content in canola meal. Using aerated chambers, *A. niger* NRC 401121 reduced the phytic acid content of canola meal by 40% and *A. niger* NRC 5765 was able to reduce it by 35% in 96 hours of fermentation (Fig. 4.8). Better reduction of phytic acid content was achieved when the experiments were performed in flasks than in aerated chambers. After 96 hours of fermentation, *A. niger* NRC 401121 reduced the phytic acid content of canola meal by 51% and 40% in flask and chamber respectively. For the same period of time, *A. niger* NRC 5765 reduced only 45% and 35% of phytic acid, respectively in a flask and a chamber.

For both strains of *A. niger*, the dry weight reductions were similar (Fig. 4.9). Approximately 21% of the total dry weight was lost during 120 hours of fermentation in flasks. The pH of the system changed rapidly from 5.0 to 8.3 when *A. niger* NRC 5765 was used. But when *A. niger* NRC 401121 was used, the pH changed from 5.0 to only 6.2 (Fig. 4.9). This probably is one of the reasons for the lower amount of reduction in phytic acid by NRC 5765. (Note that the optimum pH for phytase activity is between 4.8 and 5.6 (Graf, 1986)).

The amount of protein in the medium decreased considerably during the first 48 hours of fermentation, but as the microbial biomass increased, there was slight increase in the amount of protein. However, after 96 hours of fermentation, a net loss of 5% of protein was observed in both cases of *A. niger* (Fig. 4.10). Fifty percent reduction in total carbohydrates was also observed in 96 hours of fermentation. Both strains of *A. niger* consumed almost the same amount of total carbohydrates.

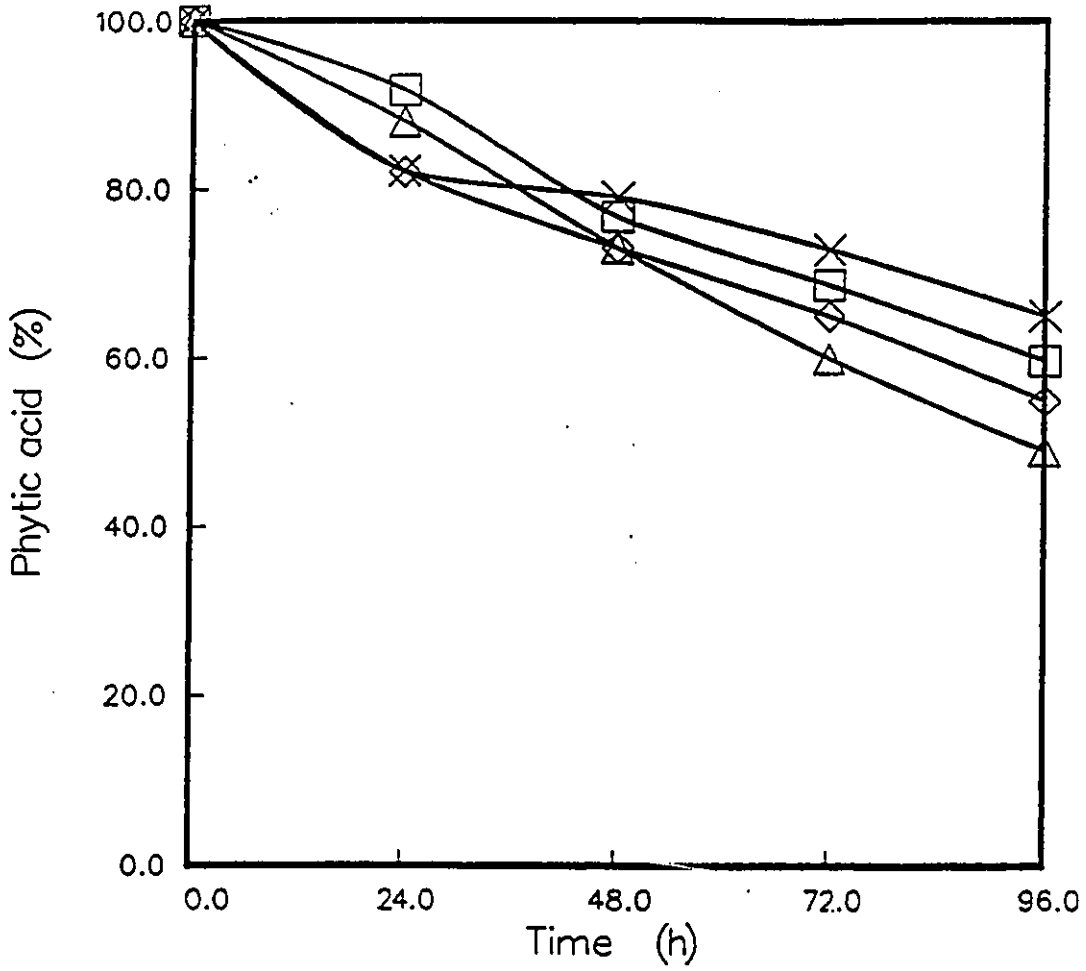


Figure 4.8: Phytic acid content reduction in canola meal using *A. niger* strains. (NRC 401121 : □ - chamber, △ - flask, NRC 5765 : X - chamber, ◇ - flask)

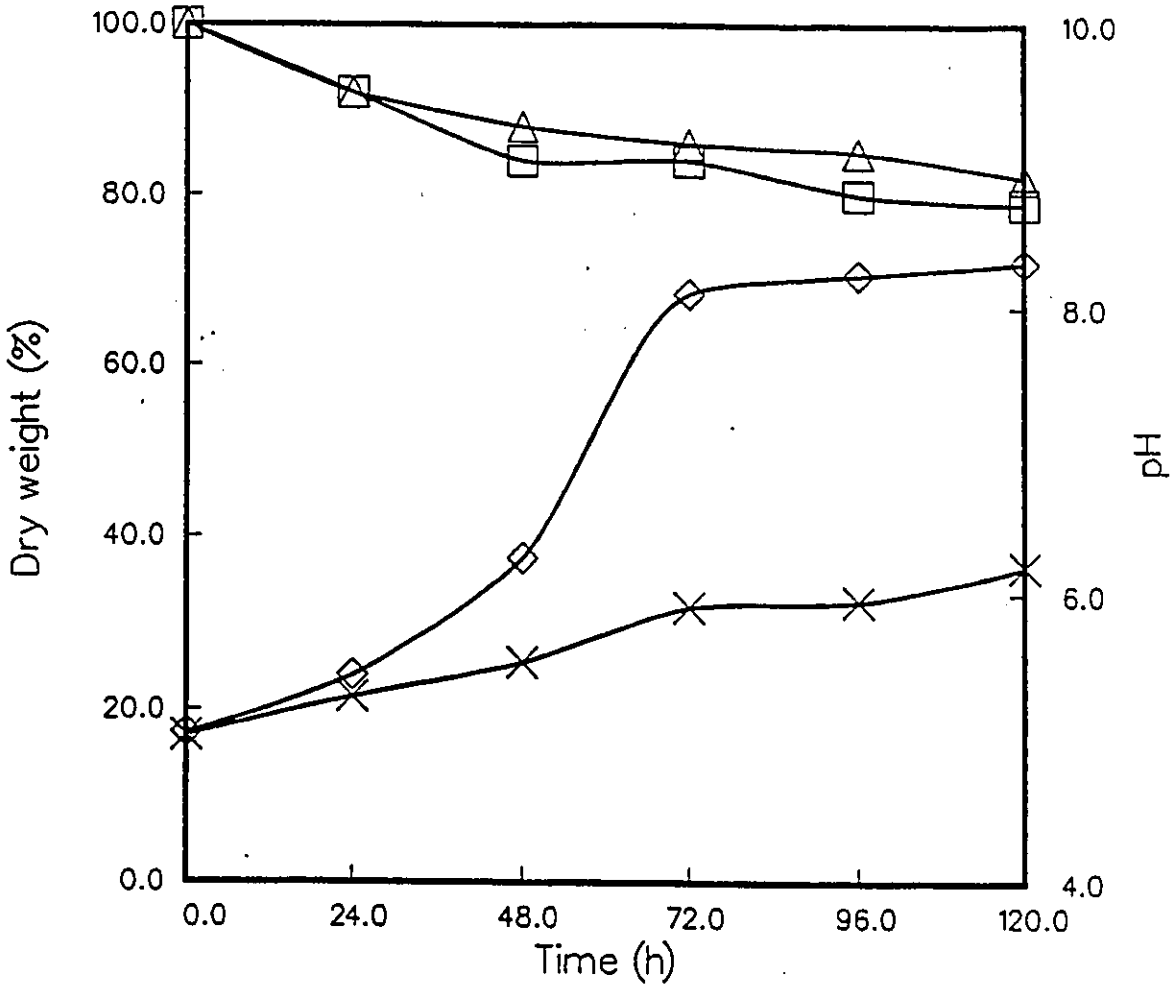


Figure 4.9: Dry weight and pH change during the incubation of canola meal with *A. niger* strains in flasks. (Dry weight : □ - NRC 401121, △ - NRC 5765, pH : X - NRC 401121, ◇ - NRC 5765)

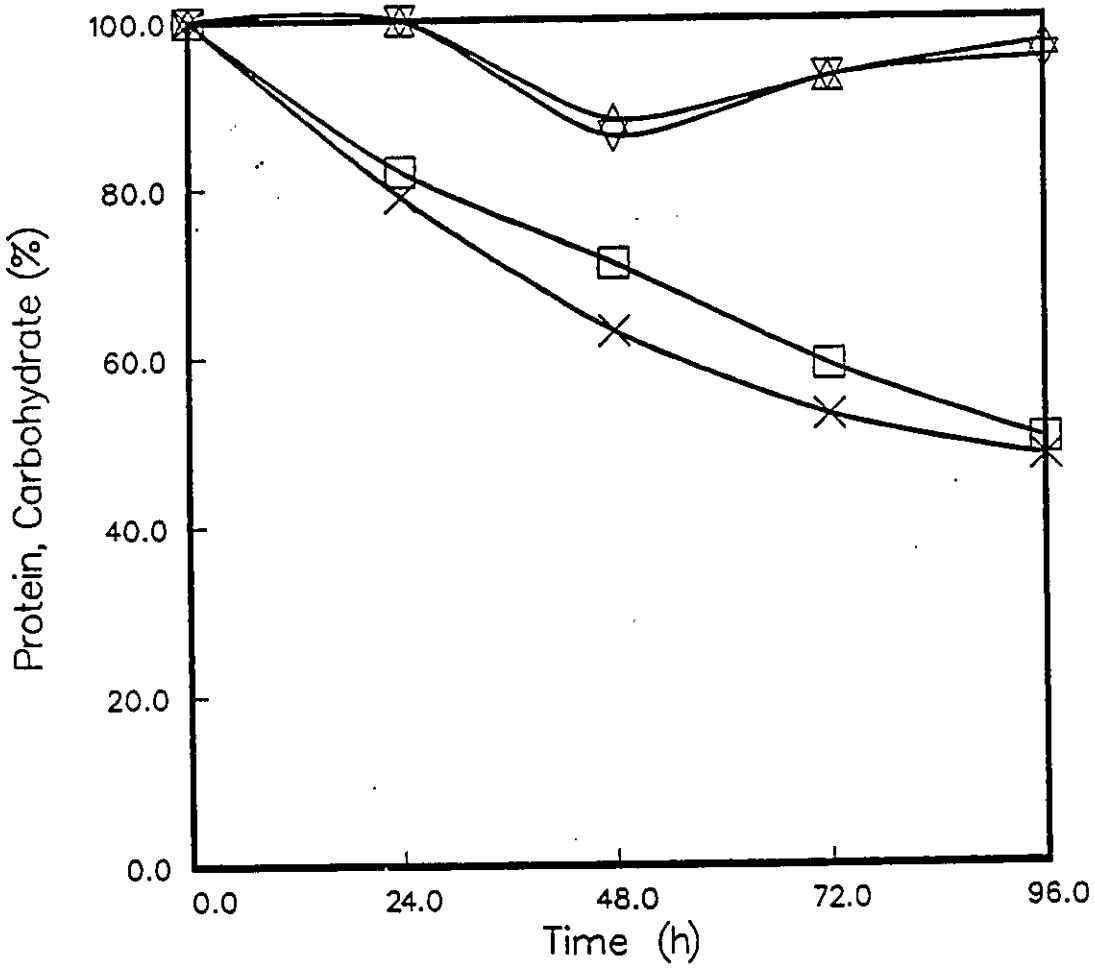


Figure 4.10: Protein and carbohydrate reduction in canola meal using *A. niger* strains. (CHO : □ - NRC 401121, X - NRC 5765, Protein : △ - NRC 401121, ▽ - NRC 5765)

Observing that *A. niger* NRC 401121 was better for the reduction of phytic acid content in canola meal than *A. niger* NRC 5765, further experiments were performed using NRC 401121.

The addition of buffer in order to keep the pH of the system at a lower value yielded better reduction in phytic acid when *A. niger* NRC 401121 was used. Sixty eight percent of the phytic acid content was reduced in the buffered system as opposed to 43% reduction in the unbuffered system (Fig. 4.11). It was observed that the addition of buffer resulted in the reduction of 96% of the total soluble carbohydrates (Fig. 4.12). Without the addition of buffer, only 80% of the total soluble carbohydrates were consumed.

The effects of spore concentration in the inoculum were studied using *A. niger* NRC 401121. The results (Fig. 4.13) show that an increase in spore concentration resulted in an increase in the reduction of phytic acid. The highest concentration ($2x10^9$ sp./100 g) of spores reduced 66% of the phytic acid content in 120 hours of fermentation. The lowest concentration ($4.7x10^8$ sp./100 g) yielded only 44% reduction. In all cases, buffer was added to the system.

It was observed that the mycelial growth was rather poor in the first 24 hours, but excellent growth was noticed after 48 hours of fermentation. There was a net loss of protein content in 24 hours of fermentation, but as the biomass production increased, there was a net gain of up to 10% protein in 72 hours of fermentation (Fig. 4.14) depending on the spore concentration. It should be noted that there was a loss of protein content after 72 hours of fermentation. It was observed that the final protein content of the medium after 96 hours of fermentation was more or less equal to the initial value. In the case of *Rhizopus*, there was a 10% loss in the amount of protein using the inoculum containing a similar amount of spores.

Carbohydrate utilization was similar to the case when *R. oligosporus* was used. Total carbohydrate reduction was almost 40% which was independent of spore concentration (Fig. 4.15). Most of the carbohydrate consumption occurred during the first 48 hours of fermentation, when the microbial growth was taking place.

Increase in spore concentration did not have a significant effect on the pH of the medium (Fig. 4.16). In all cases the pH of the system increased from about 5.4 to 8.0.

It was observed that *A. niger* NRC 401121 yielded approximately the same results as *Rhizopus oligosporus* NRRL 2990 in the reduction of the phytic acid content in canola meal. The maximum reduction attained by *A. niger* NRC 401121 was about 66% in 120 hours using 2.0×10^9 spores. In order to achieve better results, few other microorganisms were studied.

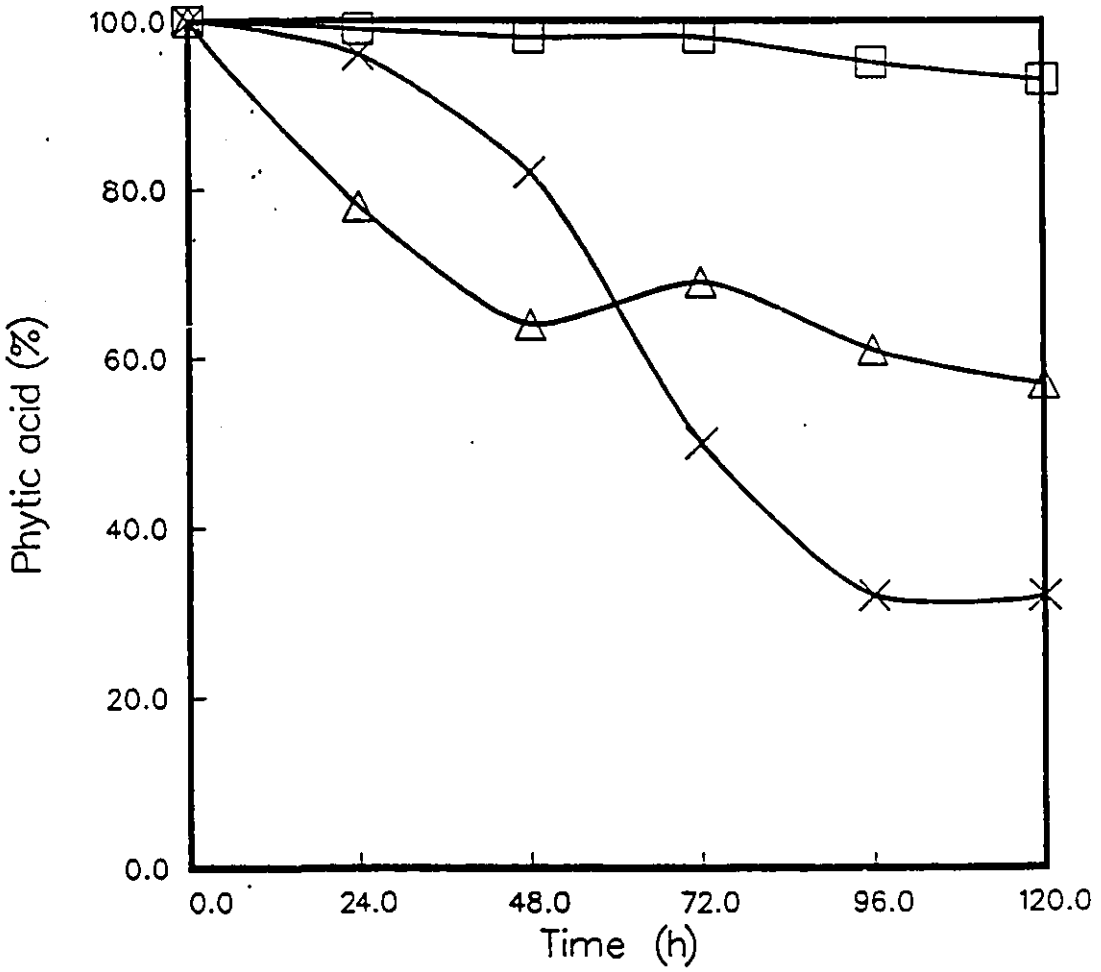


Figure 4.11: Effect of buffer on the reduction of phytic acid content in canola meal by *A. niger* NRC 401121. (□ - control, △ - Without buffer, X - With buffer)

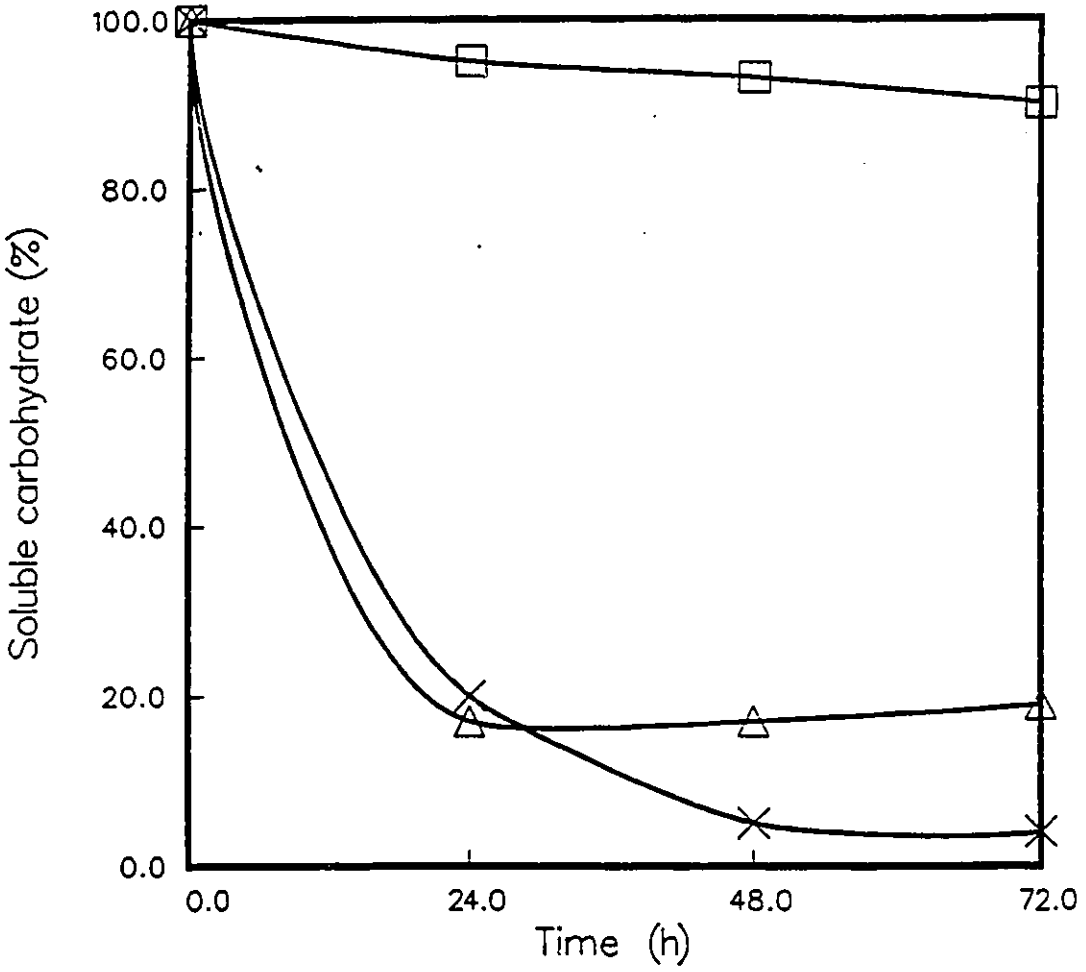


Figure 4.12: Effect of buffer on the reduction of soluble carbohydrate using *A. niger* NRRL 401121. (□ - control, △ - Without buffer, X - With buffer)

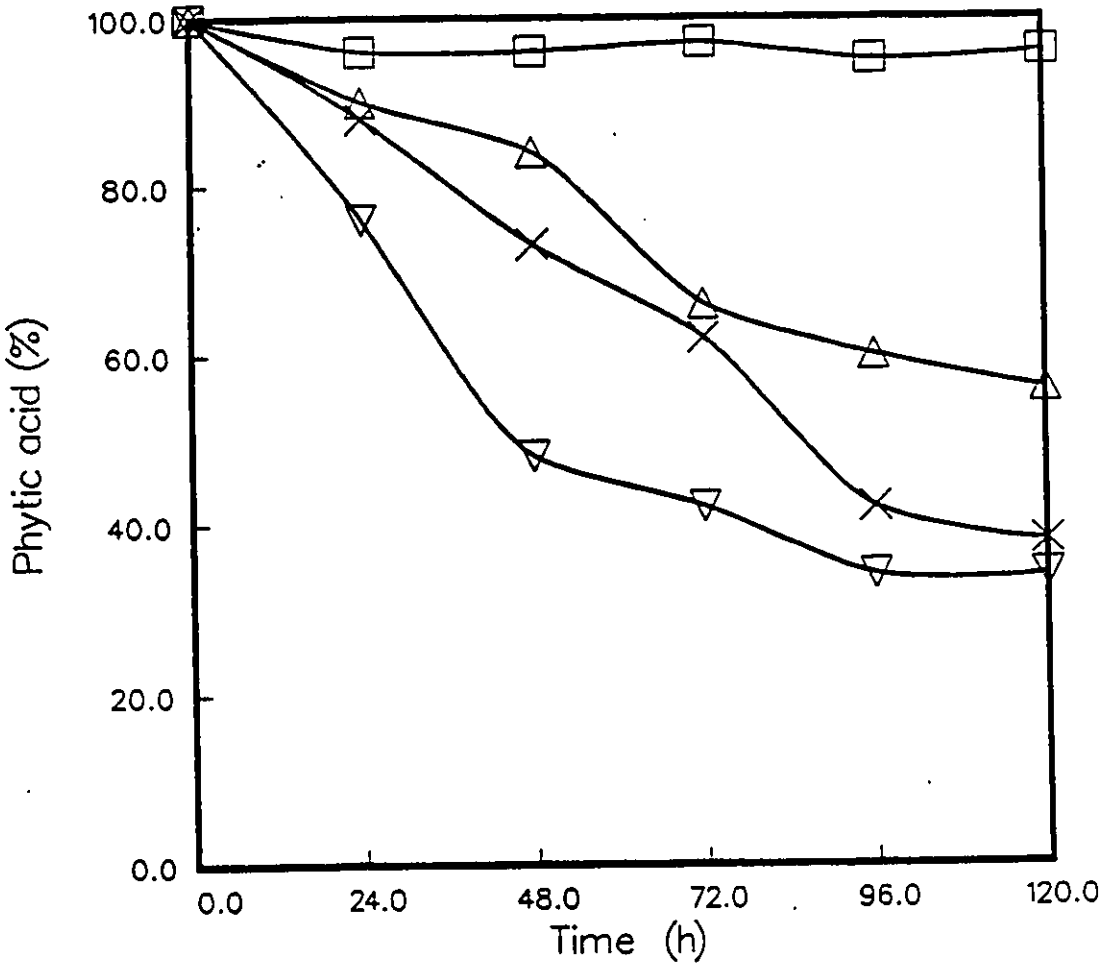


Figure 4.13: Effect of spore concentration in inoculum on the reduction of phytic acid content in canola meal using A. niger NRRL 401121. (Spores : □ - 0 (control), △ - 4.7×10^8, X - 8.0×10^8, ▽ - 2.0×10^9)

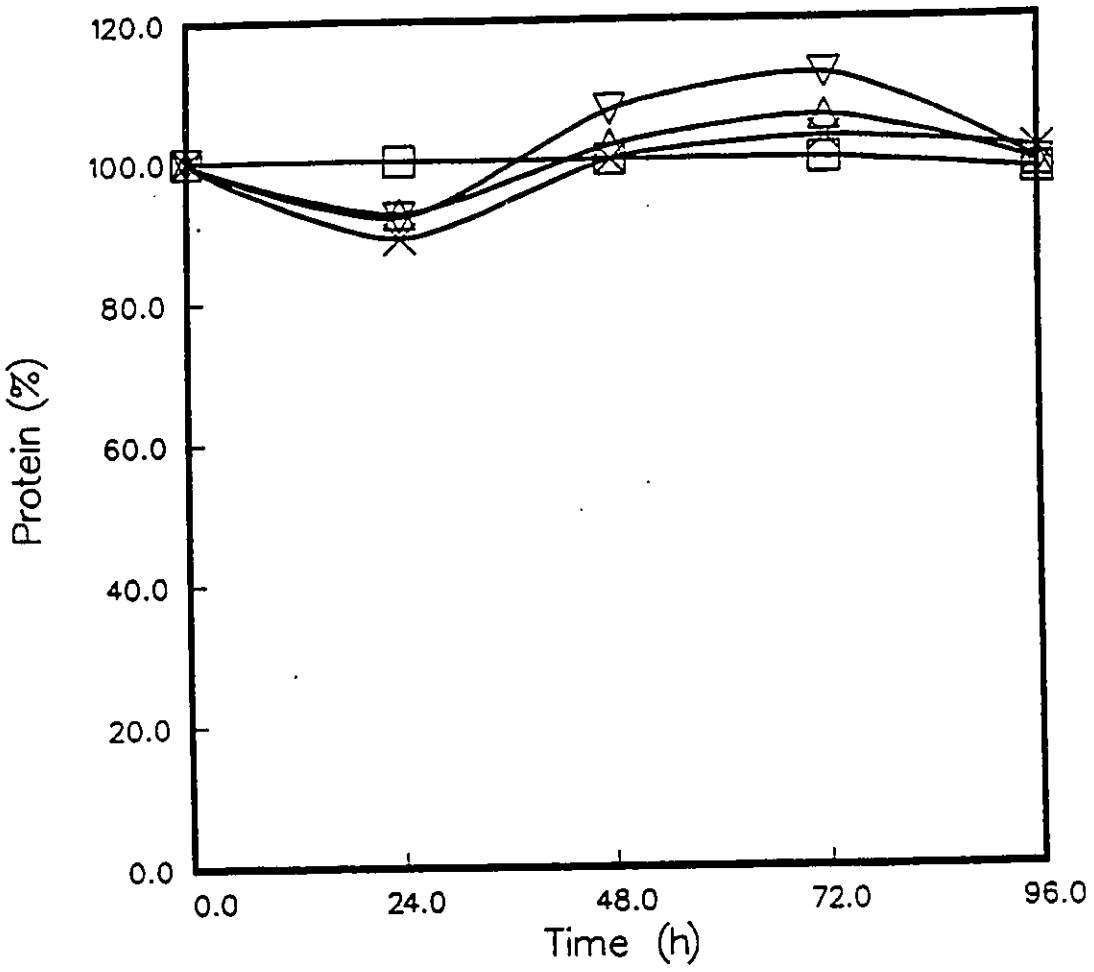


Figure 4.14: Effect of spore concentration in inoculum on the amount of protein in canola meal by A. niger NRRL 401121. (Spores : □ - 0 (control), △ - 4.7×10^8, X - 8.0×10^8, ▽ - 2.0×10^9)

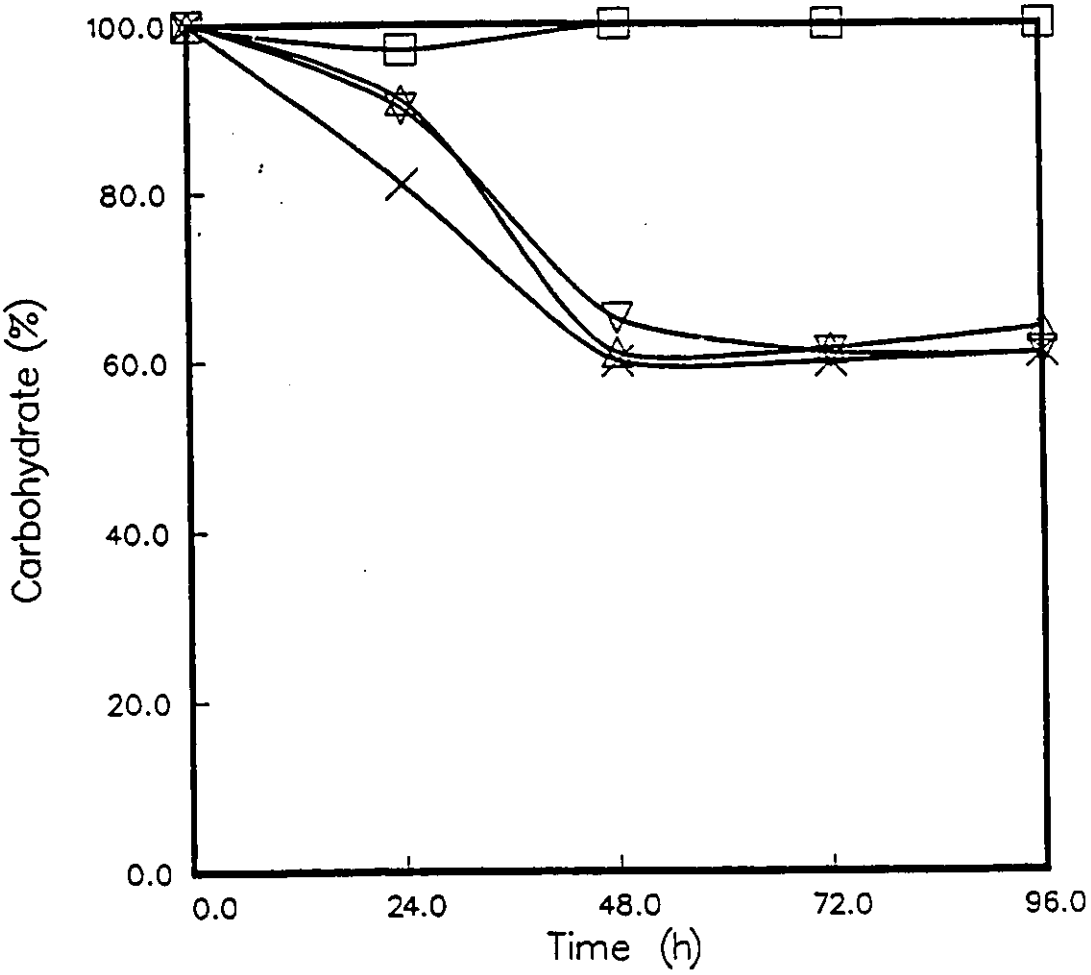


Figure 4.15: Effect of spore concentration in inoculum on the consumption of total carbohydrate in canola meal by A. niger NRRL 401121. (Spores : □ - 0 (control), △ - 4.7×10^8, X - 8.0×10^8, ▽ - 2.0×10^9)

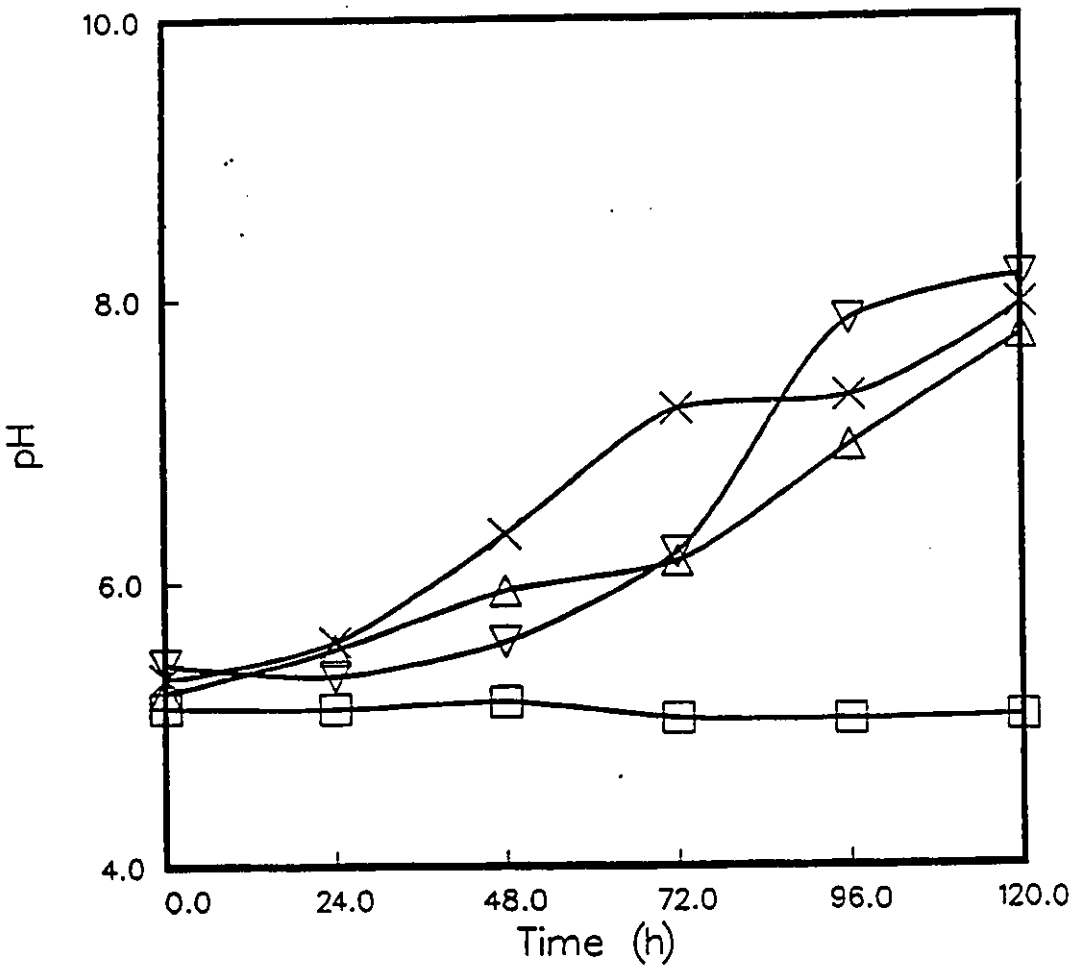


Figure 4.16: Effect of spore concentration in inoculum on the pH of the canola meal medium by A. niger NRRL 401121. (Spores : □ - 0 (control), △ - 4.7×10^8, X - 8.0×10^8, ▽ - 2.0×10^9)

4.3 *S. cerevisiae* and *A. carbonarius* NRC 401124

Preliminary tests were also carried out for the reduction of phytic acid content in canola meal using *Saccharomyces cerevisiae* (wild strain) and *Aspergillus carbonarius* NRC 401124. All these experiments were performed in 500 mL Erlenmeyer flasks in the presence of 0.2 M acetate buffer (pH 4.7). *S. cerevisiae* was found to be a poor producer of phytase, under the conditions in which the experiments were performed. The results (Fig. 4.17) show that only 26% of phytic acid was hydrolyzed in 120 hours using this microorganism. A mixture of *R. oligosporus* NRRL 2990 and *S. cerevisiae* was also tested as the inoculum; however, the results show that when the mixed inoculum was used, the amount of phytic acid hydrolyzed was lower (44%) than the case when only *R. oligosporus* NRRL 2990 was used (60%). The probable reason would be that some compounds produced by the yeast may have had an inhibitory effect on the phytase production by *R. oligosporus* NRRL 2990. Another reason would be that both microorganisms were competing for the available nutrients in the medium and thus the growth of *Rhizopus* was diminished.

Solid state fermentation using *A. carbonarius* NRC 401124 yielded good results. Complete reduction in phytic acid content of canola meal was achieved in 120 hours (Fig. 4.17). It should be noted that no growth of microbe was physically observed during the first 48 hours of fermentation. This may be the reason for the lower rate of reduction of phytic acid during this period. However, further study is needed to determine optimum conditions for the reduction of the phytic acid content in canola meal using this microorganism.

From these preliminary studies, it could be speculated that *S. cerevisiae* is a poor producer of phytase. *A. carbonarius* NRC 401124 seems to be an excellent source of phytase; however, another microorganism has been reported to have five times higher phytase activity than *A. carbonarius* NRC 401124 and therefore attention was focussed on the new microorganism.

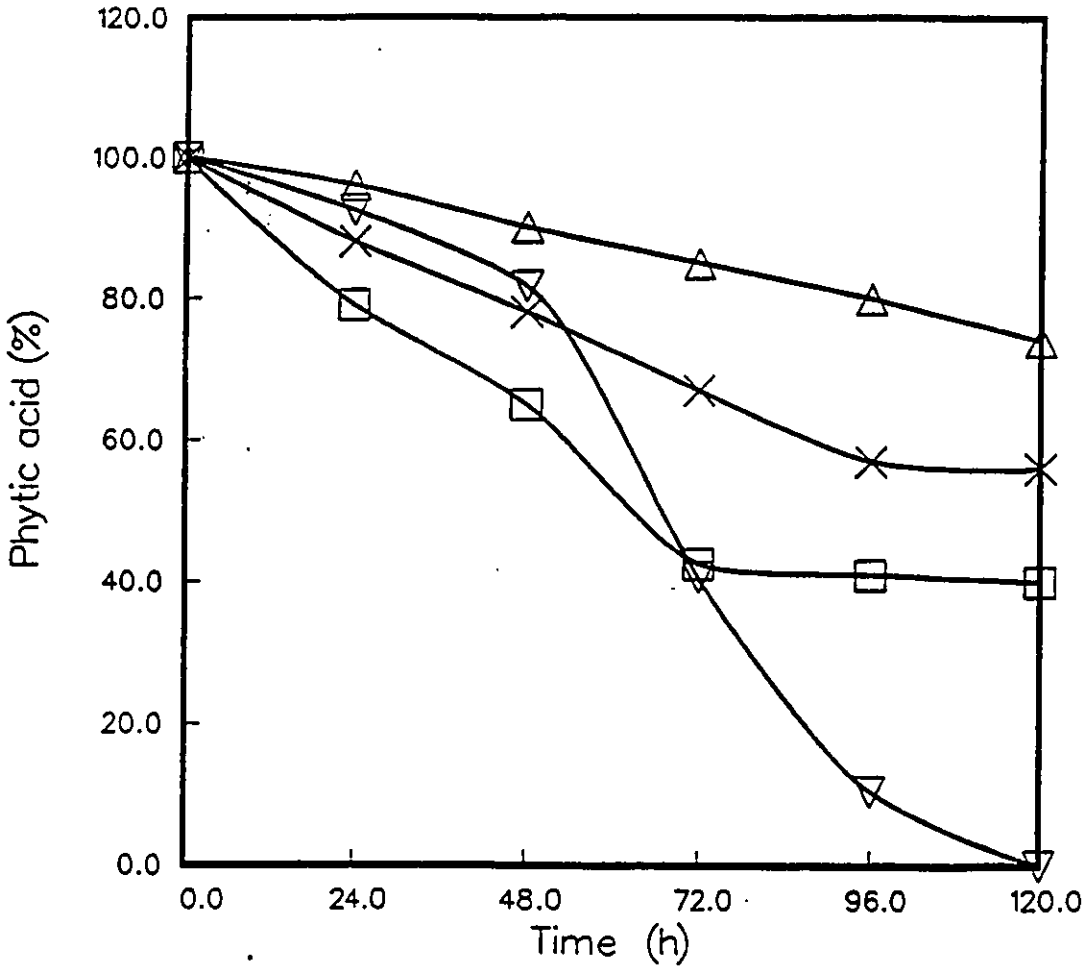


Figure 4.17: Phytic acid content reduction in canola meal by various microorganisms. (□ - *R. oligosporus*, △ - *S. cerevisiae*, X - *R. oligosporus* + *S. cerevisiae*, ▽ - *A. carbonarius*)

4.4 *Aspergillus ficuum* NRRL 3135

4.4.1 Biomass and Phytase Production

Aspergillus ficuum has been reported to have a phytase activity ten times higher than several *A. niger* strains and five times higher than some *A. carbonarius* strains (Shieh and Ware, 1968). For this reason, *A. ficuum* NRRL 3135 has been studied extensively. A few experiments were carried out in the submerged culture in order to determine the growth conditions and the enzyme production.

Aspergillus ficuum NRRL 3135 was grown in the liquid medium containing glucose to study the effect of the carbon source concentration on the biomass and phytase production in a submerged culture. The results show that an increase in glucose concentration resulted in the production of a larger amount of biomass (Table 4.2). However, enzyme production was suppressed by higher glucose concentrations. An increase in glucose concentration from 0.5% to 5.0% yielded an 85% increase in biomass concentration and a 64% decrease in enzyme concentration. The specific enzyme productivity was decreased by 78% in this case.

Noticing that an increase in glucose concentration suppressed the phytase production substantially, a fed batch technique was applied to enhance the production of the enzyme by eliminating the effect of high glucose concentrations. Batch processes with two different glucose concentrations were carried out as well. In the fed batch test, the initial glucose concentration was 0.5% and every 24 hours an amount of glucose which corresponds to its initial quantity was added to the medium. This culture technique resulted in a higher biomass and phytase production than in the batch processes (Fig. 4.18, 4.19, Table 4.2). All the above tests were carried out for 7 days. After the third day of growth, the biomass started to autolyze in the medium containing 0.5% glucose while the enzyme concentration was steadily increasing, suggesting the release of intracellularly confined enzyme. The final enzyme concentration in this medium was 0.12 units per mL, while in the medium containing 5% glucose it was only about 0.05 units per mL. The medium to which glucose

was added every 24 hours contained twice as much enzyme (0.25 units per mL) as that in the medium with 0.5% glucose. These results show that a higher enzyme concentration can be attained in a fed batch process than in a batch process due to the inhibition of the enzyme production at high glucose concentrations.

Table 4.2: Effect of glucose concentration on biomass and phytase production in a submerged culture

Initial glucose conc. (% w/v)	After 96 hours			
	Biomass (mg/mL)	Enzyme (units/mL)	Specific enzyme productivity (units/mg biomass)	Final pH
0.5	3.00	0.070	0.023	5.60
1.5	4.30	0.046	0.011	4.75
3.0	4.50	0.028	0.006	3.30
5.0	5.60	0.025	0.005	3.00

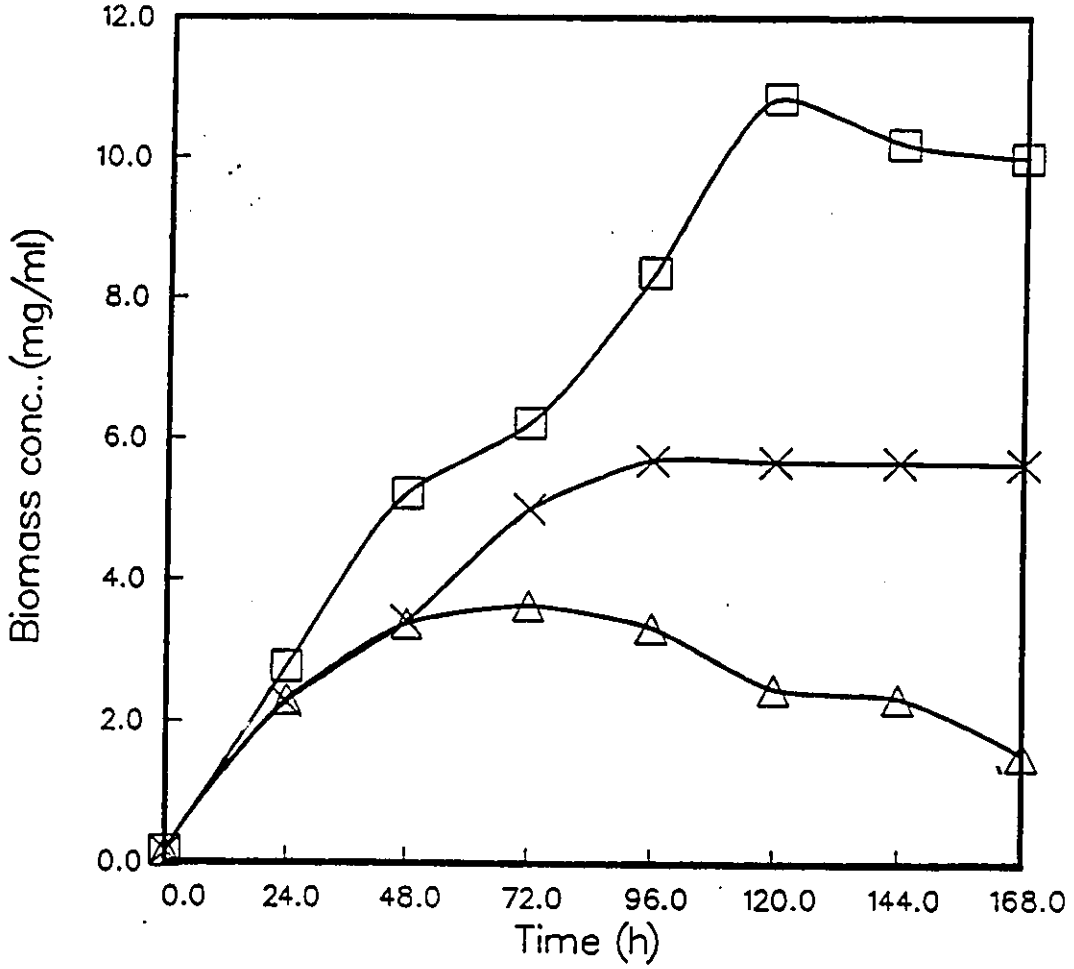


Figure 4.18: Biomass production in batch and fed batch processes using *A. ficuum* NRRL 3135. (□ - 0.5% glucose initially plus 0.5% glucose every 24 h., △ - 0.5% glucose, X - 5% glucose)

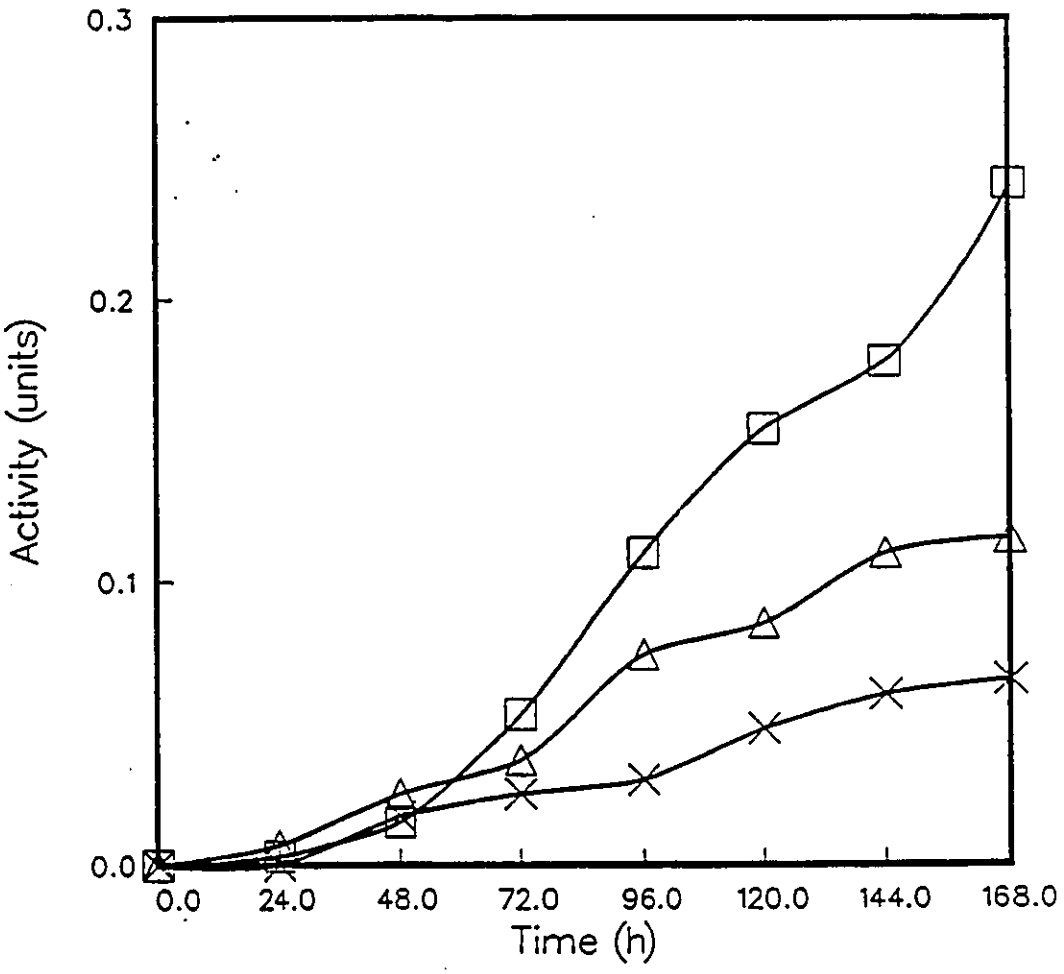


Figure 4.19: Phytase production in batch and fed batch processes using *A. ficuum* NRRL 3135. (□ - 0.5% glucose initially and 0.5% glucose every 24 h., △ - 0.5% glucose, X - 5% glucose)

The effect of aeration on the enzyme and biomass production was evaluated in 500 mL Erlenmeyer flasks filled with various amounts of the medium containing 0.5% glucose. The results show that different levels of aeration did not cause a significant difference in the amount of biomass produced but did affect the rate of its production (Fig. 4.20).

The systems with a better aeration resulted in higher enzyme concentrations (Fig. 4.21). It was also noted that, in the best aerated medium, the enzyme started to appear one day earlier than in those with a lower level of aeration. The effect of aeration is important for the production of phytase in a liquid medium, and this fact has to be taken into consideration in the development of a process for the reduction of phytic acid content in canola meal by a solid state technique.

4.4.2 Phytase Characteristics

To study some phytase characteristics, the enzyme extract, which was obtained after the growth of *A. ficuum* NRRL 3135 by the solid state technique on canola meal, was used because this extract had a higher enzyme activity than the enzyme preparation produced in the submerged culture. To find suitable conditions for enzyme determination, experiments were carried out with the systems having different incubation times and different substrate and enzyme concentrations.

Figure 4.22 shows the effect of incubation time on phytase activity. Up to 45 minutes of incubation time, the relationship between the time of incubation and the activity was almost linear. On the basis of these results, 30 min. of incubation time was chosen for further studies so as to compromise between the time of incubation and the activity. A longer incubation period would not be economically feasible.

The effect of substrate concentration on the phytase activity is shown in Fig. 4.23. Sodium phytate was used as the substrate. The enzyme system was saturated with the substrate when its concentration was 2.0 mM. The Lineweaver-Burk plot of the results (Fig. 4.24) yielded a K_m value of 0.27 mM, and a V_{max} value of 0.46 units/h for this enzyme.

Two quite different K_m values have been reported in the literature for the phytase of *A. ficuum* NRRL 3135 (Table 4.3). The K_m value obtained in this work is between the two of them. The higher reported value was obtained when calcium phytate was used as the substrate, while the lower value was obtained using sodium phytate. The authors who have reported the lower K_m value claimed that the higher K_m value was probably influenced by a high concentration of calcium ions introduced into the system in the form of the substrate used. Their explanation was based on the inhibition of the phytase from *Pseudomonas* species by magnesium ions, which resulted in an increase in K_m value of the enzyme (Irving and Cosgrove, 1971a,b). Although sodium phytate was used in this work, the obtained K_m value does not agree with either of these values. However, this value is within the range of K_m values (0.018 to 0.65 mM) reported for phytases from various plant sources.

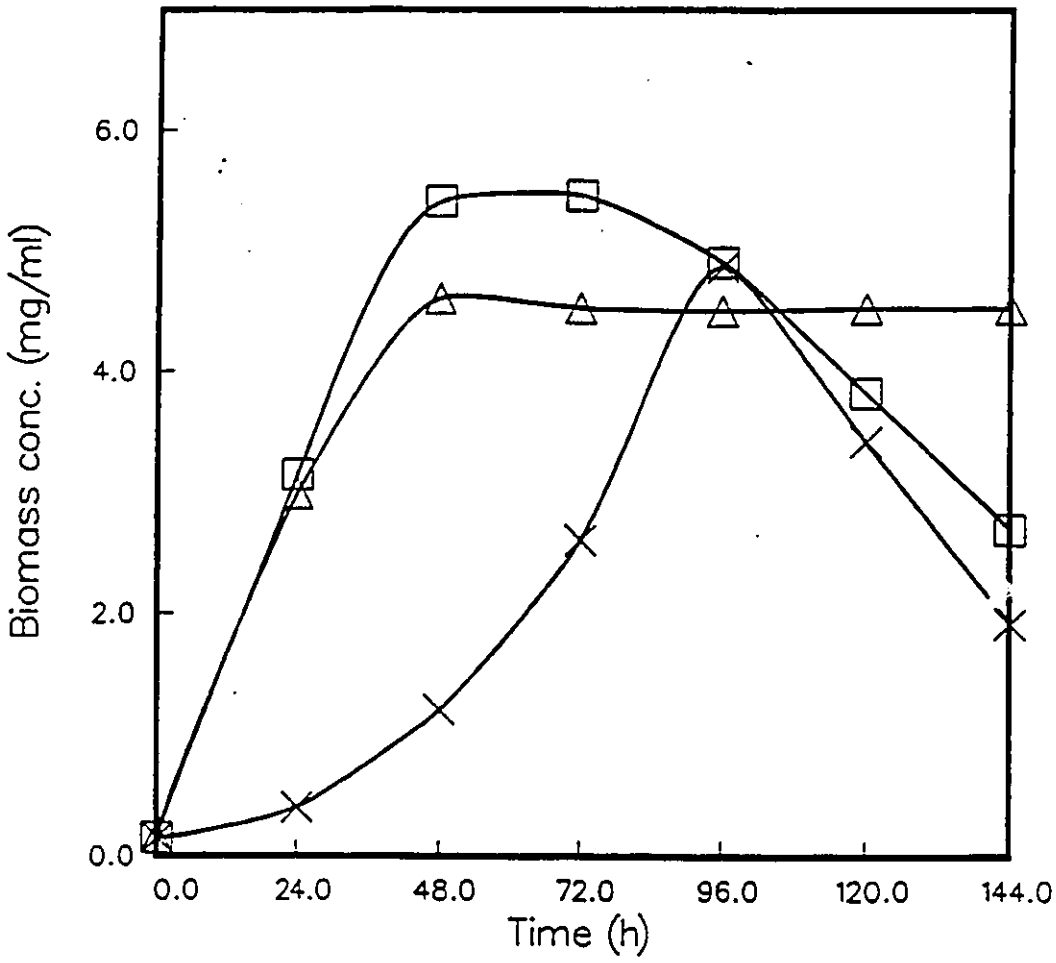


Figure 4.20: Effect of aeration on biomass production using *A. ficuum* NRRL 3135. (The amount of medium in a flask: □ - 50 mL, △ - 100 mL, X - 150 mL)

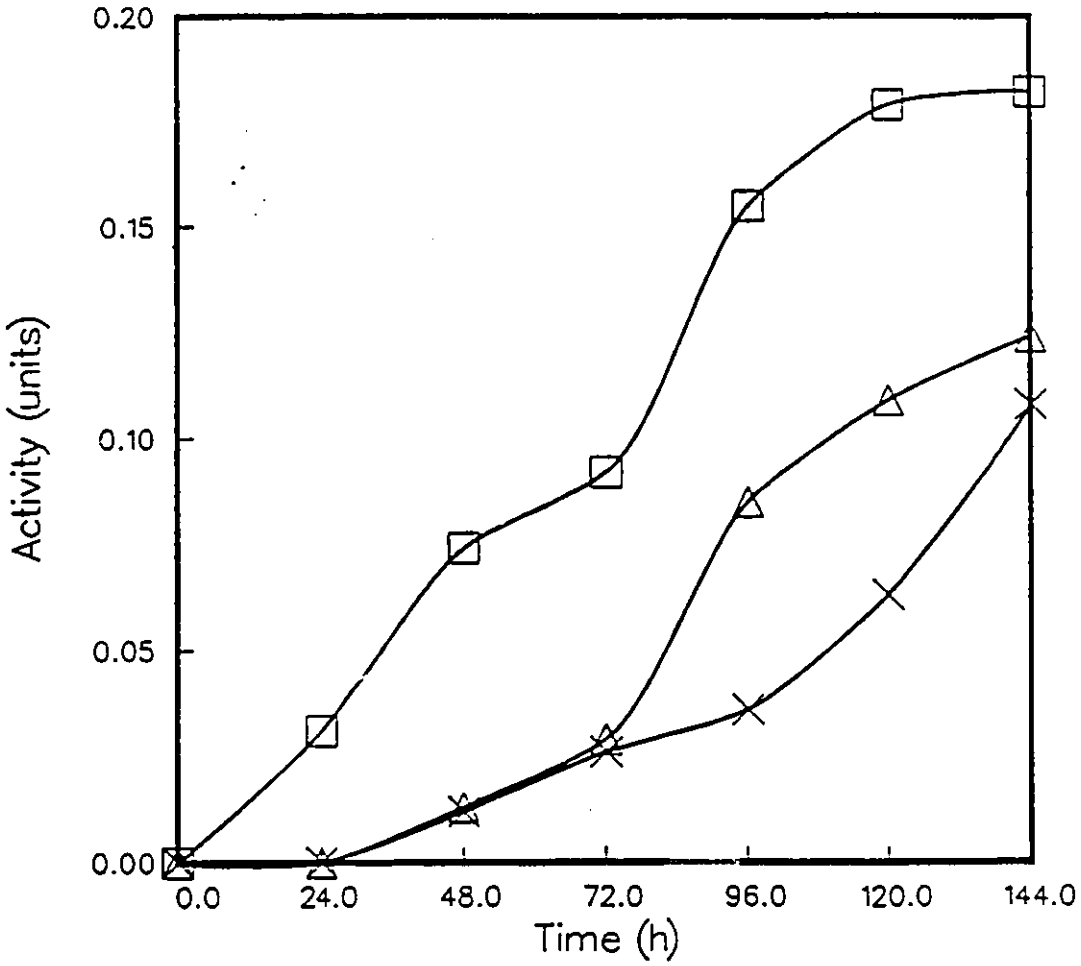


Figure 4.21: Effect of aeration on phytase production using *A. ficuum* NRRL 3135. (The amount of medium in a flask: □ - 50 mL, △ - 100 mL, X - 150 mL)

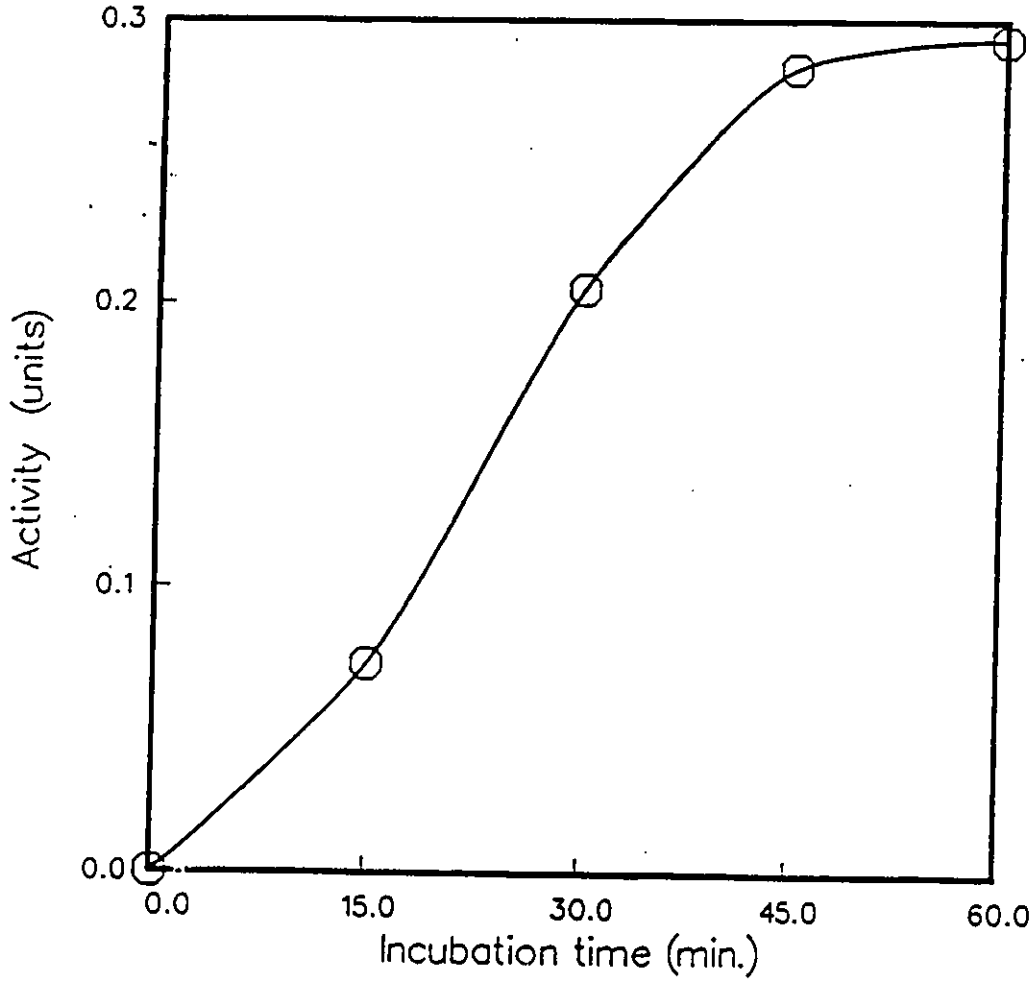


Figure 4.22: Effect of incubation time on phytase activity at 50°C and pH 5.0.

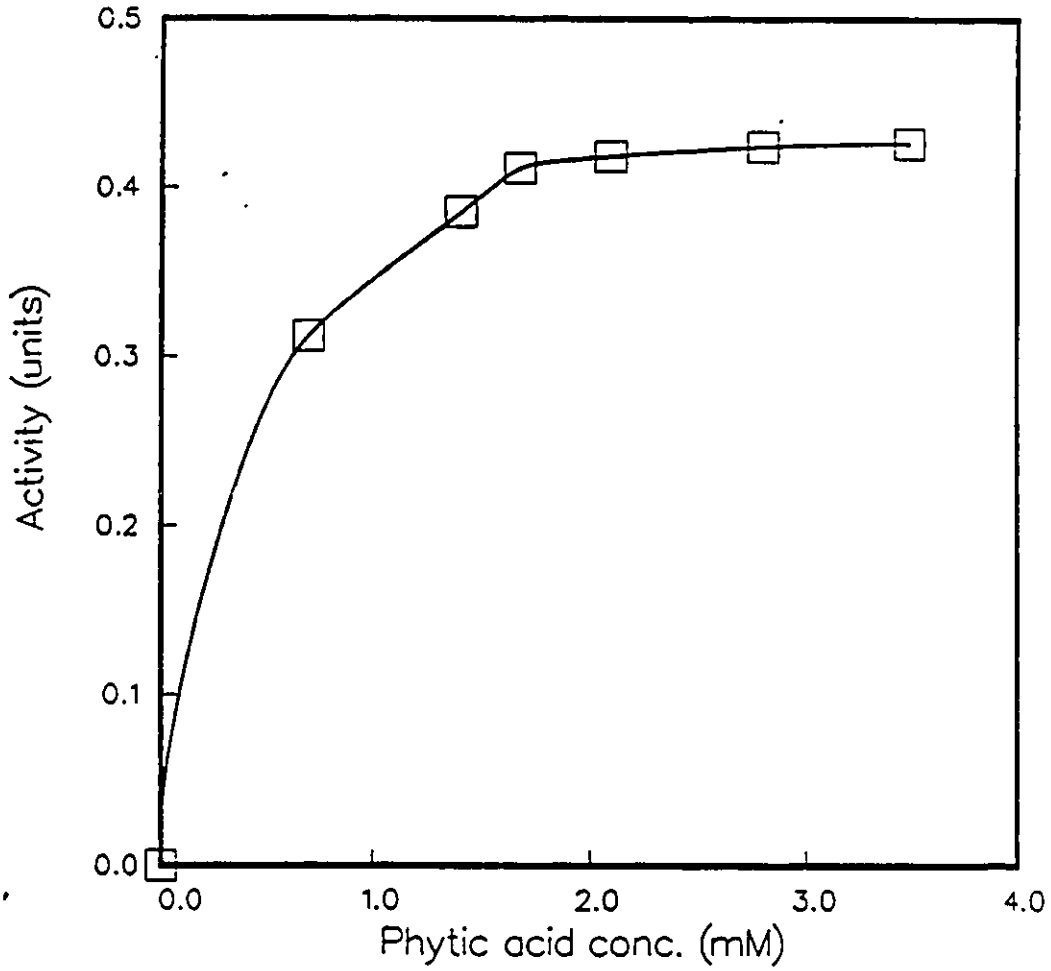


Figure 4.23: Effect of phytic acid concentration on phytase activity at 50°C and pH 5.0

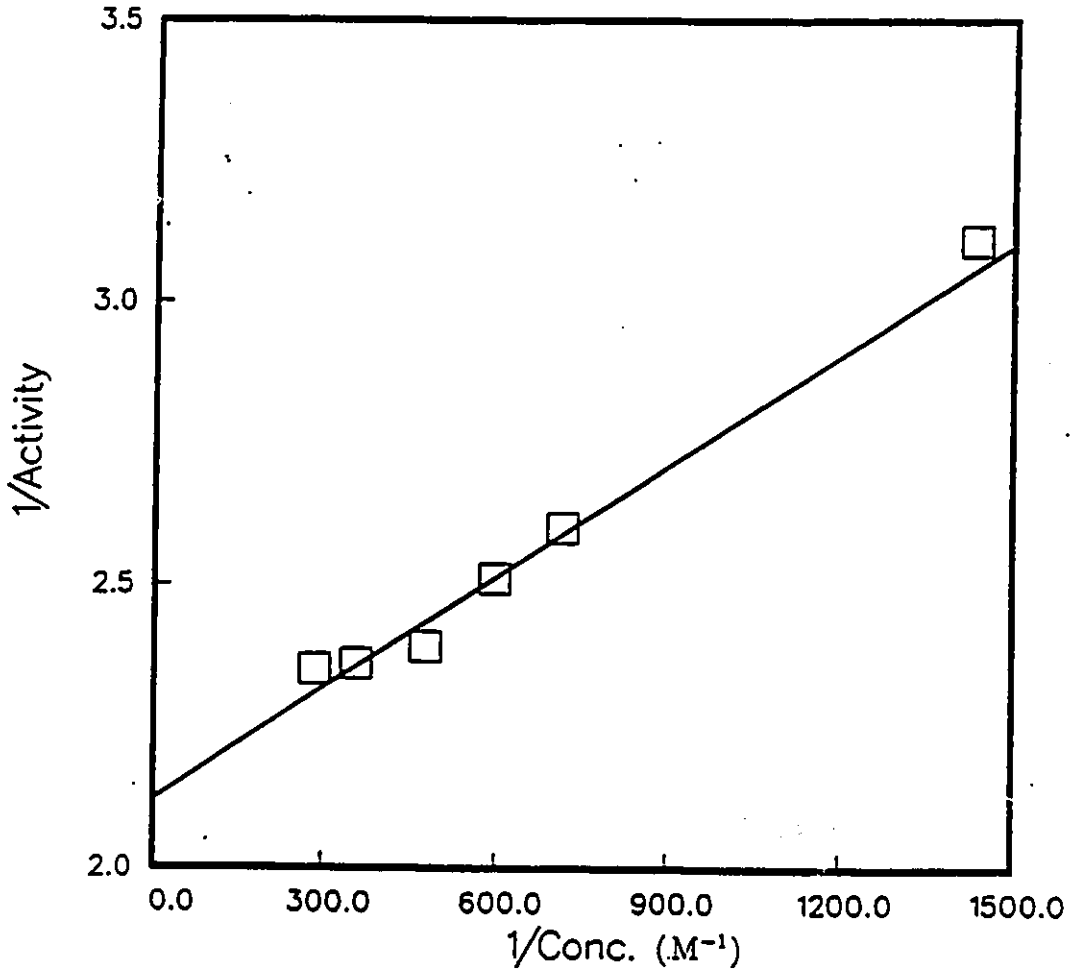


Figure 4.24: Determination of K_m and V_{max} for the phytase preparation.

Table 4.3: Some characteristics of phytases from various origins

Phytase origin	Optimum pH	Optimum temp. (°C)	K_m (mM)	References
Wheat	5.2	55	0.33	Peers, 1953
Corn	5.6	50	0.09	Chang, 1967
Beans	5.2	-	0.15	Gibbins and Norris, 1963
Mung beans	7.5	57	0.65	Mandal et al., 1972
Navy beans	5.3	50	0.018	Lolas and Markakis, 1977
Intestinal mucosae				
-rat .	7.0	-	-	Bitar and Reinhold, 1972
-human	7.4	-	-	"
-chicken	8.2	-	-	"
-calf	8.7	-	-	"
R. oligosporus	5.6	-	0.28	Sudarmadji and Markakis, 1977
A. ficuum	5.5	-	1.25	Shieh et al., 1969
A. ficuum	5.3	-	0.013	Irving and Cosgrove, 1972; 1974
A. ficuum	5.0	60	0.27	This work

Different enzyme concentrations were tested for their effect on its activity. Almost a linear relationship exists between the amount of enzyme preparation and its activity up to 1 mL of the enzyme preparation in the systems having phytate concentration of 2.0 mM (Fig. 4.25). With further increase in enzyme concentration, the activity increased at a much lower rate. One mL of enzyme preparation was used for subsequent assays, since it would be better to use the minimum amount of enzyme to achieve the maximum activity.

Phytase activity was determined for a pH range between 4.2 and 5.9. The result shows that the optimum pH for the enzyme activity is 5.0 (Fig. 4.26). This result is comparable to those obtained for the same microorganism by other researchers and to the values reported for the phytases of wheat and beans (Table 4.3). The enzyme seems to be more stable at lower than at higher pH; its activities obtained at pH 5.9 and 4.2 are 75% and 20% respectively lower than the value obtained at the optimum pH.

The effect of temperature on the phytase activity was studied for a temperature range between 30°C and 70°C (Fig. 4.27). Three different enzyme preparations were used for this purpose and as expected, all of them yielded the same result. The optimum temperature for the enzyme activity was found to be 60°C and it is higher than the reported values for phytases of other origins (Table 4.3).

It was necessary to establish the stability of the enzyme at different temperatures because the intention was to use such enzyme preparations in a process for the reduction of phytic acid content in canola meal. It was found that preincubating this enzyme at 60°C for half an hour does not cause a significant reduction in phytase activity; however, almost 70% of the activity was lost during the preincubation for three hours (Fig. 4.28). The preincubation at 50°C for three hours resulted in the reduction of only 5% of the activity.

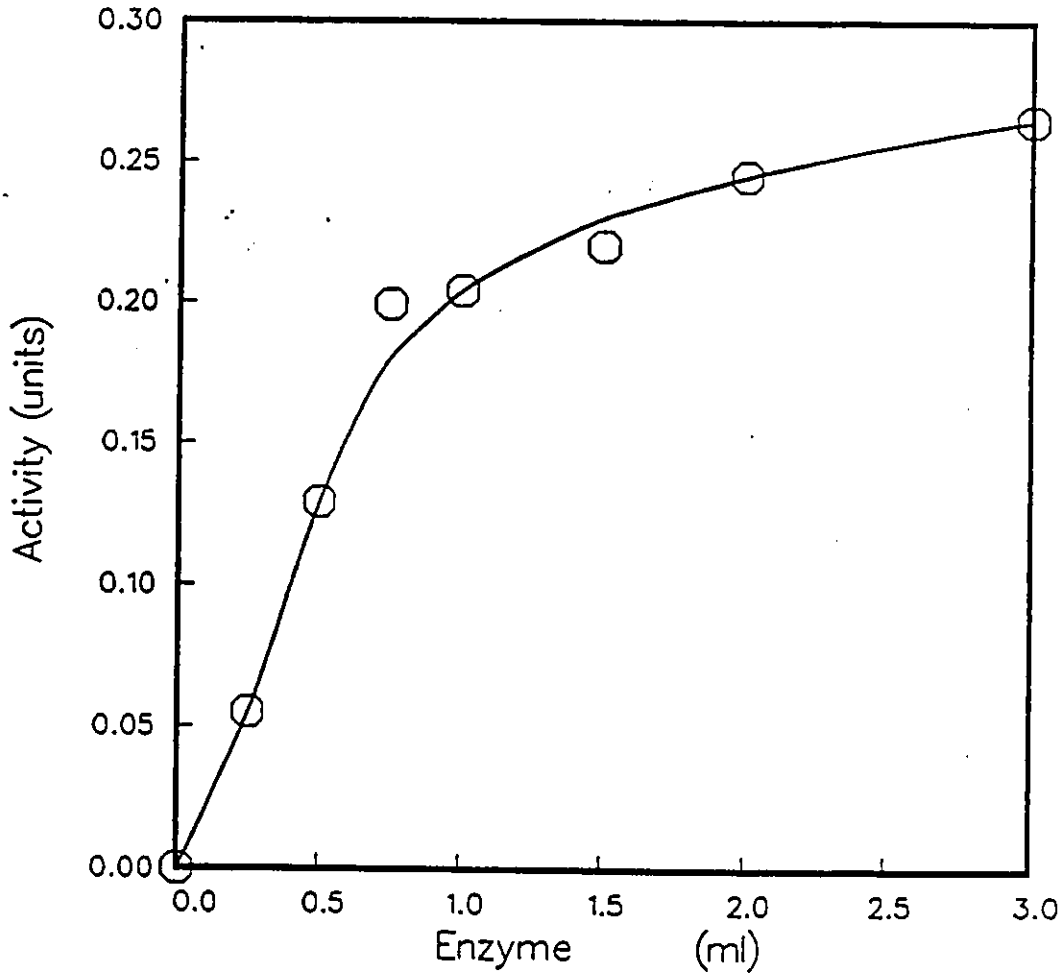


Figure 4.25: Effect of enzyme concentration on phytase activity at 50°C and pH 5.0

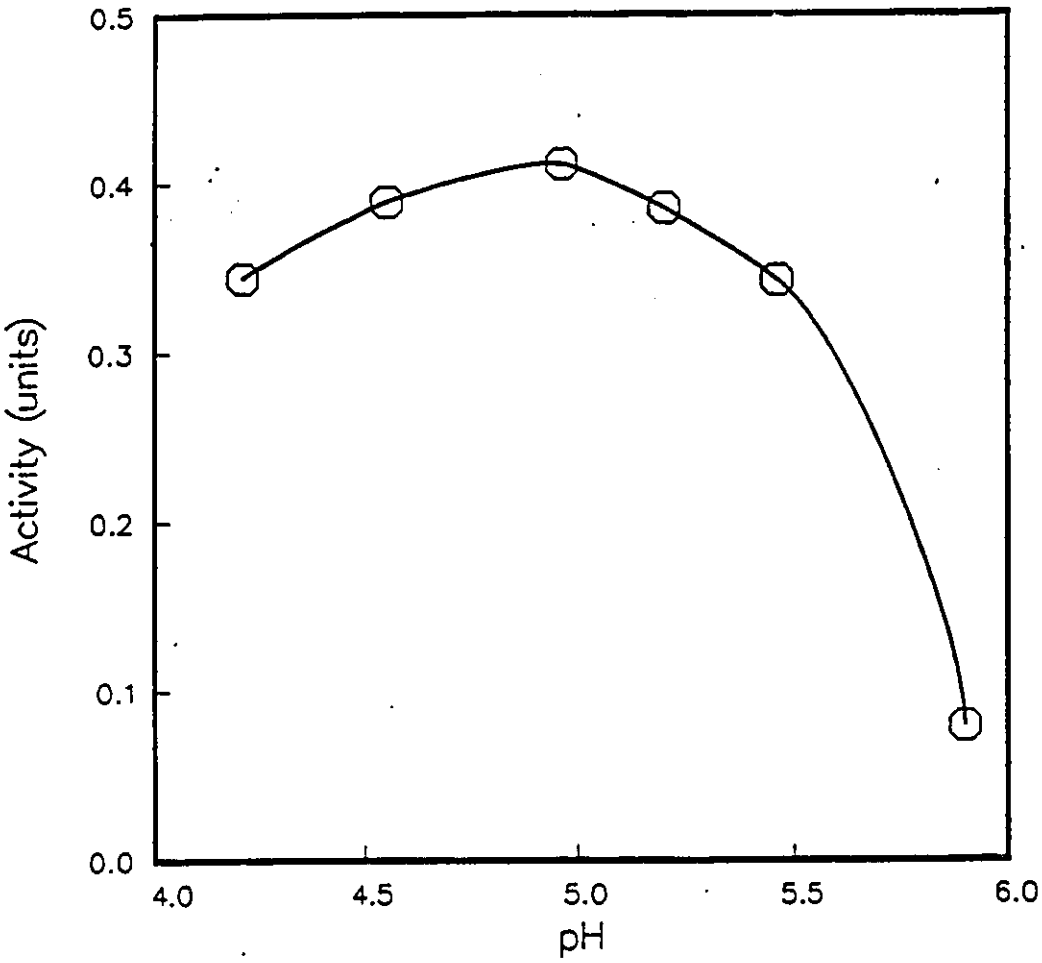


Figure 4.26: Effect of pH on phytase activity at 50°C.

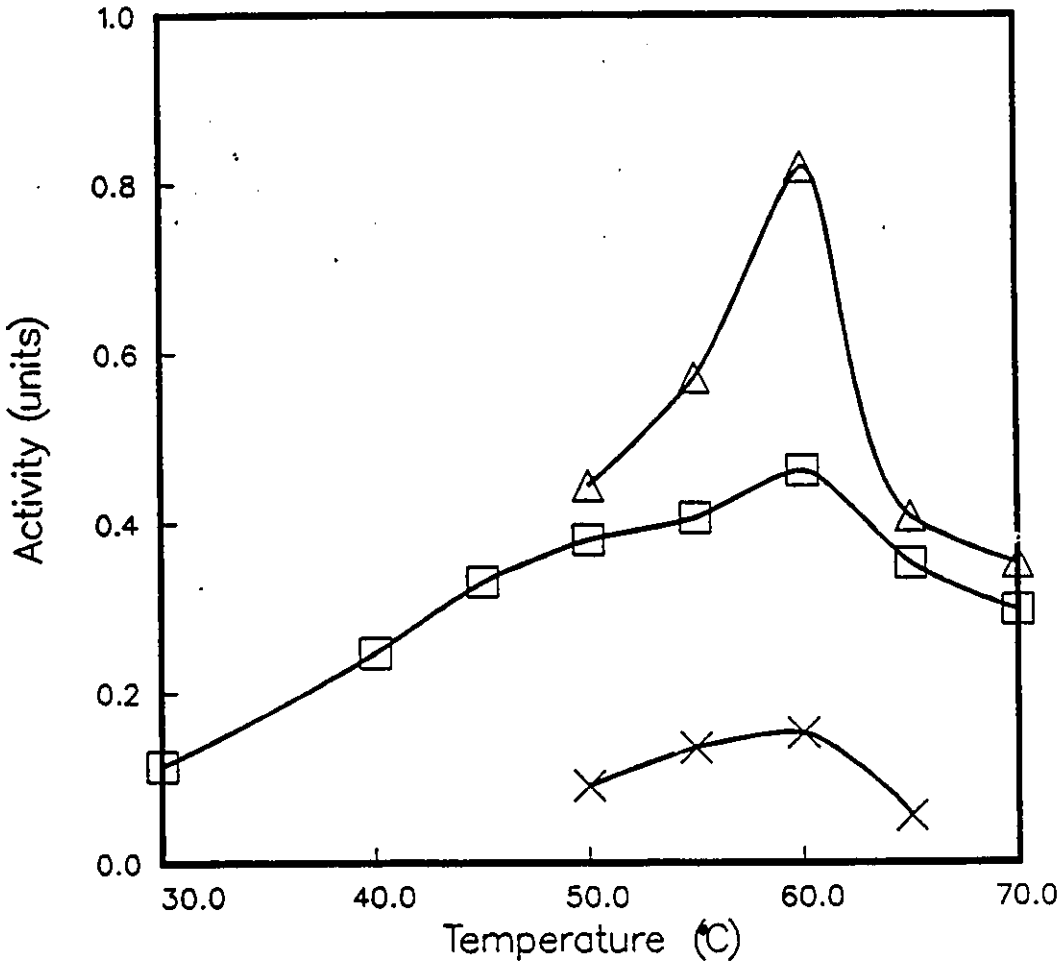


Figure 4.27: Effect of temperature on phytase activity at pH 5.0 (□ - 1:2 extract, △ - 1:1 extract, X - liquid broth)

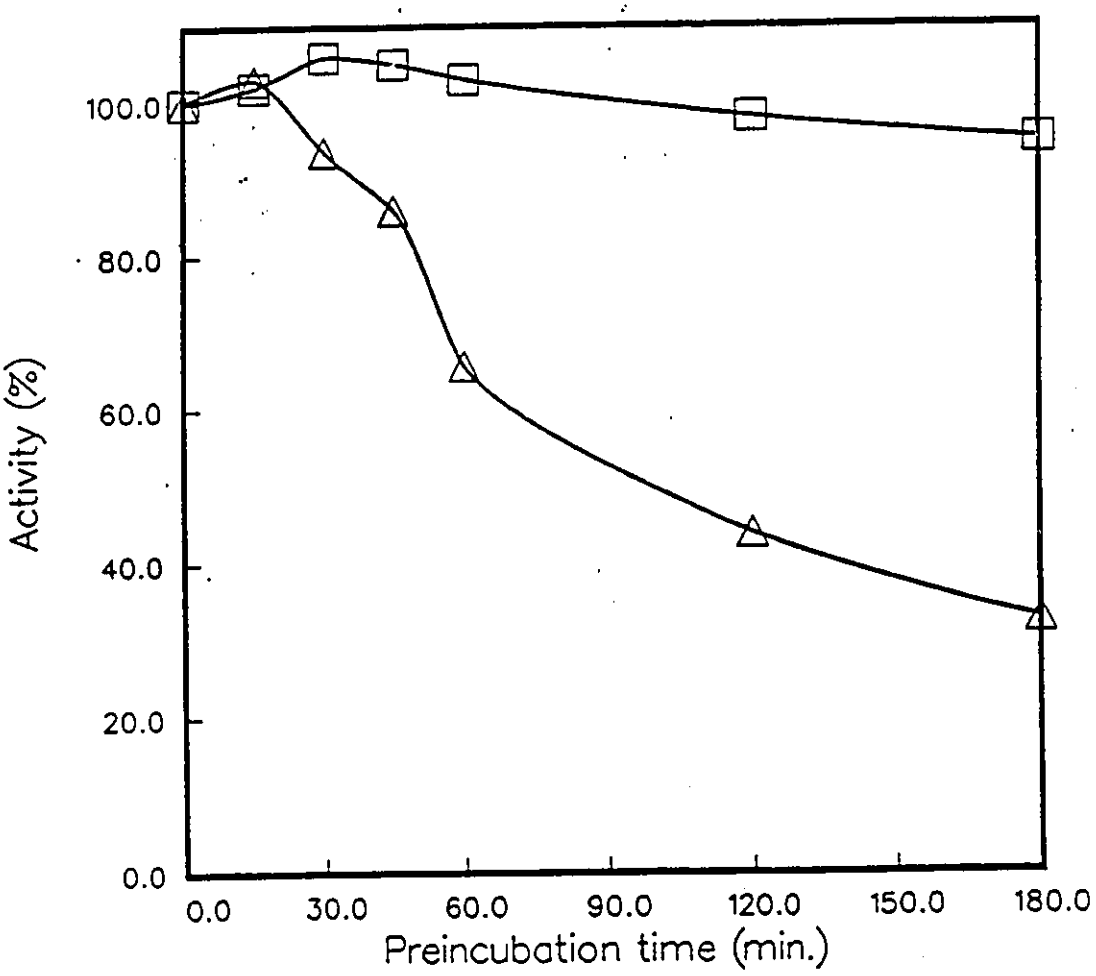


Figure 4.28: Effect of preincubation of enzyme preparation at 50°C and 60°C on its activity. (□ - 50°C, △ - 60°C)

To establish the period of time for which the enzyme can be stored at a lower temperature, a concentrated enzyme preparation was kept for five weeks at 4°C and its activity measured during that time. It was noticed that its activity decreased by about 10% in a week (Fig. 4.29); after that time, the activity continued decreasing but at a much lower rate. The total loss of activity in five weeks of storage at 4°C was about 15%. These results show that the enzyme preparation can be stored for a longer period of time without a significant loss in its activity.

4.4.3 SSF Process

To examine the possibility of the reduction of phytic acid content in canola meal by *A. ficuum* NRRL 3135, this microorganism was grown by a solid state technique on a canola meal medium for 5 days at 30°C. During that period of time, 91% of the phytic acid content was reduced (Fig. 4.30). During the execution of this test, the change in carbohydrate and protein contents in the meal were also measured. It was found that about 50% of total carbohydrates present in canola meal were in soluble and the rest in insoluble forms. The results show that about 80% of the soluble fraction of carbohydrates were used up in the first 24 to 48 hours of growth while the amount of insoluble fraction remained the same. The carbohydrates in the canola meal were consumed for the growth and the production of phytase and other by-products. It was noticed that the protein content of canola meal increased by 25% after 48 to 72 hours of growth of the microorganism (Fig. 4.30). Since canola meal is used as a feed stuff, the increase in protein content plays a significant role in the upgrading of the material. The amount of protein started to decrease after the third day of incubation. Taking this fact into account, it is desirable to design the process in such a way that the phytic acid content is completely reduced during the first two to three days to avoid the loss of protein.

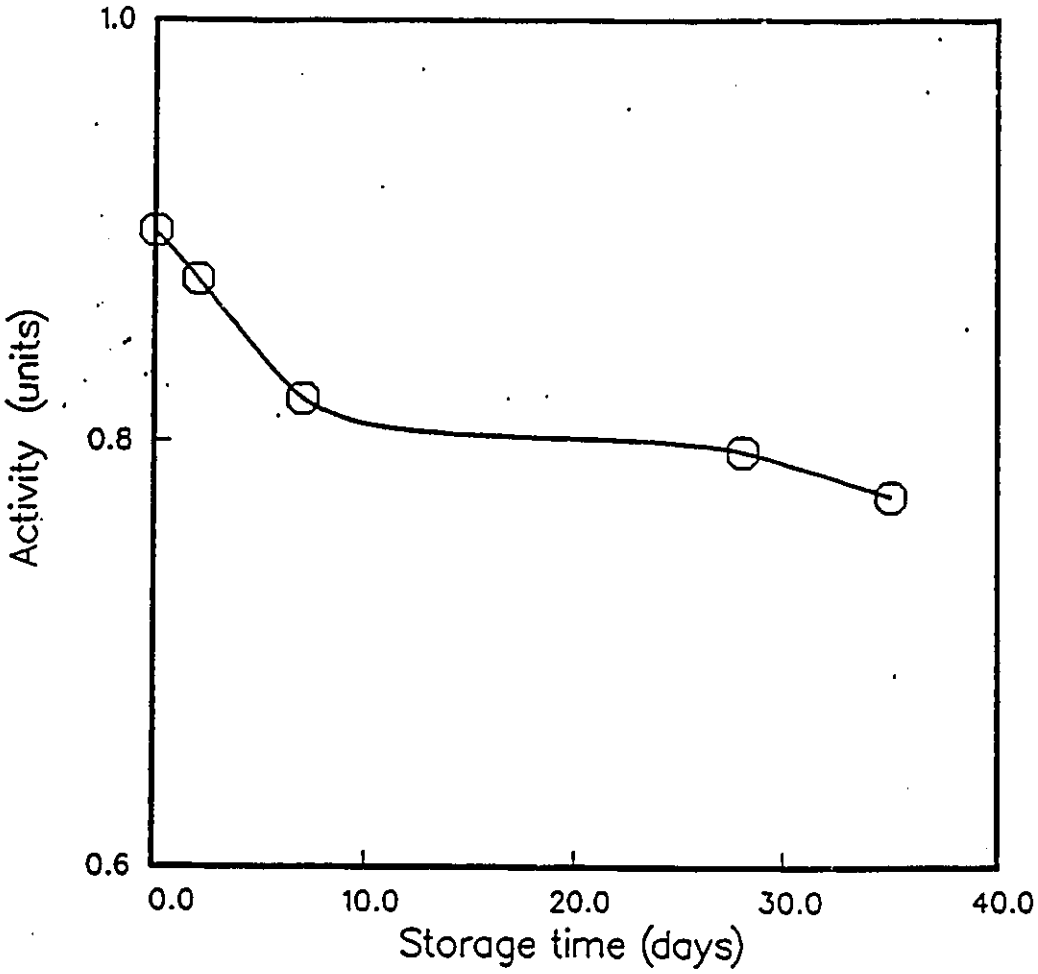


Figure 4.29: Effect of storage time at 4°C on the phytase activity of a concentrated enzyme preparation at pH 5.0 and 60°C

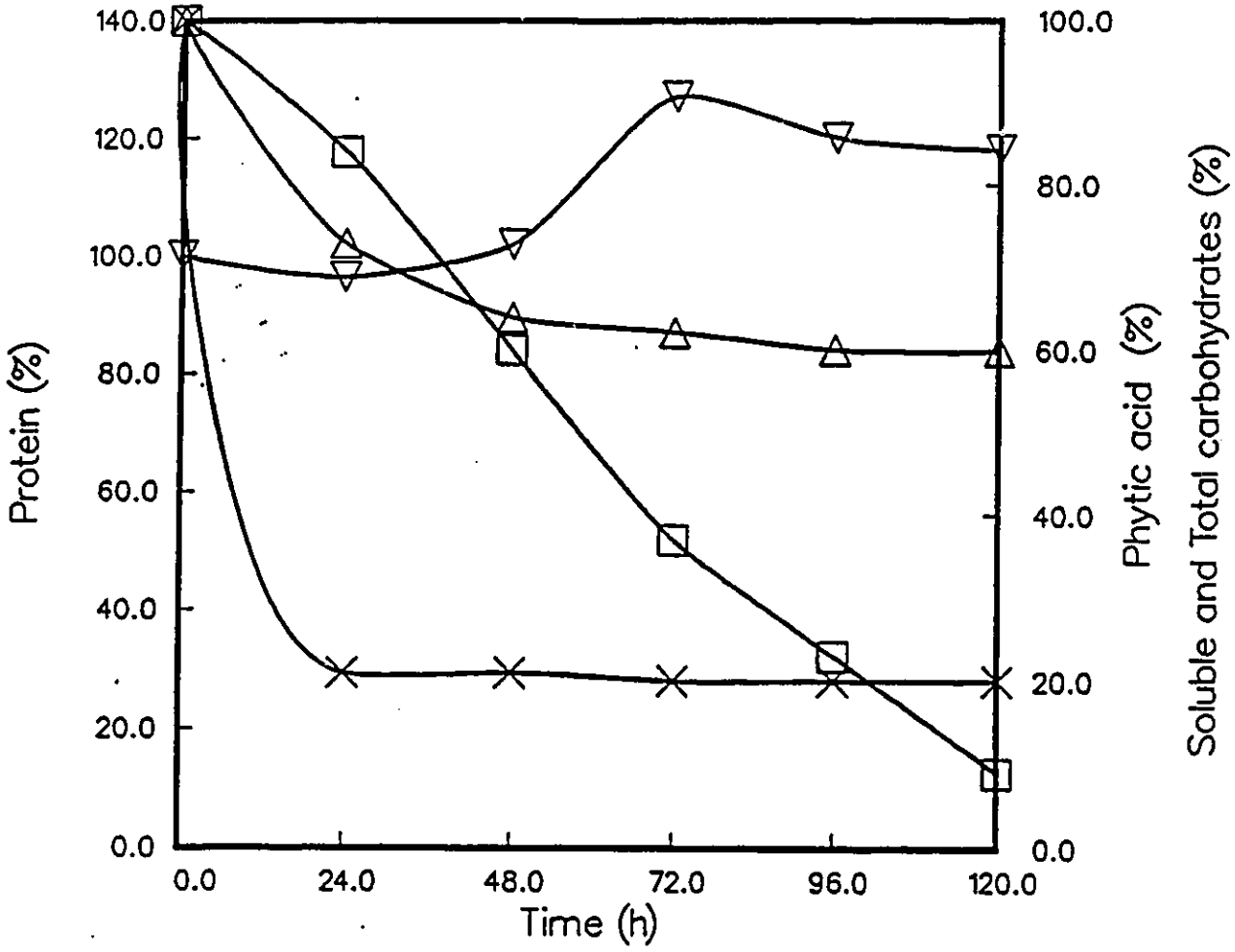


Figure 4.30: Relative changes in phytic acid, protein and soluble and total carbohydrates in canola meal using *A. ficuum* NRRL 3135 in a solid state technique (□ - phytic acid, △ - total carbohydrates, ▽ - protein, X - soluble carbohydrates)

The effect of the amount of biomass in the inoculum on the reduction of phytic acid content is shown in Fig. 4.31. The results show that the phytic acid content of the meal was reduced to zero in each of the systems regardless of the amount of biomass in the inoculum, but the rates of decrease differed. When the inoculum contained 101 mg of biomass, six days of incubation were required for complete hydrolysis of phytic acid in the meal. With an increase in the amount of biomass used for inoculation (proportionally larger amount of inocula were transferred), the time required for complete reduction of phytic acid content decreased. The phytic acid content was reduced to zero in two days in the system inoculated with 390 mg of biomass in the inoculum. Bearing in mind that various volumes of inocula were used and that they contained some phytase, it is obvious that the amount of the enzyme transferred to the canola meal medium was different in each of the above systems. It could be speculated that the amount of enzyme has a significant effect on the reduction of the phytic acid content in the meal. After measuring the phytase, it was found that the inoculum containing 101 mg of biomass also contained 1.25 units of the enzyme. The inocula containing larger amount of biomass contained proportionally larger amounts of the enzyme. The largest amount of the enzyme that was transferred to 50 g of the meal was 4.7 units. Taking into account that the amounts of transferred enzyme, although different in each system, were relatively small compared to the amount of enzyme required to completely reduce the phytic acid content (this will be shown later in this work), it can be concluded that the effect of the transferred enzyme in these tests was negligible and that the difference in the rates of reduction in phytic acid content was predominantly due to the amount of the biomass used for inoculation.

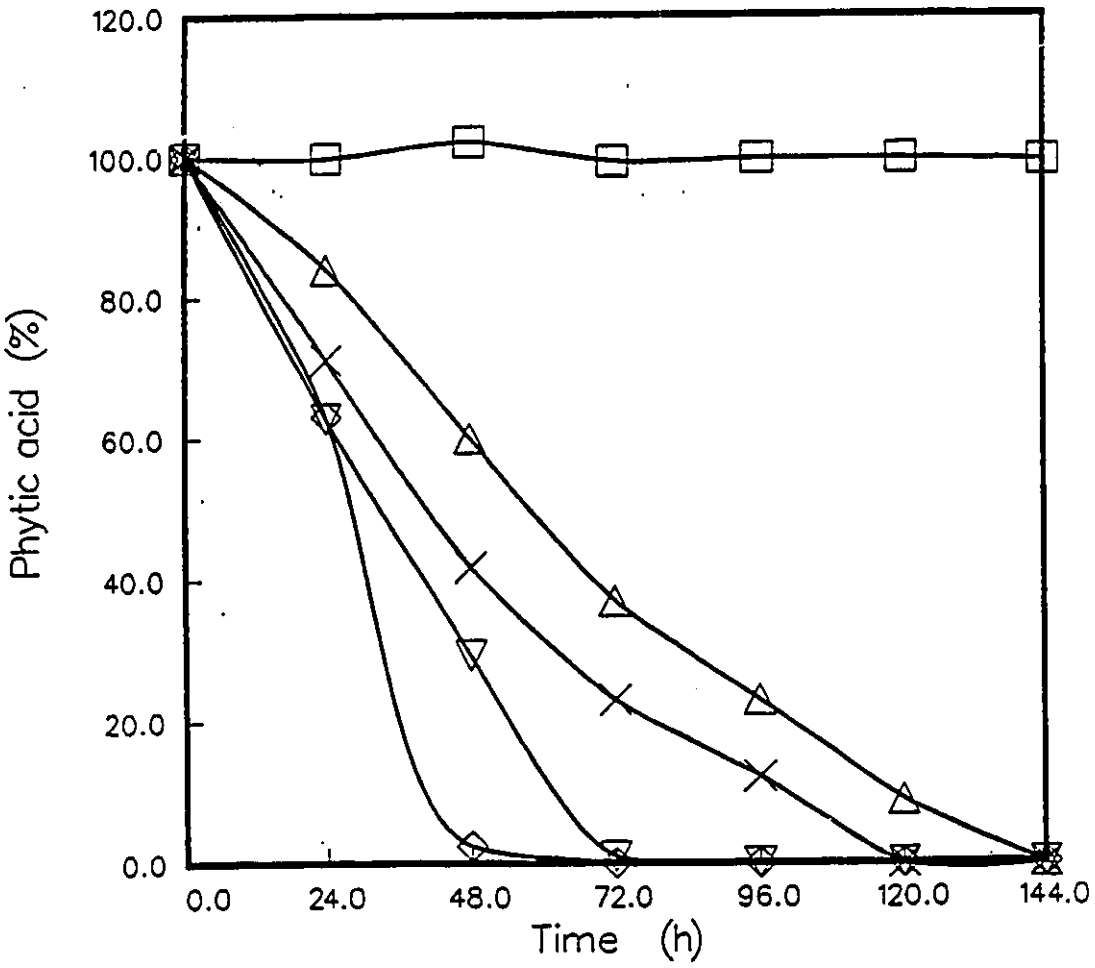


Figure 4.31: Effect of the amount of biomass in the inoculum on the rate of phytic acid content reduction in canola meal using *A. ficuum* NRRL 3135. (Biomass in inoculum : □ - 0.0 mg (control), △ - 101 mg, X - 202 mg, ▽ - 330 mg, ◇ - 390 mg)

Observing the development of the mold, it was noticed that during the first 24 hours of incubation, the growth was visually unnoticeable in all the systems; moderate and excellent growth was observed within 48 and 60 hours respectively. The colour of the canola meal changed with the growth of the microorganism from brownish to whitish; i.e. the intensity of the colour change was related to the intensity of the growth of the microorganism.

Examining the effect of the inoculum age on the phytic acid content reduction, it was found that older inocula were more efficient (Fig. 4.32). The system inoculated with a two days old inoculum still retained about 22% of the initial amount of phytic acid after five days of incubation, while the system with a six days old inoculum had almost none of the phytic acid after 48 hours. For inoculation of the meal in each of these systems, the same initial amount of biomass was used regardless of its age. However, the total amount of phytase transferred to the meal was different in each system due to the difference in the amount of inoculum broth used. It ranged from 0.37 units to 3.6 units for inocula two to six days old respectively. In this case, the effect of the amount of the transferred enzyme on the rate of reduction of phytic acid content in the canola meal can be neglected for the same reason which was discussed in the previous set of tests.

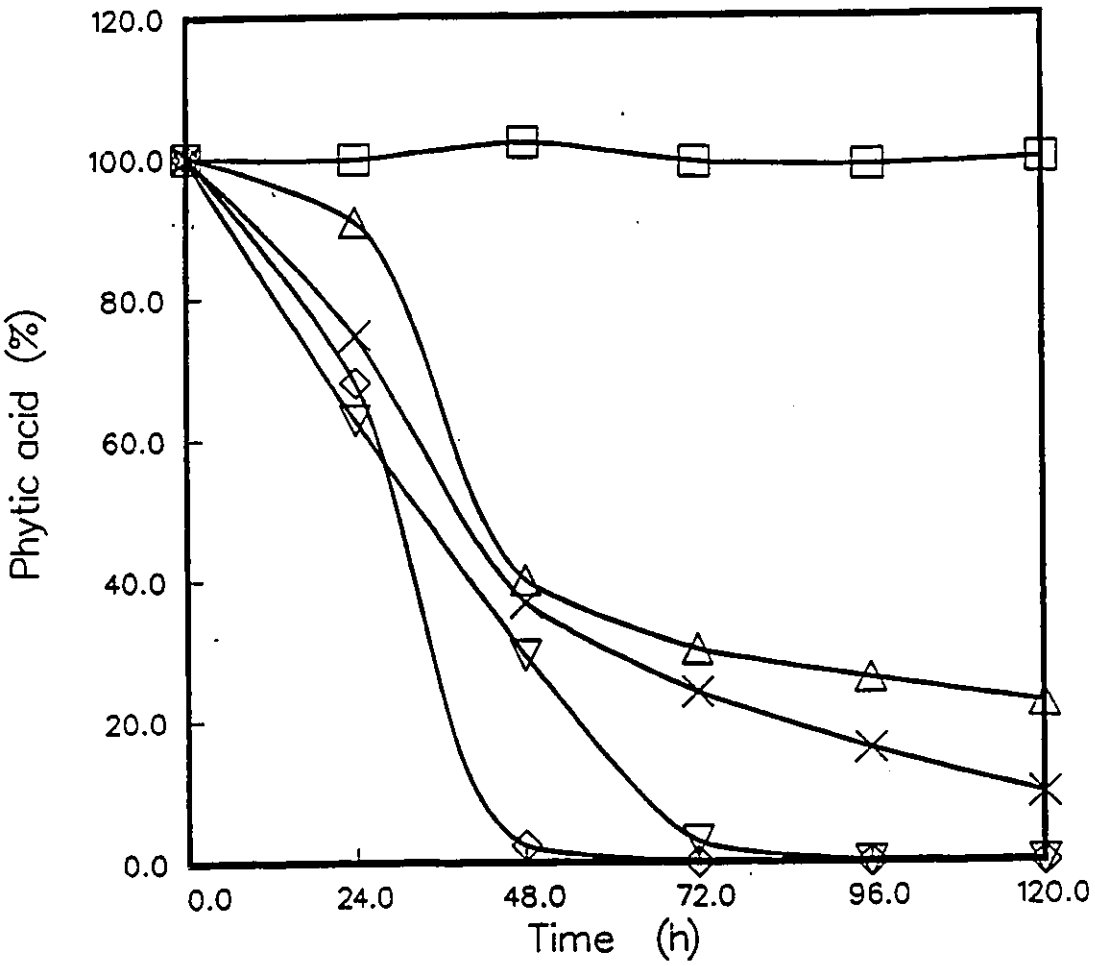


Figure 4.32: Effect of age of inoculum on phytic acid content reduction using *A. ficuum* NRRL 3135. (Age of inoculum : □ - Control (no inoculum), △ - 2.0 days, X - 3.0 days, ▽ - 4.5 days, ◇ - 6.0 days)

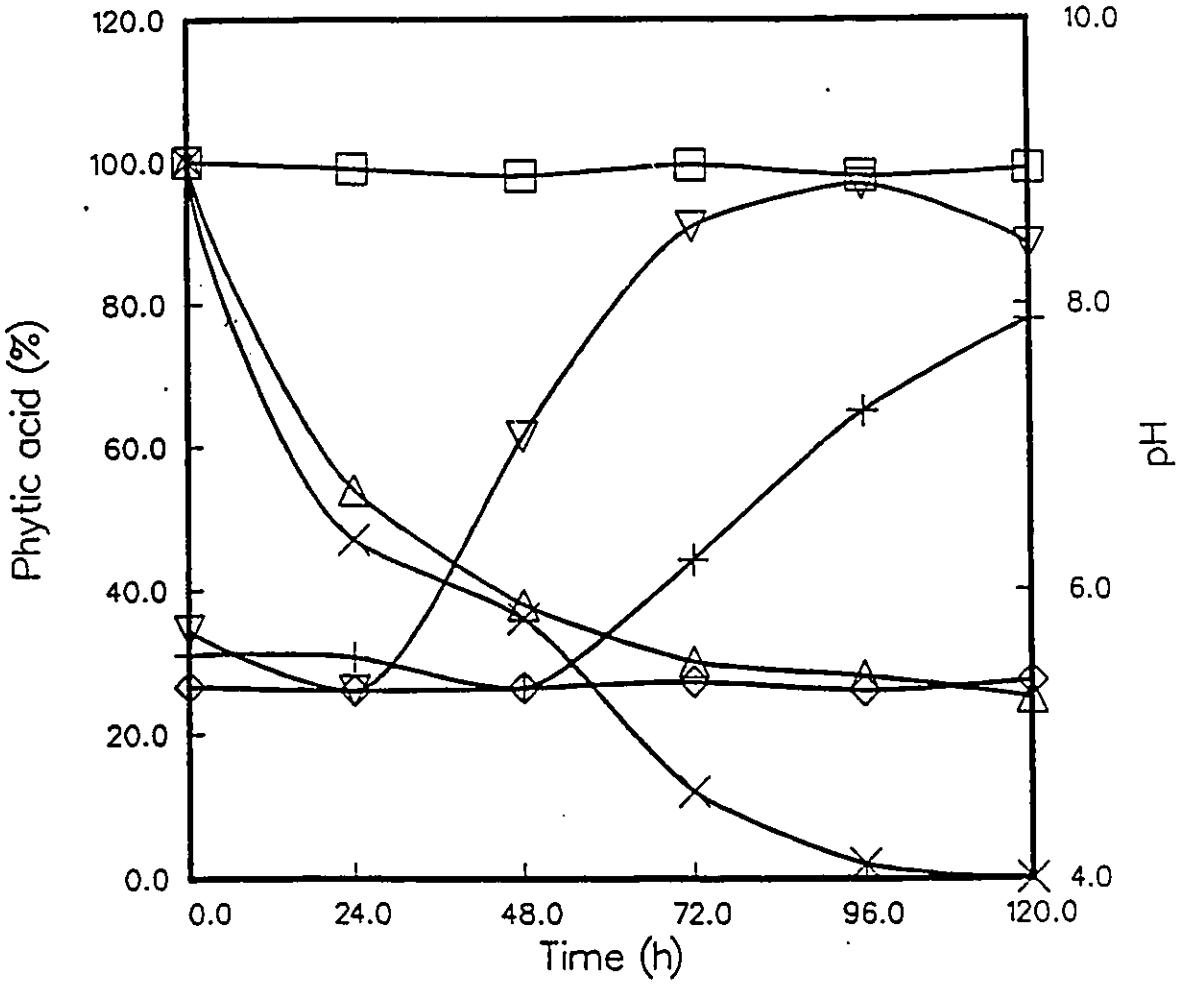


Figure 4.33: Effect of buffer on the reduction of phytic acid content using *A. ficuum* NRRL 3135. (System without buffer : △ - phytic acid, ▽ - pH; System with buffer : X - phytic acid, + - pH; Control (no inoculum) : □ - phytic acid, ◇ - pH)

During the growth of *A. ficuum* NRRL 3135 on the meal, the pH value increased from about 5.6 to 8.2 in five days of incubation (Fig. 4.33). Knowing that the pH optima for microbial phytases are between 4.8 and 5.6 (Graf, 1986), the SSF of a canola meal medium supplemented with water was compared with the one in which water was substituted with the same amount of 0.2 M acetate buffer (pH 4.7). The results show (Fig. 4.33) that the pH of the systems without and with buffer remained constant for the first 24 hours and 48 hours respectively. After these periods of time, the pH started increasing in both systems, but as expected, the increase was higher in the system without buffer than in the one with buffer. Almost complete reduction of phytic acid content occurred in the buffered system in four days, while in the unbuffered system, only 71% of phytic acid was hydrolyzed in the same period of time. It can also be seen from this figure that in the first 24 hours of fermentation, there was no significant difference in the reduction of phytic acid content between the two systems, because the pH values were similar in both of them during that period of time.

During the preparation of inocula in the liquid medium, *A. ficuum* NRRL 3135 grew in the form of pellets. The next tests were designed to investigate the effect of homogenization of inoculum by treating it in a blender for ten seconds at a low speed. In spite of the fact that the biomass can be partially damaged by the treatment, it was assumed that this process could increase the rate of reduction of phytic acid content in the meal due to the generation of more centres of growth. The results show (Fig. 4.34) that homogenization shortened the period of time required for the complete reduction of phytic acid content. It can also be seen that the amount of phytic acid content reduced within the first 24 hours was lower in the system with homogenized inoculum than in the non-homogenized one. This can be explained by a lower initial activity of the homogenized biomass, because this operation caused partial damage of the biomass and it needed some time to recover.

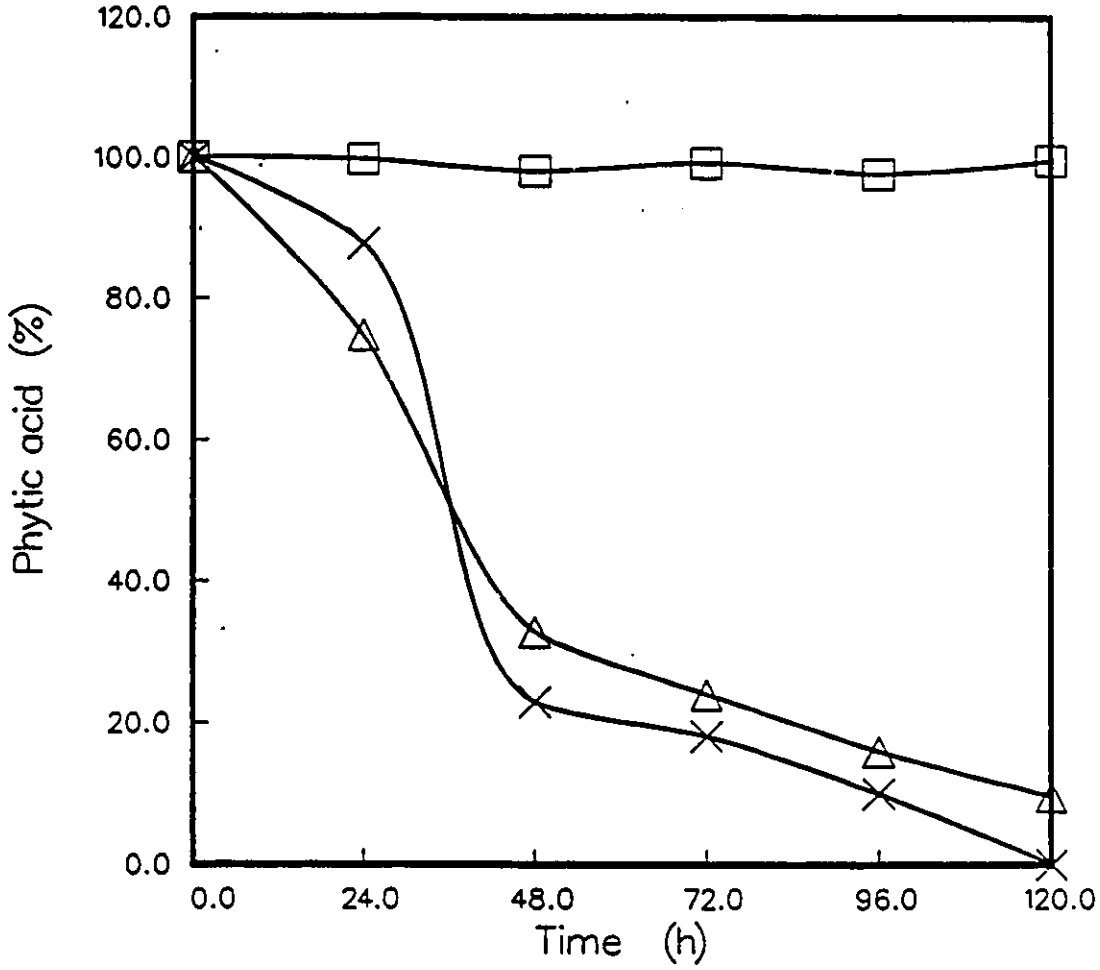


Figure 4.34: Effect of homogenization of inoculum on the reduction of phytic acid content using *A. ficuum* NRRL 3135. (□ - Control (no inoculum), △ - non-homogenized inoculum, X - homogenized inoculum)

It has been known that the moisture content of the medium in SSF is very important for the growth of microorganisms. For example, Raimbault and Alazard (1980) have reported that the optimum moisture for biomass production in SSF was 55%. To examine the effect of moisture content on the reduction of phytic acid content, tests were carried out with the meal containing between 52% and 78% moisture. The results show (Fig. 4.35) that the highest rate of reduction was achieved when the medium contained 67% moisture. With a higher or lower moisture content, the rate of the reduction of phytic acid content decreased. A certain lag in the decrease of phytic acid content during the first 48 hours of fermentation was noticed in the two systems with the lowest amount of moisture. The reason may be a slower growth of the microorganism and subsequently lower amount of phytase produced. After that time, the amount of biomass probably increased and even in those two systems, the rate of phytic acid content reduction increased substantially.

Partial reduction in phytic acid content of canola meal occurred even when uninoculated non-sterile medium was incubated (Fig. 4.36). Growth of the microorganisms already present in the canola meal is a possible reason for it. To see the effect of inoculation, a non-sterile medium was inoculated with *A. ficuum* NRRL 3135 and the results were compared with those obtained with an uninoculated non-sterile medium and an inoculated sterile medium. The results show (Fig. 4.36) that there was only 25% reduction in phytic acid content in the uninoculated non-sterile medium, while a better reduction was achieved in both cases where an inoculum was used. The best results were obtained when the sterile medium was inoculated with *A. ficuum* NRRL 3135. *A. ficuum* in non-sterile medium had to compete with the microorganisms already present in the canola meal. This probably resulted in a smaller amount of phytase produced. It can also be assumed that some of the microorganisms in the canola meal produced some proteolytic enzymes which affected the *A. ficuum* NRRL 3135 phytase. Another possibility is that by sterilization, some compounds in the canola meal became more accessible to the microorganism for its growth.

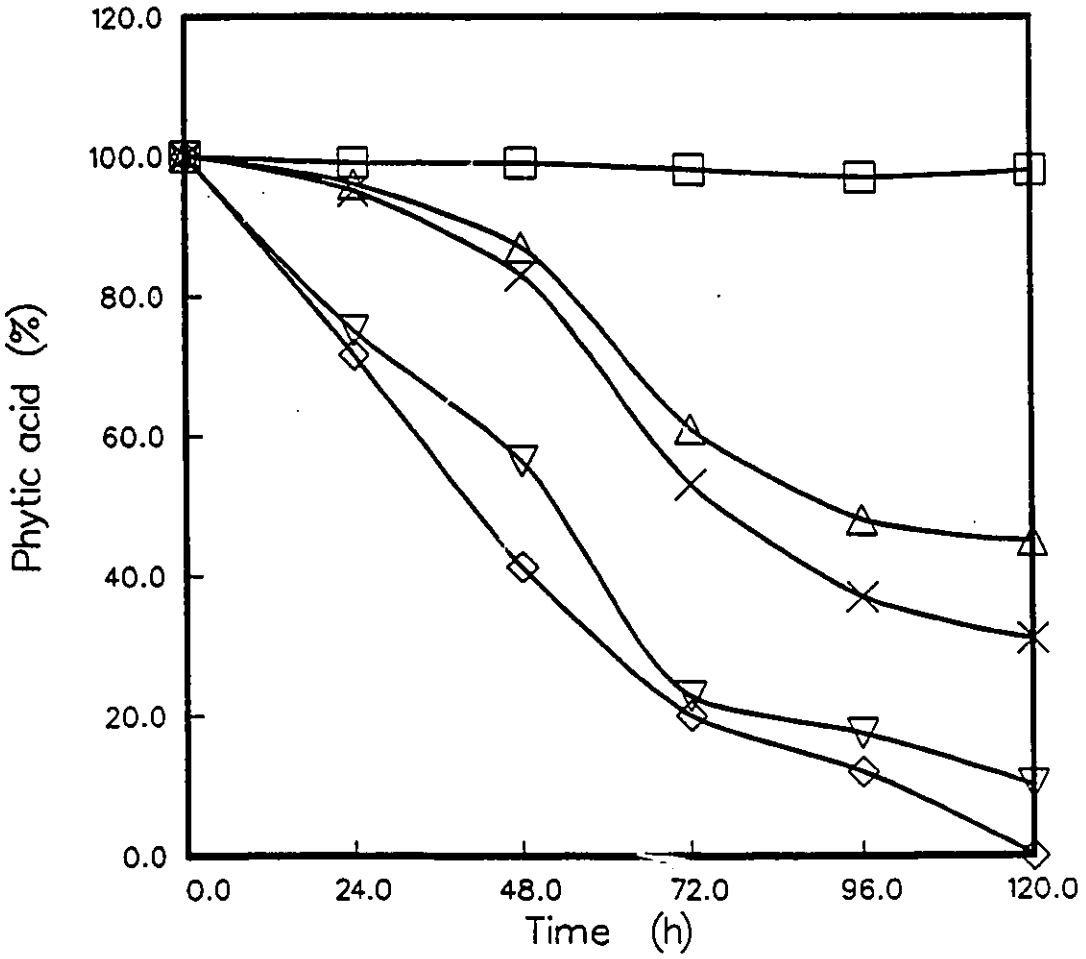


Figure 4.35: Effect of moisture content on the reduction of phytic acid content using *A. ficuum* NRRL 3135. (Moisture : □ - 65%, Control (no inoculum), △ - 52%, X - 60%, ◇ - 67%, ▽ - 78%)

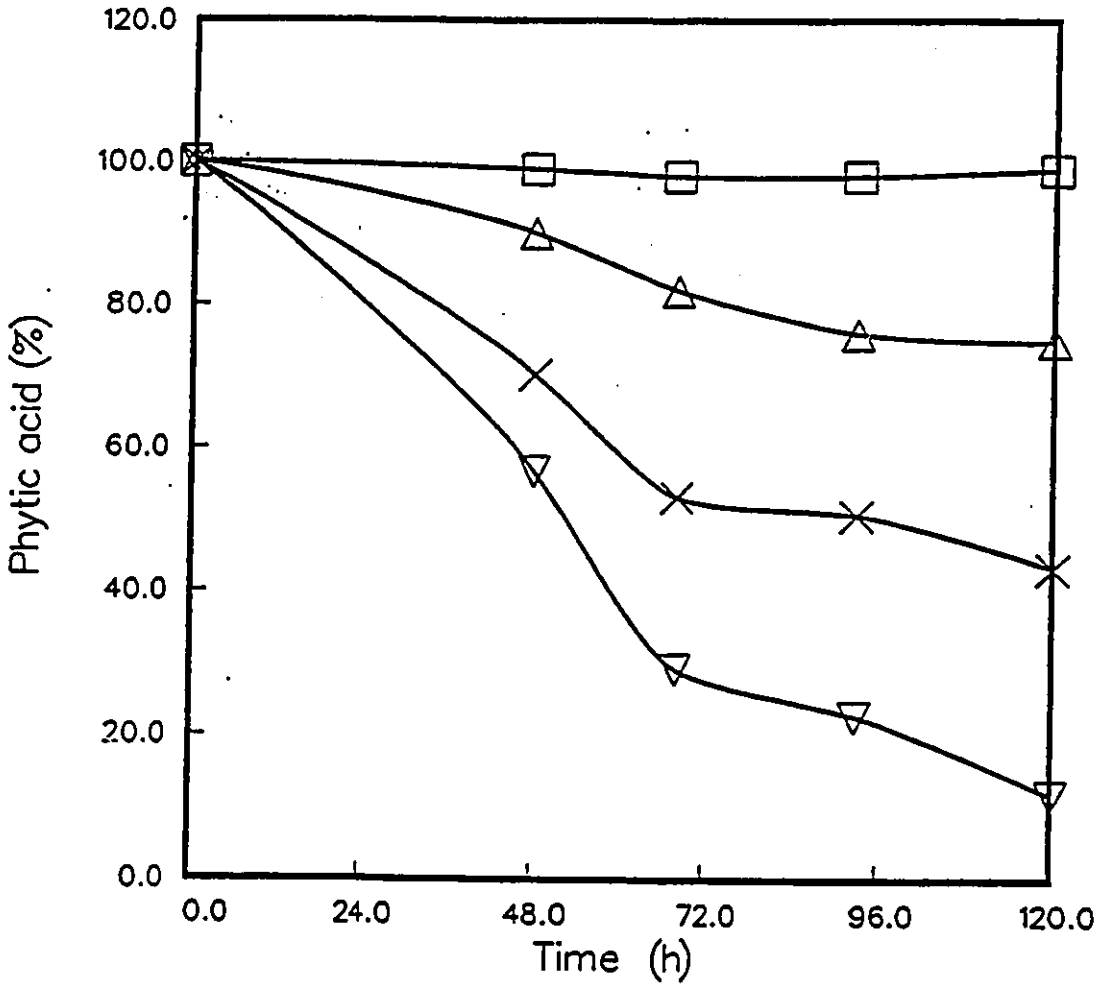


Figure 4.36: Reduction of phytic acid content in the sterile and non-sterile media using *A. ficuum* NRRL 3135. (Medium : □ - sterile, uninoculated, △ - non-sterile, uninoculated, X - non-sterile, inoculated, ▽ - sterile, inoculated)

In order to study the kinetics of phytase production on canola meal medium in a SSF process, an amount of canola meal was inoculated with *A. ficuum* NRRL 3135; samples were removed at regular intervals and the enzyme was extracted using a 2% aqueous solution of $CaCl_2$. The meal to the extractant ratio was 1:2. The results show (Fig. 4.37) that the amount of enzyme produced increased with the fermentation time, and it reached a maximum value after 7 to 8 days of fermentation.

To test the efficiency of repeated extractions of the enzyme produced on canola meal in a SSF process, 200 g of canola meal was inoculated with 1200 mg of *A. ficuum* NRRL 3135 (3 days old); after 180 hours of fermentation at 30°C the canola meal was treated with the extractant. The results of three consecutive extractions are given in Table 4.4. It shows that 65% of the total enzyme was extracted in the first step.

A reduction of phytic acid content in canola meal was also carried out using the enzyme preparation extracted from the canola meal treated in a SSF process. After the addition of the enzyme preparation, the moisture content of the meal was about 62%. In these tests, the concentration of enzyme was about 1.2 units/g of canola meal. The process was carried out at different temperatures. The results in Table 4.5 show that 53% of phytic acid content was reduced in 20 hours at 30°C. The highest (58%) and the lowest (44%) degree of reduction occurred at 45°C and 60°C respectively. A faster enzyme denaturation at 60°C was the reason for the lower reduction of phytic acid content at this temperature. It was observed earlier that 70% of the phytase activity was lost by preincubating the enzyme at 60°C for three hours.

Table 4.4: Extraction of the enzyme from canola meal treated in a SSF process

	Extraction of enzyme		
	First	Second	Third
Ratio of meal:extractant	1:2	2:3	1:1
Volume collected(mL)	450	350	250
Enzyme (units/mL)	0.697	0.308	0.248
Total enzyme (units)	314	108	62
% of total	65	22	13

Table 4.5: Reduction of phytic acid content in canola meal by enzyme preparation[2] in 20 hours of incubation

Temperature (°C)	Reduction (%)
30	53.0
45	58.0
60	44.0

[2]contains 1.2 units/g of meal

Similar tests carried out at 30°C and 50°C with the enzyme preparation added to a suspension of 12% canola meal in acetate buffer resulted in a much faster reduction in phytic acid content (Fig. 4.38) than in the previous case when the moisture content of the medium was only 62% (Table 4.5), although the same amount of enzyme preparation per gram of the meal was used. Ninety percent of phytic acid content was reduced in 24 hours of incubation at 30°C, and complete reduction was attained in 12 hours at 50°C. A better mass transfer in the suspension of canola meal than in the solid medium was probably the reason for the faster reduction of phytic acid content in that material.

The results from these experiments using *A. ficuum* NRRL 3135 have shown that this microorganism is an excellent source of phytase. Not only does *A. ficuum* NRRL 3135 reduce the phytic acid content in canola meal completely, but it also increases the protein content of the medium. It was also observed that the enzyme preparation extracted from canola meal after the treatment in a SSF process can be used effectively for the reduction of the phytic acid content in canola meal.

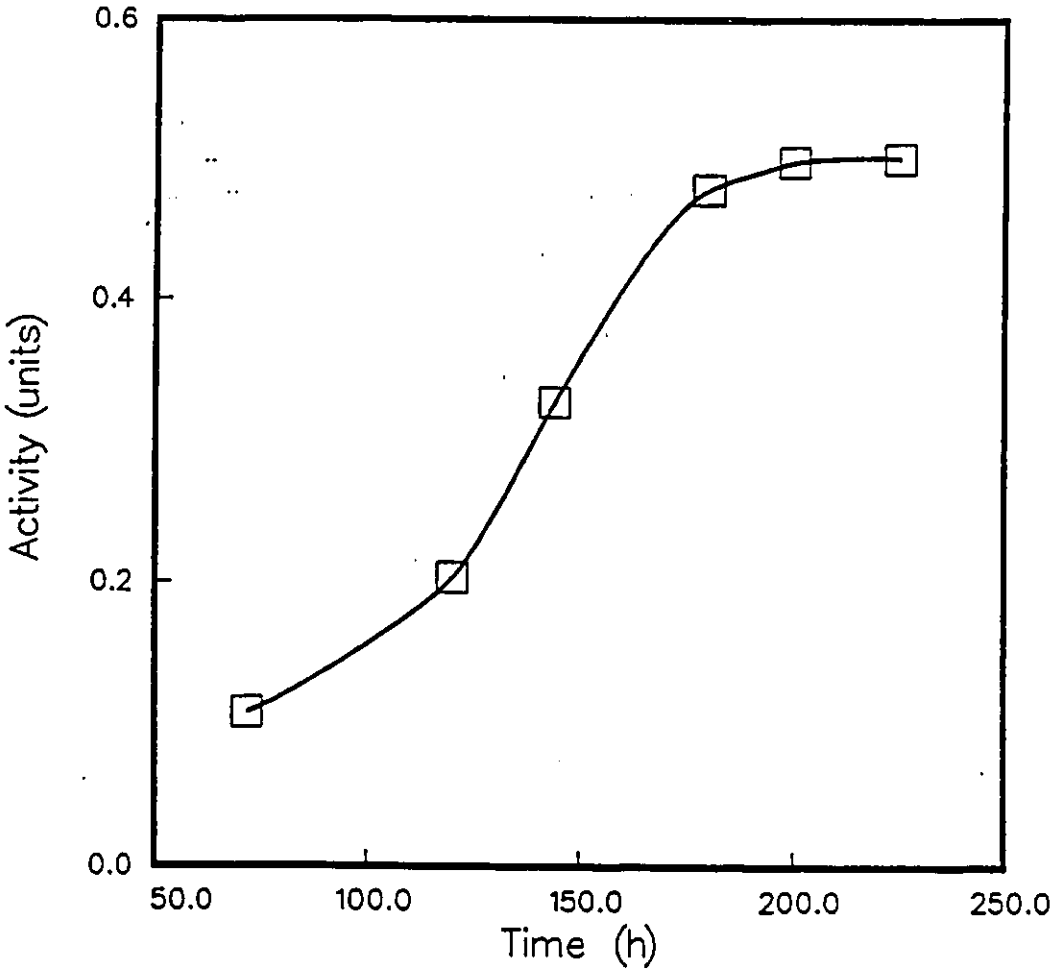


Figure 4.37: Phytase production on canola meal using *A. ficuum* NRRL 3135 in a SSF process

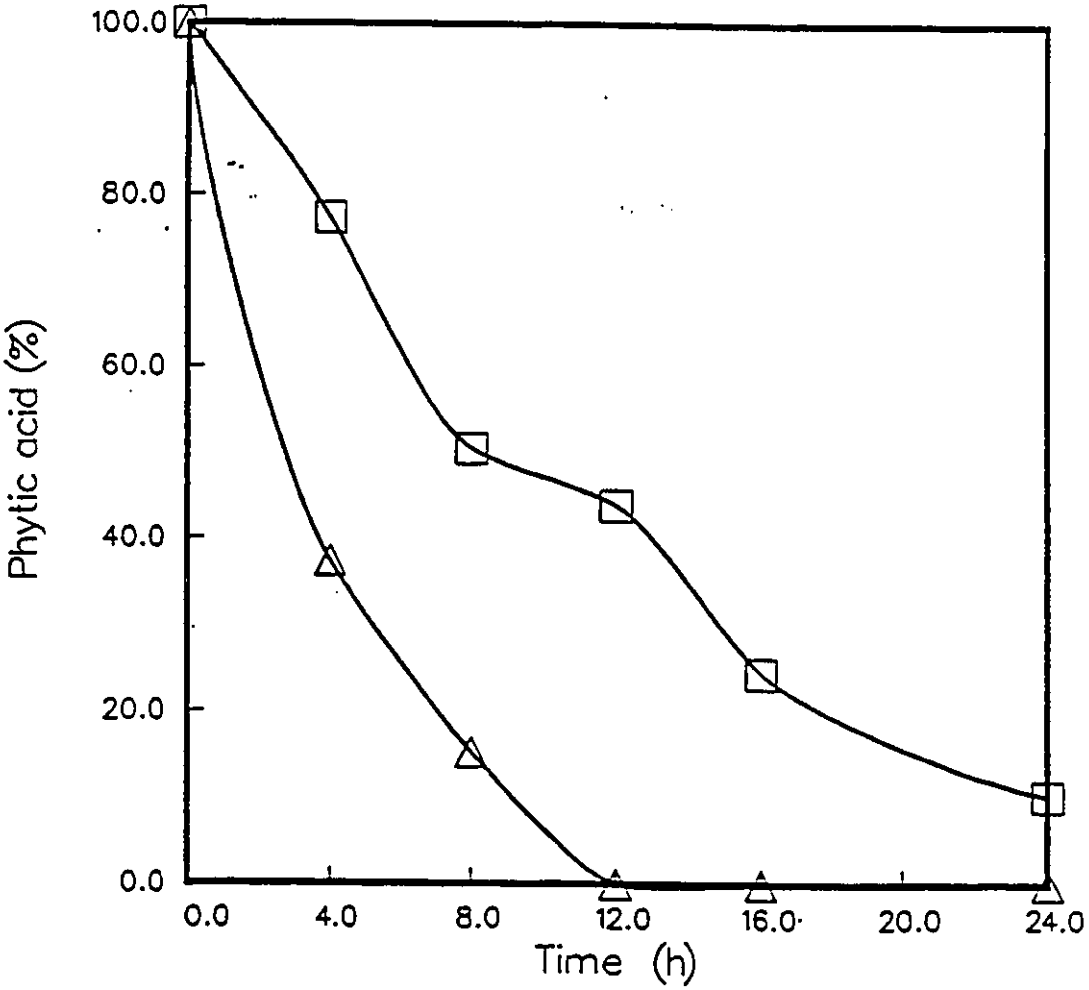


Figure 4.38: Reduction of phytic acid content in a 12% canola meal suspension by the enzyme preparation (Temperature : □ - 30°C, △ - 50°C; 1.2 units of enzyme per g of canola meal).

Chapter 5

Conclusions and Recommendations

5.1 Conclusions

To study the reduction of phytic acid content in canola meal, the following microorganisms were tested:
S. cerevisiae, *R. oligosporus* NRRL 2990, *A. niger* NRC 5765 and NRC 401121, *A. carbonarius* NRC 401124 and *A. ficuum* NRRL 3135.

1. The results showed that all these microorganisms were capable of reducing the phytic acid content in canola meal in a solid state fermentation process.

2. An increase in inoculum concentration (spores or biomass) or inoculum age increased the rate of the reduction of phytic acid content. The addition of a buffer solution which prevented a rapid change in the pH of the medium, was also beneficial in this process.

3. Among the microorganisms surveyed, *A. ficuum* NRRL 3135 was found to be the most efficient in the reduction of the phytic acid content. In certain tests, complete reduction of phytic acid content was achieved in 48 hours of incubation at 30°C.

4. The optimum moisture content for the reduction of the phytic acid content in canola meal was 67%.

5. *A. ficuum* NRRL 3135 was used for the study of phytase production in a submerged culture. It was established from this study that the production of the enzyme was partially inhibited by a higher concentration of glucose. Application of fed batch technique prevented the inhibition.

6. Aeration had a beneficial effect on the phytase production in a submerged culture.

7. The study of some of the characteristics of the enzyme produced in a submerged culture and in a solid state fermentation process revealed that the values of the K_m and the V_{max} were 0.27 mM and 0.46 units/h respectively when sodium phytate was used as the substrate. The optimum pH and temperature for the phytase activity were 5.0 and 60°C respectively.

8. The enzyme preparation was quite stable at 4°C; it lost only 15% of the activity in 5 weeks at this temperature.

9. The enzyme preparation can be used directly for the reduction of the phytic acid content in canola meal; complete reduction of phytic acid content was

achieved in 12 hours of incubation at 50°C. Using the same amount of the enzyme preparation, a better reduction of phytic acid content was achieved in the suspended medium than in the solid medium.

5.2 Recommendations

Many difficulties were experienced during this study. At first, sterile Petri dishes were used for the experiments. The amount of canola meal that can be fermented was limited in this case. Also, in order to keep the medium at relatively constant moisture level, water had to be added under asceptic conditions. This proved rather inefficient and tedious. To avoid this problem, aerated chambers were designed, but contamination from the environment was unavoidable. To deal with this problem, it is recommended that the experiments be carried out in an asceptic environment where samples can be removed from the chamber without contaminating the rest of the medium.

The preliminary conditions for the experiments were adopted from published research papers dealing with several solid state fermentation processes. Modifications are necessary in order to obtain the optimum conditions for the growth of the microorganism and for the production of phytase. Examples of such modifications are;

0.2 M acetate buffer (pH 4.7) was used in this study, but this solution was not capable of keeping the pH constant for the duration of fermentation. A higher concentration of acetate buffer, or even a different buffer system should be used.

The incubation temperature for the experiments was 30°C. Different temperatures should be tried for the optimum growth of the microorganism.

Only preliminary experiments were carried out using *S. cerevisiae* and *A. carbonarius*. More experiments have to be carried out in order to establish better conditions for the phytic acid content reduction.

For *A. ficuum* NRRL 3135, biomass and phytase should be separated and their

effect on phytic acid hydrolysis should be determined separately.

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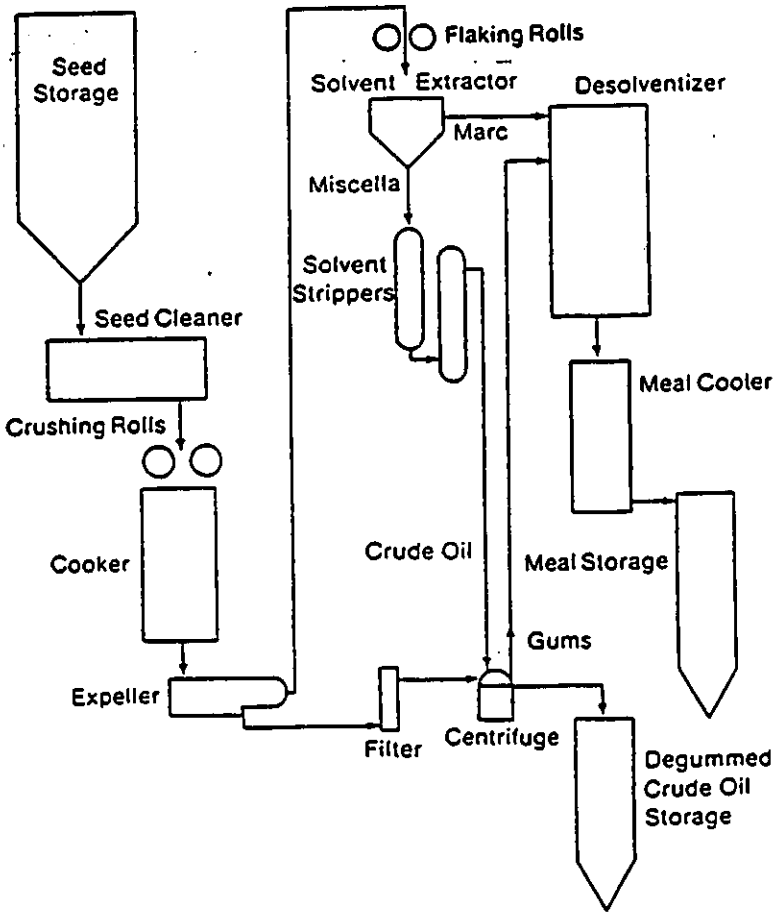
Appendix A

Canola oil and Canola meal



A.1 Schematic Flowsheet For Prepress Solvent Processing

(Taken from Clandinin, 1986)

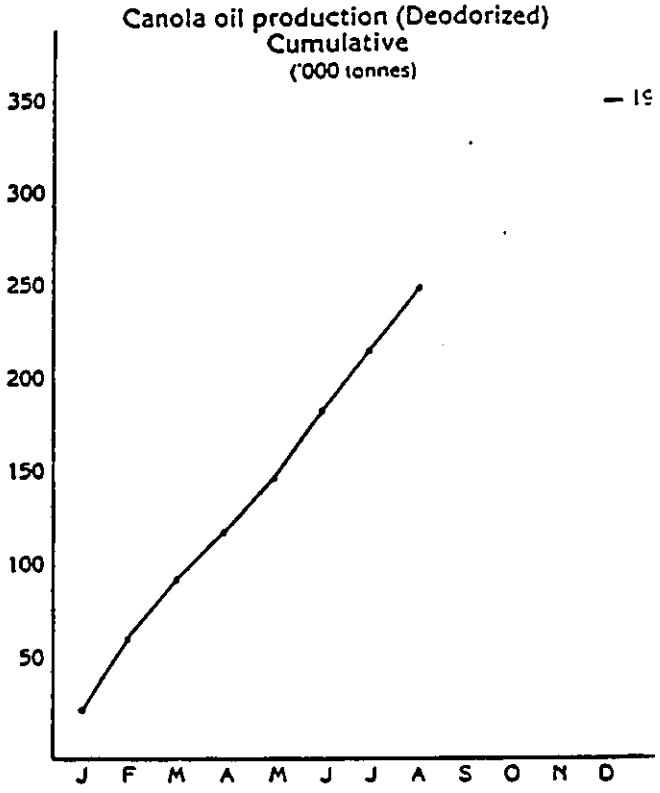
A.2 Canola oil production in Canada

Statistics

Measures of Merit – October

	October No. 1 Canada	October No. 2 Canada
OIL CONTENT (8.5% moisture basis)		
Current month	41.4	40.3
Crop year-to-date	41.9	41.0
87/88 crop year	42.3	42.0
PROTEIN CONTENT (%) (oil-free, 8.5% moisture basis)		
Current month	37.7	39.0
Crop year-to-date	37.5	38.1
87/88 crop year	36.6	37.1
LINOLENIC ACID (%)		
Current month	10.7	10.3
Crop year-to-date	11.0	10.7
87/88 crop year	11.1	11.3
ERUCIC ACID (%)		
Current month	0.5	0.5
Crop year-to-date	0.7	0.5
87/88 crop year	0.7	0.6
GLUCOSINOLATES μmoles/g (oil-free, air dry)		
Current month	25	21
Crop year-to-date	23	19
87/88 crop year	22	20
CHLOROPHYLL (ppm seed)		
Current month	20	31
Crop year-to-date	19	28
87/88 crop year	18	28
FREE FATTY ACID (%)		
Current month	0.3	0.5
Crop year-to-date	0.3	0.5
87/88 crop year	0.4	0.6
IODINE VALUE		
Current month	117	117
Crop year-to-date	117	117
87/88 crop year	118	118

During the month of October ten No. 1 CC cargo samples, representing 157,192 tonnes, were tested by the Grain Research Laboratory of the Canadian Grain Commission. To date 29, No. 1 CC samples representing 427,107 tonnes have been tested in the crop year.



(Taken from Clandinin, 1986)

A.2 Canola oil production in Canada (Cont'd)

Overseas clearances – September

The Canadian Grain Commission reported the following canola export clearances (in thousands of tonnes).

	Japan	W. Europe EEC	Africa	Asia others	Mexico	U.S.	Total
September	158.4	—	—	—	21.4	—	179.8
Crop y.t.d. - 88/89	294.8	—	—	—	21.4	1.2	317.5
Previous crop y.t.d.	243.0	—	—	1.6	85.1	—	330.4

Canola crushing totals – September

	Total crush	Oil production	Meal production
September	130,579	51,671	74,844
Crop year-to-date	203,300	81,289	116,158
Calendar year-to-date	1,145,281	466,638	653,529

At the end of September stocks of oil were 24,877 tonnes and meal stocks recorded 40,638 tonnes.

Deodorized oil production – July/August

Statistics Canada data on July and August production are as follows:

(All figures in tonnes. An "x" means confidential while a dash (—) means Nil or not applicable.)

Source	Margarine Oil July	Margarine Oil August	Shortening Oil July	Shortening Oil August	Salad Oil July	Salad Oil August	July Total	August Total	Year-to-date Total	% share of all Veg. Oils 1988	% share of all Veg. Oils 1987
Canola	3,358	5,300	9,536	10,295	16,748	16,360	29,642	31,955	247,686	64.1	60.1
Soybean	3,857	5,845	3,109	4,329	983	1,654	7,949	11,828	81,689	21.1	25.4
Corn	x	x	172	x	x	x	1,017	1,858	15,978	4.1	3.3
Palm	182	347	627	835	—	4	809	1,186	8,498	2.2	2.0
Sunflower	x	x	312	x	x	x	1,882	1,048	14,261	3.7	3.5
Coconut	32	64	855	1,233	—	—	887	1,297	8,873	2.3	2.6
Peanut	—	—	206	361	—	—	206	361	2,109	0.5	1.4
Others	26	40	286	790	91	80	403	910	7,143	1.8	1.6
Total Veg. oils	8,199	12,641	15,103	18,437	19,493	19,365	42,795	50,443	386,237		
Animal oils	57	74	1,945	2,714	—	—	2,002	2,788	18,361		
Total All oils	8,256	12,715	17,048	21,151	19,493	19,365	44,797	53,231	404,598		

(Taken from Clandinin, 1986)

A.3 Composition of Canola meal and Soybean meal

	Canola meal[1]		Soybean meal[2]	
	as fed %	in protein %	as fed %	in protein %
Proximate composition				
Moisture	8.01		11.00*	
Crude fiber	11.41		7.30*	
Ether extract	3.35		0.80*	
Protein (Nx6.25)	37.15		45.21	
Amino acid composition				
Alanine	1.73	4.65	1.95	4.31
Arginine	2.26	6.08	3.03	6.71
Aspartic acid	3.03	8.16	5.27	11.66
Cystine	0.90	2.48	0.71	1.61
Glutamic acid	6.46	17.38	8.43	18.65
Glycine	1.92	5.18	2.06	4.55
Histidine	1.04	2.79	1.12	2.48
Isoleucine	1.28	3.44	1.82	4.03
Leucine	2.57	6.92	3.36	7.44
Lysine	2.21	5.95	2.82	6.24
Methionine	0.76	2.10	0.70	1.59
Phenylalanine	1.45	3.90	2.13	4.72
Proline	2.48	6.67	2.27	5.03
Serine	1.69	4.55	2.38	5.27
Threonine	1.68	4.52	1.74	3.85
Tryptophan	0.44	1.19	0.52	1.15
Tyrosine	0.97	2.62	1.33	2.95
Valine	1.64	4.42	1.89	4.18

All values except those marked with asterisks and except those for cystine and methionine were computed from published and unpublished data of D.R. Clandinin, A.R. Robblee, Y.K. Goh and J. Heard, Department of Animal Science, University of Alberta, Edmonton, Alta. Those marked with asterisks were taken from Nutrient Requirements of Poultry, Publ. 1, 7th Revision, National Academy of Sciences, Washington, D.C. 1977. Those for cystine and methionine were, as indicated in the text, computed from Canadian and European data.

1 Averages based on eighteen samples of canola meal except for arginine, histidine and lysine where the averages were based on eighty-one samples of canola meal and except for cystine and methionine where the averages were based on nineteen samples of canola meal and European low glucosinolate-type rapeseed meal (1 to 8).

2 Protein and amino acid values based on ten samples of soybean meal except for cystine and methionine where the averages were based on five samples of soybean meal (1,2,8).

(Taken from Clandinin, 1986)

A.4 Phytate contents of cereals

Phytate Concentration and Distribution in Morphological Components of Cereals

Cereal	Morphological component	Phytate phosphorus (%)	Phytate[a] (%)	Distribution[b] (%)
Corn	Commercial hybrid	0.25	0.89	—
	Endosperm	0.01	0.04	3.20
	Germ	1.80	6.39	88.00
	Hull	0.02	0.07	0.04
Corn	High lysine	0.27	0.96	—
	Endosperm	0.01	0.04	3.00
	Germ	1.61	5.72	88.90
	Hull	0.07	0.25	1.50
Wheat	Soft	0.32	1.14	—
	Endosperm	0.001	0.004	2.20
	Germ	1.10	3.91	12.90
	Aleurone layer	1.16	4.12	87.10
Rice	Brown	0.25	0.89	—
	Endosperm	0.004	0.01	1.20
	Germ	0.98	3.48	7.60
	Pericarp	0.95	3.37	80.00
Pearl millet	Whole	0.25	0.89	—
	Endosperm	0.09	0.32	—
	Germ	0.75	2.66	—
	Bran	0.28	0.99	—

[a] Phytate content is calculated by assuming that it contains 28.20% phosphorus.
[b] Percentage of phytate in the component part.

(Taken from Reddy et al., 1989)

Appendix B

Standard Curves

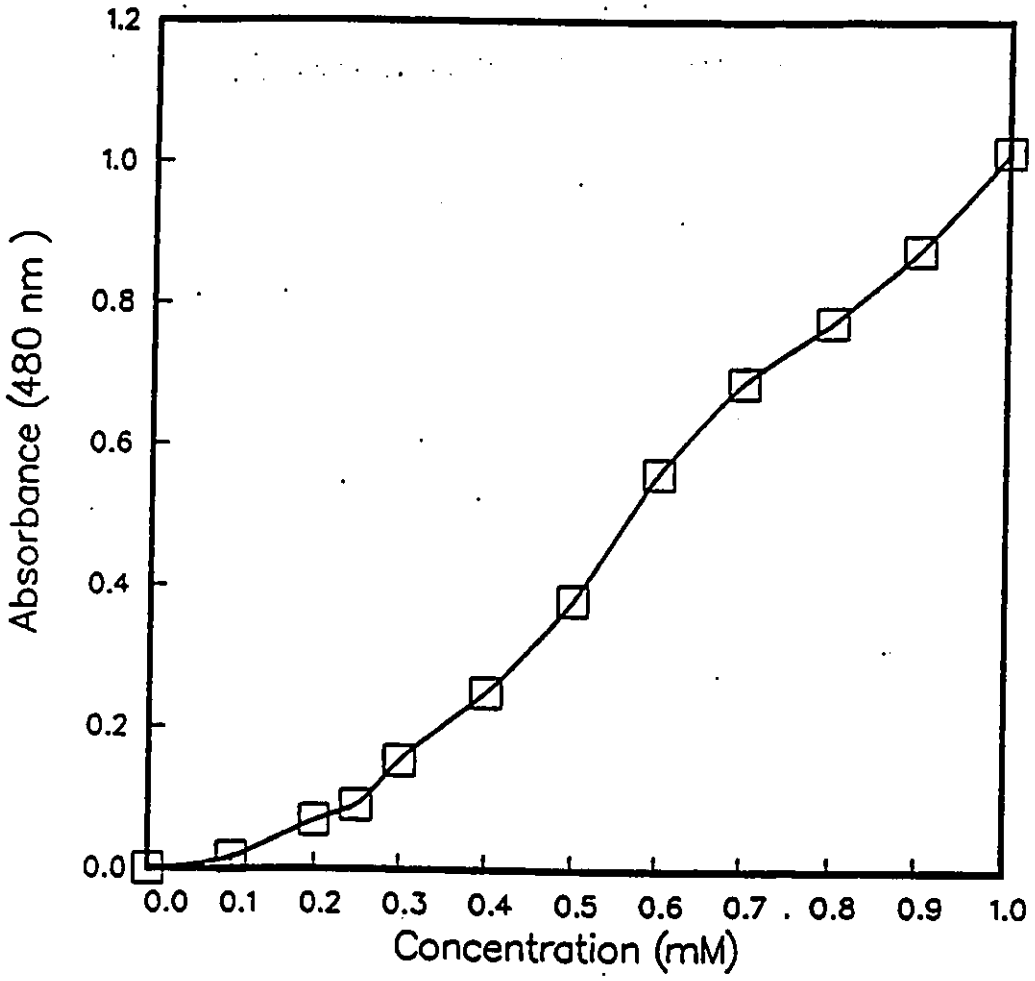


Figure B1. Phytic acid determination by the method described by Wheeler and Ferrel (1971). (Concentration of Fe is determined from the curve).

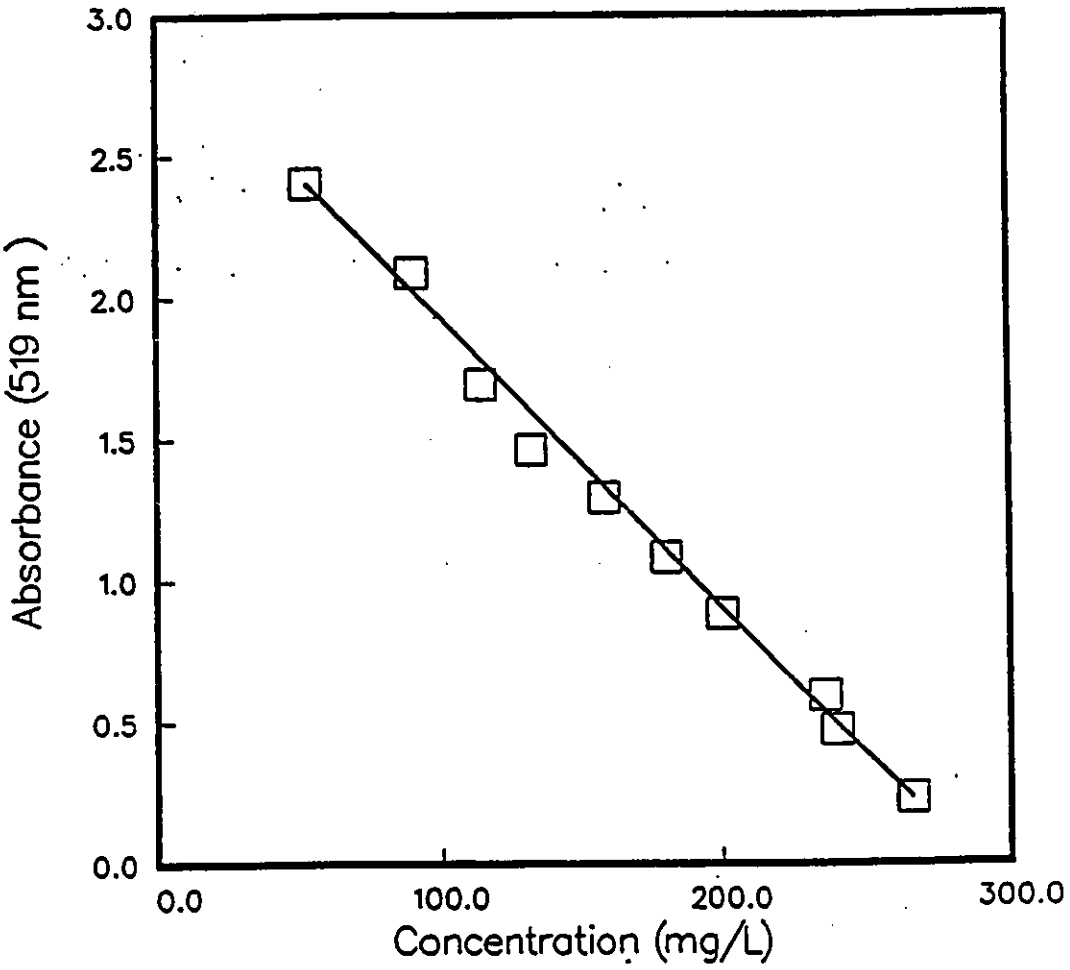


Figure B2. Phytic acid determination by the method described by Haug and Lantzsch (1983). (Concentration of phytic acid is determined directly).

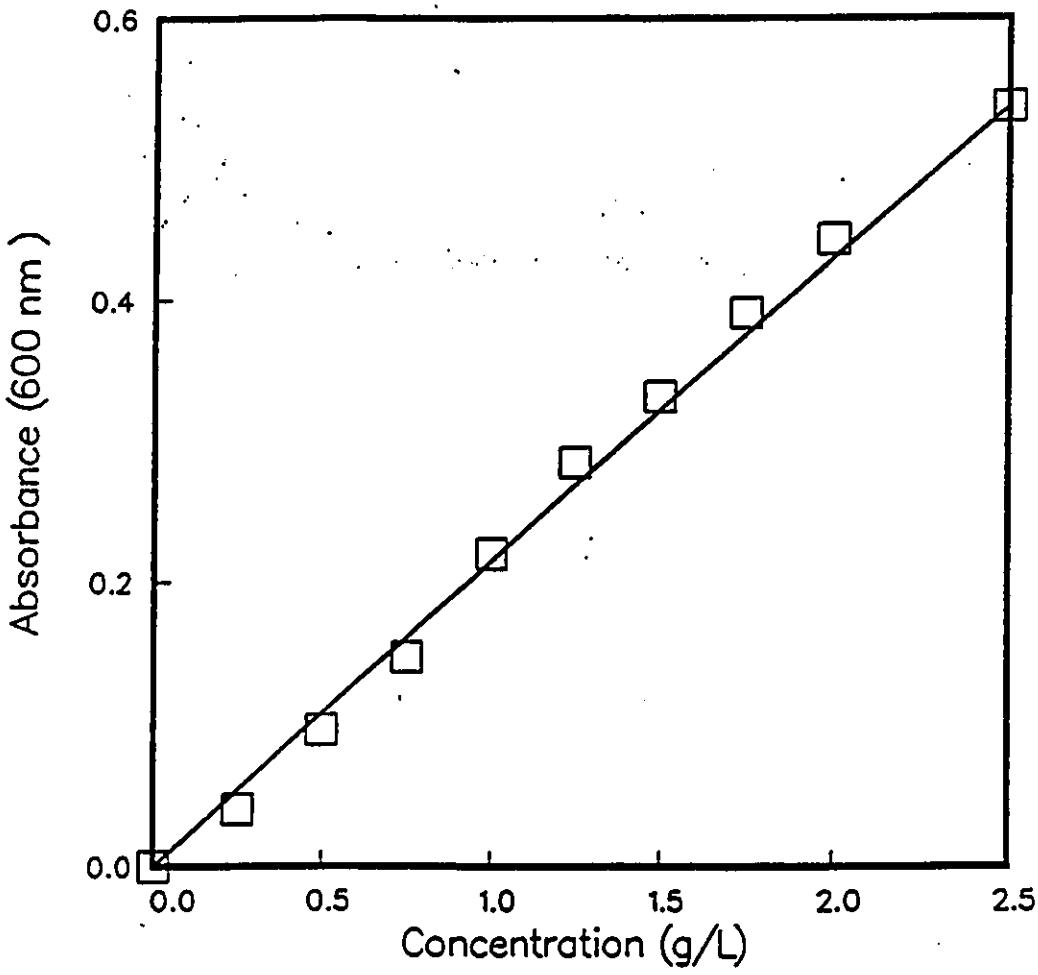


Figure B3. Reducing sugar determination by the method described by Weiner (1978). (Concentration of glucose is determined directly).

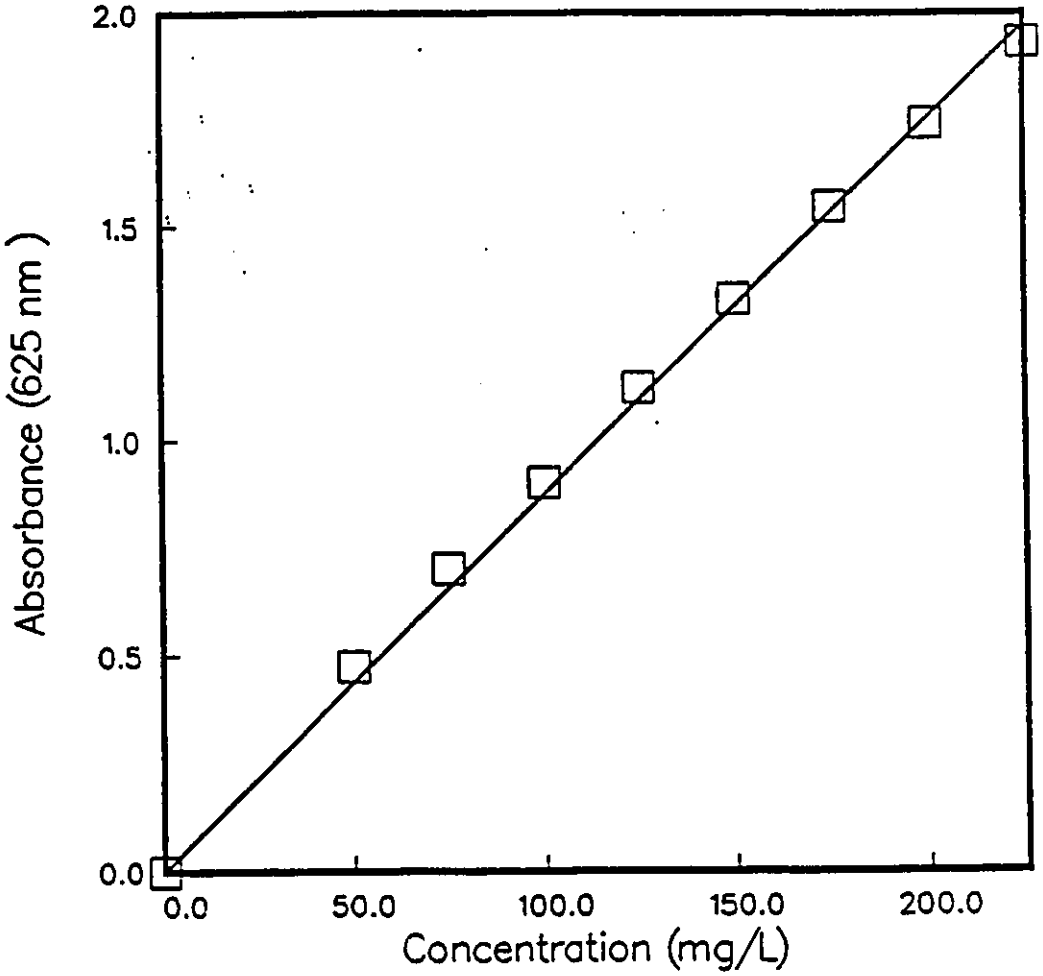


Figure B4. Determination of total carbohydrates (AOAC, 1975) using sucrose as the standard.

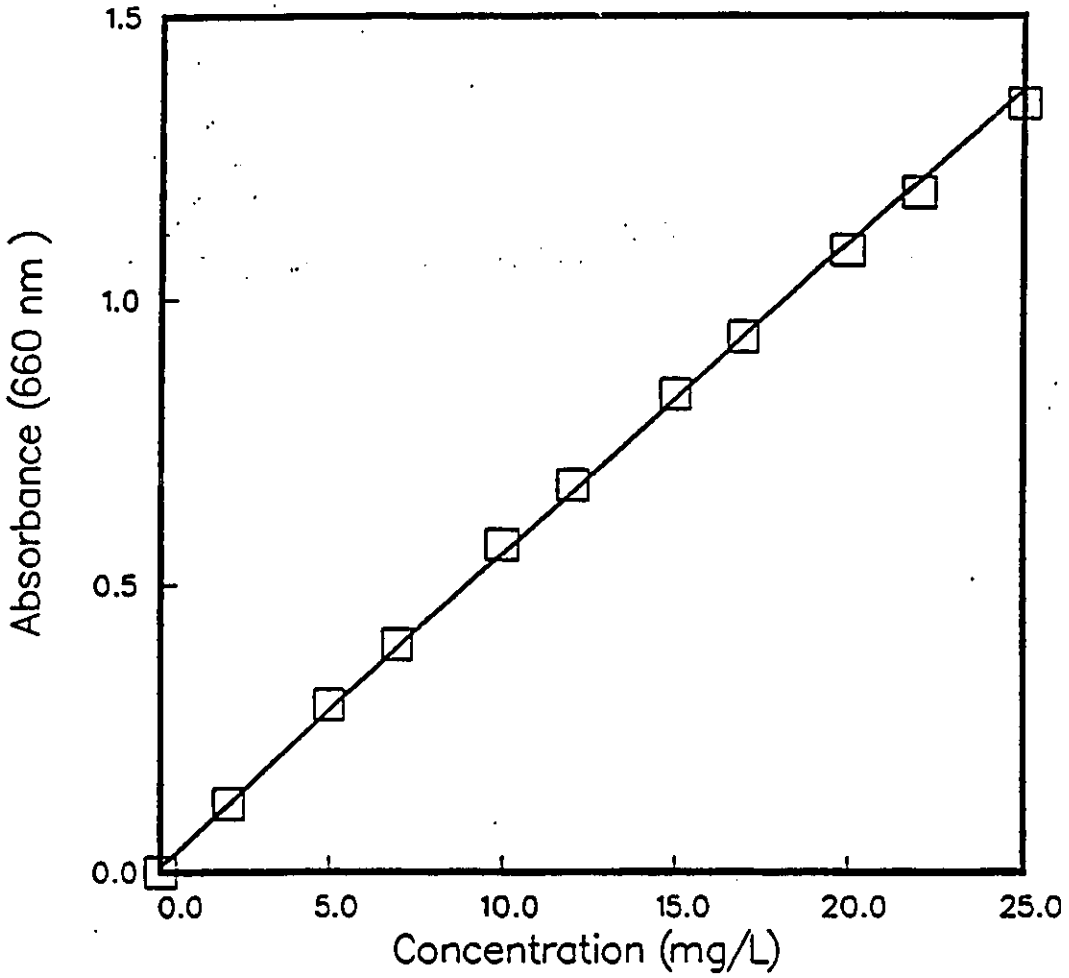


Figure B5. Determination of inorganic phosphorus by the method described by Harland and Harland (1980).

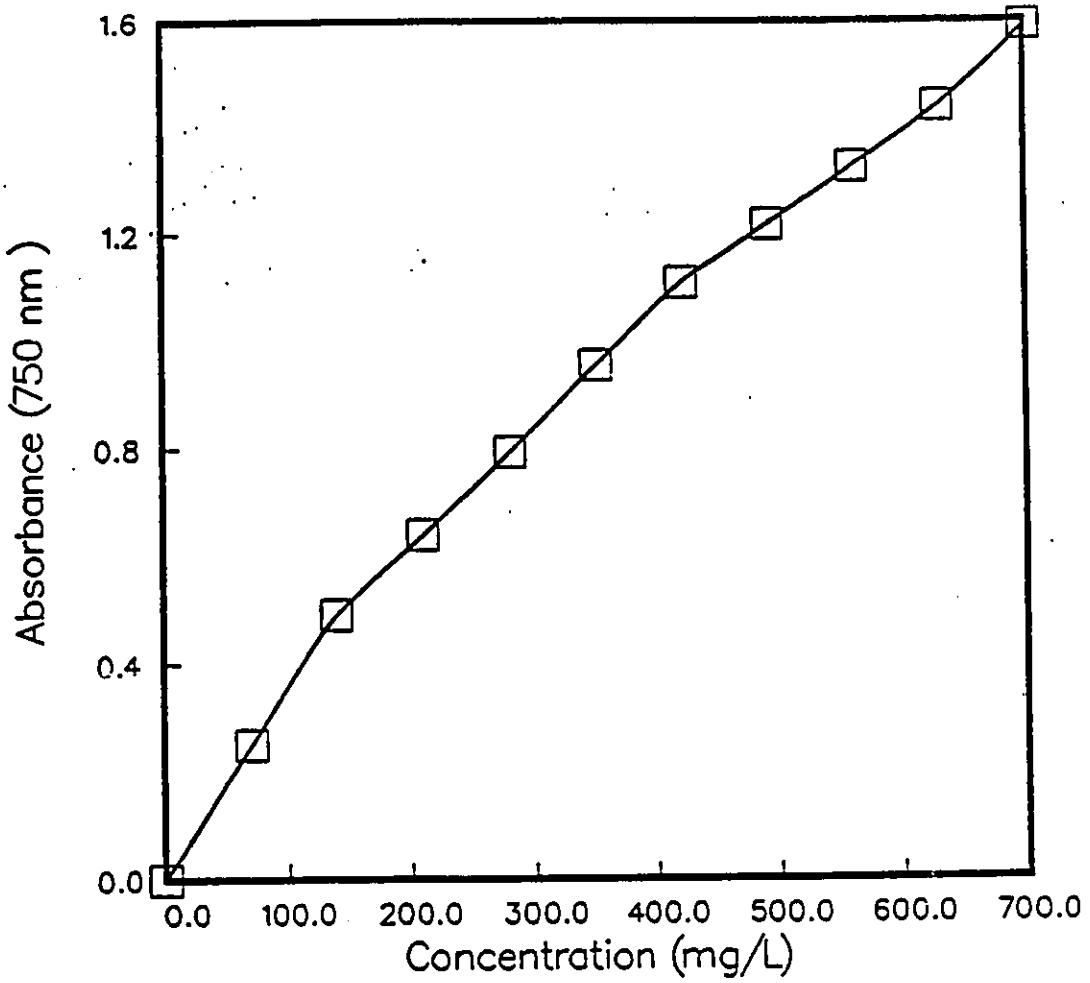


Figure B6. Determination of protein by the method described by Lowry et al. (1951).

Appendix C

Experimental Data

C.1 *R. oligosporus*

Table C1. Moisture and Dry weight reduction in canola meal medium

Time (h)	Moisture (%)	Dry weight (%)
0.0	124.6	100.0
18.0	103.8	99.8
24.0	104.0	97.0
52.0	89.5	87.0
69.0	84.0	82.0
92.0	65.7	80.0
144.0	50.0	72.0

Table C2. Effect of addition of nutrients to the medium on phytic acid and carbohydrate contents of canola meal

Time	With Nutrients		Without Nutrients	
(h)	PA	CHO	PA	CHO
0.0	100.0	100.0	100.0	100.0
48.0	77.3	39.6	85.0	86.9
72.0	66.4	34.5	79.2	67.6
96.0	50.4	32.0	58.3	59.7
139.0	45.8	32.9	52.5	48.5

(Note: PA = phytic acid, CHO = total carbohydrates)

Table C3. Effect of aeration and spore concentration on phytic acid content reduction in canola meal

Time	With Aeration		Without Aeration
(h)	3.1×10^8 sp.	6.2×10^8 sp.	6.2×10^8 sp.
0.0	100.0	100.0	100.0
24.0	85.7	85.7	88.0
48.0	79.0	75.0	79.4
72.0	69.7	51.2	66.3
96.0	62.9	50.8	57.4
120.0	61.6	49.0	45.8

Table C4. Effect of aeration and spore concentration on the amount of protein in canola meal medium

Time	Petri dish	With Aeration		Without Aeration
(h)	$3.1x10^8$	$3.1x10^8$ sp.	$6.2x10^8$ sp.	$6.2x10^8$ sp.
0.0	100.0	100.0	100.0	100.0
24.0	96.0	102.0	106.0	106.0
48.0	92.2	88.0	86.4	96.3
72.0	88.1	85.0	85.1	91.4
96.0	78.0	80.4	83.7	88.5

Table C5. Change in pH of the canola meal medium during the fermentation in petri dish and chamber (aerated and non-aerated)

Time	pH		
(h)	Petri dish	With aeration	Without aeration
0.0	5.75	5.75	5.75
24.0	6.61	7.65	7.55
48.0	8.56	8.39	8.24
72.0	8.75	8.56	8.38
96.0	8.76	8.61	8.55
120.0	8.70	8.64	8.62

Table C6. Comparison of experiments in Chamber and Flask; the effect of addition of buffer on the reduction of phytic acid content in canola meal

Time (h)	Phytic acid (%)			
	Without buffer		With buffer	
	sterile	non-sterile	Chamber	Flask
0.0	100.0	100.0	100.0	100.0
24.0	88.0	93.0	59.0	57.0
48.0	78.0	92.0	46.0	49.0
72.0	67.0	90.0	41.0	45.0
96.0	57.0	76.0	36.0	40.0
120.0	58.0	75.0	31.0	35.0

Table C7. Effect of sterilization time on the reduction of phytic acid content in canola meal

Time (h)	Phytic acid (%)			
	Control	25 min.	45 min.	120 min.
0.0	100.0	100.0	100.0	100.0
24.0	96.0	88.0	81.0	82.0
48.0	95.0	70.0	69.0	69.0
72.0	96.0	59.0	52.0	45.0
96.0	94.0	52.0	53.0	42.0

C.2 *A. niger*

Table C8. Phytic acid reduction by two strains of *A. niger* - Comparison of experiments in Flask and Chamber

Time (h)	Phytic acid (%)			
	NRC 401121		NRC 5765	
	Chamber	Flask	Chamber	Flask
0.00	100.0	100.0	100.0	100.0
24.0	91.7	88.0	82.1	82.0
48.0	76.7	73.0	79.0	73.0
72.0	68.7	60.0	72.9	65.0
96.0	59.7	49.0	65.0	54.9

Table C9. Dry weight reduction and change in pH during the fermentation of canola meal

Time	NRC 401121		NRC 5765	
(h)	Dry weight (%)	pH	Dry weight (%)	pH
0.0	100.0	5.02	100.0	5.04
24.0	92.0	5.29	92.0	5.44
48.0	84.0	5.53	88.0	6.25
72.0	84.0	5.91	86.0	8.10
96.0	80.0	5.95	85.0	8.23
120.0	79.0	6.19	82.0	8.32

Table C10. Protein and carbohydrate reduction in canola meal

Time	NRC 401121		NRC 5765	
(h)	CHO (%)	PRO (%)	CHO (%)	PRO (%)
0.0	100.0	100.0	100.0	100.0
24.0	82.0	100.0	79.0	100.0
48.0	71.0	88.0	63.0	86.0
72.0	59.0	93.0	53.0	93.0
96.0	50.0	97.0	48.0	95.0

(Note: CHO = total carbohydrate, PRO = protein)

Table C11. Effect of addition of buffer on the reduction of phytic acid

Time	Phytic acid (%)				
	Control	NRC 401121		NRC 5765	
(h)		No buffer	Buffer	No buffer	Buffer
0.0	100.0	100.0	100.0	100.0	100.0
24.0	99.0	78.0	96.0	93.0	100.0
48.0	98.0	64.0	82.0	83.0	96.0
72.0	98.0	69.0	50.0	75.0	85.0
96.0	95.0	61.0	32.0	61.0	75.0
120.0	93.0	57.0	32.0	63.0	72.0

Table C12. Effect of buffer on the reduction of soluble carbohydrates in canola meal

Time	Protein (%)				
	Control	NRC 401121		NRC 5765	
(h)		No buffer	Buffer	No buffer	Buffer
0.0	100.0	100.0	100.0	100.0	100.0
24.0	100.0	91.0	99.0	102.0	100.0
48.0	98.0	80.0	88.0	86.0	104.0
72.0	99.0	80.0	93.0	93.0	96.0
96.0	96.0	91.0	97.0	92.0	100.0

Table C13. Effect of spore concentration in the inoculum on the reduction of phytic acid content in canoal meal

Time	Phytic acid (%)				
(h)	Control	$4x10^8$ sp.	$5x10^8$sp.	$8x10^8$ sp.	$2x10^9$sp.
0.0	100.0	100.0	100.0	100.0	100.0
24.0	96.0	86.0	95.0	88.0	76.0
48.0	96.0	80.0	88.0	73.0	48.0
72.0	97.0	68.0	64.0	62.0	42.0
96.0	95.0	67.0	54.0	42.0	34.0
120.0	96.0	56.0	56.0	38.0	34.0

Table C14. Effect of spore concentration in the inoculum on the amount of protein in canoal meal

Time (h)	Protein (%)				
	Control	$4x10^8$sp.	$5x10^8$sp.	$8x10^8$sp.	$2x10^9$sp.
0.0	100.0	100.0	100.0	100.0	100.0
24.0	100.0	95.0	90.0	89.0	92.0
48.0	100.0	93.0	111.0	100.0	107.0
72.0	100.0	97.0	115.0	103.0	112.0
96.0	98.0	93.0	105.0	101.0	99.0

Table C15. Effect of spore concentration in the inoculum on the amount of total carbohydrates in canoal meal

Time (h)	Total CHO (%)				
	Control	$4x10^8$sp.	$5x10^8$sp.	$8x10^8$sp.	$2x10^9$sp.
0.0	100.0	100.0	100.0	100.0	100.0
24.0	97.0	96.0	87.0	81.0	90.0
48.0	100.0	64.0	58.0	60.0	65.0
72.0	100.0	65.0	58.0	60.0	61.0
96.0	100.0	68.0	61.0	61.0	67.0

Table C16. Effect of spore concentration in the inoculum on the pH of the canoal meal medium

Time	pH				
(h)	Control	$4x10^8$	$5x10^8$	$8x10^8$	$2x10^9$
0.0	5.12	5.21	5.26	5.33	5.43
24.0	5.11	5.29	5.77	5.58	5.34
48.0	5.16	5.66	6.21	6.34	5.58
72.0	5.04	6.04	6.26	7.22	6.21
96.0	5.03	6.77	7.14	7.31	7.85
120.0	5.05	7.47	8.01	7.96	8.15

Table C17. Comparison of few microorganisms for the reduction of phytic acid content in canola meal

Time	Phytic acid (%)			
(h)	R. Oligo.	S. cerev.	S. cerev. + R. oligo.	A. carb.
0.0	100.0	100.0	100.0	100.0
24.0	79.0	96.0	88.0	92.4
48.0	65.0	90.0	78.0	81.7
72.0	42.5	85.0	67.0	40.6
96.0	41.0	80.0	57.0	10.5
120.0	40.0	74.0	56.0	0.00

C.3 *A. ficuum*

Table C18. Effects of incubation time and the pH on the phytase activity

Effect of incubation time		Effect of pH	
Time (min.)	Activity (units)	pH	Activity (units)
0.0	0.0	4.21	0.344
15.0	0.073	4.55	0.389
30.0	0.205	4.96	0.411
45.0	0.283	5.20	0.385
60.0	0.293	5.90	0.079

Table C19. Effect of substrate concentration on the phytase activity

Substrate concentration (S)		Lineweaver-Burk plot	
Conc. of PA (mM)	Activity (units)	1/conc. (mM^{-1})	1/Activity ($units^{-1}$)
0.70	0.312	0.287	2.35
1.40	0.385	0.359	2.36
1.67	0.398	0.478	2.39
2.09	0.418	0.598	2.51
2.79	0.424	0.718	2.60
3.48	0.426	1.434	3.20

Table C20. Effect of temperature on the phytase activity of three different enzyme extracts

Temperature (°)	Activity (units)		
	Extract 1	Extract 2	Extract 3
30.0	0.114		
40.0	0.246		
45.0	0.330		0.051
50.0	0.380	0.444	0.090
55.0	0.406	0.571	0.135
60.0	0.461	0.820	0.152
65.0	0.352	0.407	0.055
70.0	0.296	0.382	

Table C21. Effect of preincubation of the enzyme at two different temperatures and the amount of enzyme on the phytase activity

Time	Activity (%)		Enzyme	Activity
(min.)	50°C	60°C	(mL)	(units)
0.0	100.0	100.0	0.0	0.0
15.0	102.0	103.0	0.25	0.055
30.0	106.0	93.6	0.50	0.129
45.0	105.0	85.9	0.75	0.199
60.0	103.0	65.7	1.00	0.204
120.0	98.0	43.9	1.50	0.220
180.0	95.0	32.7	2.00	0.245
			3.00	0.265

Table C22. Effect of glucose concentration on biomass production in *A. ficuum*

Time	Biomass (mg/mL)		
(h)	0.5%	0.5% fed batch	5.0%
0.0	0.16	0.16	0.16
24.0	2.26	2.76	2.30
48.0	3.40	5.22	3.37
72.0	5.00	6.22	3.63
96.0	5.70	8.36	3.32
120.0	5.68	10.85	2.47
144.0	5.67	10.20	2.34

Table C23. Effect of glucose concentration on phytase production in *A. ficuum*

Time	Phytase (units)		
(h)	0.5%	0.5% fed batch	5.0%
0.0	0.007	0.003	0.00
24.0	0.025	0.015	0.017
48.0	0.037	0.053	0.025
72.0	0.074	0.110	0.030
96.0	0.085	0.154	0.048
120.0	0.110	0.178	0.060
144.0	0.115	0.241	0.065

Table C24. Effect of aeration on biomass production in *A. ficuum*

Time	Biomass (mg/mL)		
(h)	50 mL	100 mL	150 mL
0.0	0.15	0.15	0.15
24.0	3.14	2.98	0.40
48.0	5.40	4.60	1.20
72.0	5.45	4.52	2.60
96.0	4.90	4.50	4.86
120.0	3.82	4.52	3.42
144.0	2.69	4.52	1.91

Table C25. Effect of aeration on the production of enzyme in *A. ficuum*

Time	Activity (units)		
(h)	50 mL	100 mL	150 mL
0.00	0.0	0.0	0.0
24.0	0.031	0.0	0.0
48.0	0.074	0.013	0.012
72.0	0.092	0.029	0.026
96.0	0.155	0.085	0.036
120.0	0.179	0.109	0.063
144.0	0.182	0.124	0.108

Table C26. Effect of aeration on the pH of the medium

Time	pH		
(h)	50 mL	100 mL	150 mL
0.0	6.23	6.25	6.30
24.0	4.66	4.27	4.07
48.0	5.19	5.16	5.30
72.0	5.45	5.49	4.30
96.0	5.59	5.44	4.68
120.0	5.57	5.51	5.99
144.0	5.52	5.49	6.79

Table C27. Effect of aeration on the consumption of glucose by *A. ficuum*

Time	Glucose (%)		
(h)	50 mL	100 mL	150 mL
0.0	0.463	0.464	0.462
24.0	0.026	0.052	0.045
48.0	0.017	0.018	0.021
72.0	0.013	0.018	0.021
96.0	0.017	0.013	0.011
120.0	0.010	0.017	0.010

Table C28. Reduction of phytic acid content in canola meal by *A. ficuum*: Effect of the amount of biomass in the inoculum

Time (h)	Phytic acid (%)				
	Control	Amount of biomass (mg)			
		101	202	330	390
0.0	100.0	100.0	100.0	100.0	100.0
24.0	99.7	84.0	71.1	62.7	63.0
48.0	100.0	60.0	41.8	29.3	2.40
72.0	99.2	37.0	23.0	0.90	0.00
96.0	99.6	23.0	12.2	0.00	0.00
120.0	99.6	9.00	0.00	0.00	0.00

C29. Effect of age of inoculum on the reduction of phytic acid content in canola meal

Time (h)	Phytic acid (%)				
	Control	Age of inoculum			
		2 days	3 days	4.5 days	6 days
0.0	100.0	100.0	100.0	100.0	100.0
24.0	99.7	90.8	74.7	62.7	68.1
48.0	100.0	40.0	36.8	29.3	2.40
72.0	99.2	30.1	24.0	2.90	0.00
96.0	98.7	26.0	16.0	0.00	0.00
120.0	99.6	22.4	9.60	0.00	0.00

Table C30. Effect of age of inoculum on the amount of protein in the medium

Time (h)	Protein (%)				
	Control	Age of inoculum			
		2 days	3 days	4.5 days	6 days
0.0	100.0	100.0	100.0	100.0	100.0
24.0	100.0	96.3	103.0	91.1	100.0
48.0	100.0	102.0	105.0	109.0	97.2
72.0	99.0	127.0	117.0	110.0	108.0
96.0	98.3	120.0	101.0	117.0	124.0
120.0	105.0	110.0	100.0	103.0	103.0

Table C31. Effect of age of inoculum on the amount of total carbohydrates in the medium

Time (h)	Total CHO (%)				
	Control	Age of inoculum			
		2 days	3 days	4.5 days	6 days
0.0	100.0	100.0	100.0	100.0	100.0
24.0	92.0	72.9	67.6	69.4	68.7
48.0	94.1	63.9	61.5	61.0	57.4
72.0	100.0	62.1	67.7	58.0	63.9
96.0	94.3	59.9	54.9	55.0	60.2
120.0	96.3	60.0	52.6	52.0	57.3

Table C32. Effect of age of inoculum on the pH of the medium

Time (h)	pH				
	Control	Age of inoculum			
		2 days	3 days	4.5 days	6 days
0.0	5.36	5.37	5.37	5.37	5.37
24.0	5.29	5.25	5.13	5.17	5.05
48.0	5.24	5.15	6.39	4.72	5.07
72.0	5.24	6.20	6.83	5.83	6.23
96.0	5.11	6.83	7.00	7.38	7.26
120.0	5.31	6.76	7.98	8.03	8.17

Table C33. Effect of buffer on the reduction of phytic acid content in canola meal

Time (h)	Phytic acid (%)				
	Control	3 day old		1.5 day old	
		Without buffer	With buffer	Without buffer	With buffer
0.0	100.0	100.0	100.0	100.0	100.0
24.0	100.0	54.0	47.0	100.0	99.0
48.0	99.0	38.0	36.0	87.0	42.0
72.0	98.0	30.0	12.0	74.0	33.0
96.0	99.0	28.0	2.00	71.0	19.0
120.0	96.0	25.0	0.00	69.0	12.0

Table C34. Effect of buffer on the amount of protein in the canola meal medium

Time	Protein (%)				
(h)	Control	3 day old		1.5 day old	
0.0	100.0	100.0	100.0	100.0	100.0
24.0	105.0	110.0	111.0	108.0	93.0
48.0	98.0	117.0	117.0	112.0	112.0
72.0	103.0	111.0	121.0	108.0	100.0
96.0	99.0	101.0	112.0	108.0	95.0
120.0	98.0	87.0	106.0	102.0	98.0

Table C35. Effect of buffer on the pH of the canola meal medium

Time	pH				
(h)	Control	3 day old		1.5 day old	
0.0	5.33	5.72	5.55	5.43	5.30
24.0	5.30	5.30	5.53	5.27	5.16
48.0	5.32	7.07	5.32	7.58	5.28
72.0	5.36	8.54	6.21	8.54	6.80
96.0	5.30	8.84	7.25	8.75	8.10
120.0	5.37	8.41	7.89	8.74	8.36

Table C36. Effect of homogenization of inoculum on the reduction of phytic acid content in canoal meal

Time (h)	Phytic acid (%)		
	Control	Non-homogenized	Homogenized
0.0	100.0	100.0	100.0
24.0	99.7	74.7	87.7
48.0	100.0	32.8	22.9
72.0	99.2	24.0	18.1
96.0	90.7	16.0	10.0
120.0	99.6	9.60	0.0

Table C37. Effect of moisture level in the medium on the reduction of phytic acid content in canoal meal

Time (h)	Phytic acid (%)				
	Control	Moisture (%)			
		52	60	67	78
0.0	100.0	100.0	100.0	100.0	100.0
24.0	99.0	96.0	95.0	71.5	75.0
48.0	99.0	87.0	83.0	41.2	56.3
72.0	98.0	61.0	53.0	20.0	22.7
96.0	97.0	48.0	37.0	12.0	17.5
120.0	98.0	45.0	31.0	0.00	10.0

Table C38. Relative changes in the amount of protein, and soluble and total carbohydrates in a typical fermentation process of canola meal using *A. ficuum*

Time (h)	Protein (%)	Total CHO (%)	Soluble CHO (%)
0.0	100.0	100.0	100.0
24.0	96.3	72.9	21.0
48.0	102.0	63.9	21.0
72.0	127.0	62.1	20.0
96.0	120.0	60.0	20.0
120.0	110.0	59.9	20.0

Table C39. Effect of sterilization of canola meal on the reduction of phytic acid content

Time (h)	Phytic acid (%)		
	Non-sterile, no inoculum	Non-sterile, with A.F.	Sterile, with A.F.
0.0	100.0	100.0	100.0
48.0	90.0	70.2	56.7
68.0	82.0	53.1	29.1
93.0	76.0	50.6	22.6
120.0	75.0	43.2	11.7

(Note: A. F. = *Aspergillus ficuum*)

Table C40. Determination of phytic acid in canola meal by two methods

Trials	Phytic acid (%)	
	Method 1	Method 2
1	6.18	6.05
2	6.07	6.00
3	5.86	6.01
4	5.93	6.05
5	6.25	6.03
6	6.35	5.95
7	5.75	5.96
8	5.61	5.90
Average	6.00	5.99
Std. dev.	0.24	0.05

(Note: Method 1 - Wheeler and Ferrel (1971), Method 2 - Haug and Lantzsch (1983))

Appendix D

Chemical Equations

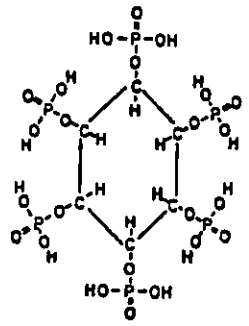
1) Buffer system (0.2M acetate; pH 4.7)

$$CH_3COOH \rightleftharpoons CH_3COO^{\ominus} + H^{\oplus}; \quad pK = 4.7$$

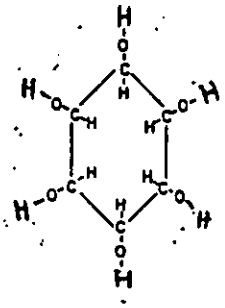
$$CH_3COONa \rightleftharpoons CH_3COO^{\ominus} + Na^{\oplus}$$

$$pH = pK + \log \frac{[CH_3COO^{\ominus}]}{[CH_3COOH]}$$

2) Hydrolysis of Phytic acid



$+ 6\,H_2O \xrightarrow{\text{Phytase}}$



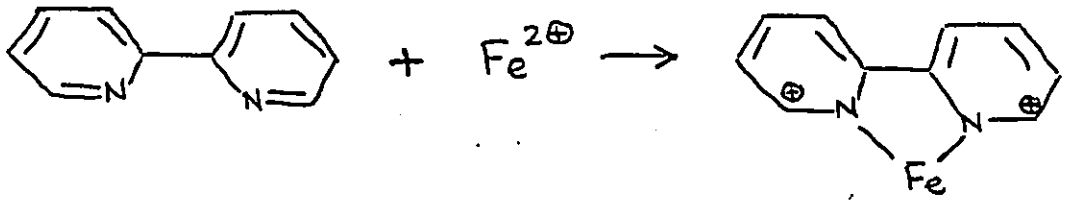
$+ 6\,H_3PO_4$

$$H_3PO_4 \rightleftharpoons H^{\oplus} + H_2PO_4^{\ominus}; \quad pK = 2.0$$

$$H_2PO_4^{\ominus} \rightleftharpoons H^{\oplus} + HPO_4^{2\ominus}; \quad pK = 6.7$$

$$HPO_4^{2\ominus} \rightleftharpoons H^{\oplus} + PO_4^{3\ominus}; \quad pK = 12.4$$

3) Complexation of Fe with bipyridine



(red complex)

4) Enzyme reaction (general)

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P$$

E = enzyme ; S = substrate ; P = product ; ES = complex

a) Michaelis-Menten equation

$$v = \frac{V_{max}\,[S]}{K_m + [S]}$$

b) Lineweaver-Burk equation

$$\frac{1}{v} = \frac{K_m}{V_{max}} \cdot \frac{1}{[S]} + \frac{1}{V_{max}}$$

$$K_m = \frac{k_{-1} + k_2}{k_1} \; ; \; V_{max} = k_2\,[E_o]$$

Appendix E

Glossary

biomass the amount of cells of the organism; usually includes living and dead cells; expressed in dry weight units.

black gram a type of lentle.

broth a liquid medium containing microbial cells or enzymes.

buffer a solution that keeps the medium at a constant pH.

cassava edible root of a plant; very rich in starch.

centrifuge an equipment for the separation of substances by using gravitational force.

enzyme a biological catalyst.

fermentation a chemical process in which microbial cells, spores enzymes are used.

fractionation mechanical separation of different components by grinding, etc.

homogenize to form a paste by grinding.

incubation keeping at a constant temperature.

inoculation introduction of microbial cells, spores or enzymes into the medium for fermentation.

mycelia long microbial cells joined together in the form of a filament.

oncom fermented peanut press cake.

reducing sugars monosaccharides such as glucose, fructose, etc.

sporulate to produce spores.

sugar cane bagasse residue after the removal of sugar cane pulp.

tempeh fermented food prepared from soy beans.

ultrafiltration a process for the separation of macromolecules using a membrane.

yeast extract a medium that contains essential minerals and vitamins required for the microbial growth.