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Reduction of phytic acid content in canola meal by *Aspergillus ficuum* in solid state fermentation process

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Summary. Solid state fermentation (SSF) of canola meal has been carried out to reduce its phytic acid content using *Aspergillus ficuum* NRRL 3135. In certain batches, a complete reduction of phytic acid content in canola meal was achieved in 48 h. A larger amount of biomass in the inoculum and older inoculum increased the rate of phytic acid hydrolysis. The optimum moisture content of the medium was found to be 67% for phytic acid hydrolysis in an SSF process. The substitution of water in the semi-solid medium with acetate buffer resulted in faster reduction of the phytic acid content. A 15% increase in the amount of protein after 120 h of incubation was observed in the treated meal. The crude phytase preparation extracted from the canola meal after it was treated in an SSF process was also used for reduction of the phytic acid content in new batches of canola meal both in semi-solid medium and in liquid medium. In the semi-solid medium, 58% of the phytic acid was hydrolysed at 45° C in 20 h, while 100% hydrolysis was recorded at 50° C in 12 h in the liquid medium. The SSF process seems to be beneficial for the upgrading of canola meal by reducing both its phytic acid content and increasing the amount of protein.

Introduction

Canola seeds contain more than 40% oil and 25% protein (Gillberg and Tornell 1976), and have been used for oil production. For this reason, canola has been produced in large amounts in many countries. In Canada alone, more than 4.3×10^6 metric tons of canola seeds were produced in the year 1988/1989. Canola meal is a by-product of canola oil production. It is used as a feed-stuff for livestock and poultry due to the richness in protein. However, the presence of phytic acid (*myo*-inositol hexaphosphoric acid) in canola meal diminishes its nutritional value.

Phytic acid is the main storage form of phosphorus in many seeds and cereals. Up to 80% phosphorus is found to be in the form of phytic acid in some cereals (Lolas and Markakis 1977). Upon its hydrolysis, phosphorus is freed and can be used in metabolic processes. If not hydrolysed, phytic acid binds to multivalent cations such as Zn^{2+}, Ca^{2+} and Fe^{3+} and so reduces their bioavailability. In addition, the enzymatic degradation of protein is obstructed by their complexation with phytic acid. It has also been reported that phytic acid inhibits enzymes such as α-amylases (Sharma et al. 1978), trypsin, tyrosinase and pepsin (Graf 1986).

Due to the nutritional implications of phytic acid, it is necessary to remove it from canola meal prior to its utilization as a feed-stuff. Some plants, such as navy beans (*Phaesolus vulgaris*), produce phytases that hydrolyse the phytic acid in them (Lolas and Markakis 1977). It has been reported that there are also plants such as canola seeds that do not contain the enzyme (Stone et al. 1984). In such cases, the phytic acid content can be reduced by chemical treatment, but a loss of nutritional value of the materials has been noticed (Harland and Harland 1980).

It is known that some microorganisms also produce phytase. After surveying many microorganisms, *Aspergillus ficuum* was found to be one of the best producers of this enzyme (Shieh and Ware 1968). Some properties of the enzyme and the conditions for its production by *A. ficuum* have been reported (Shieh et al. 1969; Irving and Cosgrove 1972, 1974). Knowing that some microorganisms produce the enzyme, application of microbial technologies for the removal of phytic acid from some materials has been taken into consideration. Fardiaz and Markakis (1981) have reported 95% reduction in phytic acid content of oncom (fermented peanut press cake) by *Rhizopus oligosporus* in solid state fermentation (SSF) process. The reduction of phytic acid in rye, white and whole wheat bread with yeast has also been reported (Harland and Harland 1980).

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Phytic acid in canola meal has been dephosphorylated in a blend of fish silage, canola meal and wheat bran in a 5-week period at room temperature by the phytase from wheat bran (Stone et al. 1984). In this work, *A. ficuum* was used to study the reduction in phytic acid content in canola meal either by growing *A. ficuum* on it by an SSF process, or by enzyme preparations extracted from the canola meal after being treated in the above process.

Materials and methods

Microorganism, media and conditions of growth

A. ficuum NRRL 3135 was used to study the reduction in phytic acid content of commercially available canola meal by an SSF process. The microorganism was maintained on solid medium composed of malt extract (3.0%)[1], glucose (0.5%), yeast extract (0.5%), agar (1.5%) and distilled water. The medium was sterilized at 115° C for 15 min. After growth of the mould, the slants were kept at 4° C.

The SSF was carried out in 500-ml erlenmeyer flasks containing 50 g canola meal. Water or 0.2 M acetate buffer (pH 4.7) was added to the meal to give a moisture content between 65% and 75% after inoculation. The meal was sterilized at 121° C for 45 min before inoculation.

The inoculum was prepared in a medium composed of glucose (0.5%), yeast extract (0.5%), nutrient broth (0.8%) and distilled water. The medium, 200 ml in a 500-ml erlenemyer flask, was inoculated with a suspension of *A. ficuum* spores and incubated in a shaker (200 rpm) at 30° C for 3 days (unless otherwise stated). If not otherwise specified, 50 ml inoculum was used for inoculation of 50 g canola meal in flasks.

In some cases, the reduction of phytic acid content in canola meal was carried out with enzyme preparations produced by canola meal SSF and extracted with 2% aqueous $CaCl_2 \cdot 2H_2O$.

In this study, the kinetics of the change in phytic acid, protein, soluble and total carbohydrate concentrations and the pH in the canola meal medium were followed during the SSF. A change in phytic acid content was also followed when the meal was treated enzymatically. Phytase activities and biomass concentrations were also measured in the broth that was used for the inoculation of canola meal. All tests were carried out in duplicate, and the results shown are average values.

Analysis of samples

Phytic acid, protein, carbohydrate, pH, and moisture were measured in samples taken every 24 h.

Phytic acid. Phytic acid was determined according to the method described by Haug and Lantzsch (1983). Approximately 3-g samples were used for the analyses. Phytic acid was extracted by shaking each sample for 1 h in the presence of 50 ml 3% trichloroacetic acid (TCA). After extraction, the suspension was centrifuged (4000 *g*, 15 min) and the supernatant collected. The supernatant (5 ml) was mixed with 10 ml of 0.1% ferrous ammonium sulphate solution in 3% TCA and boiled for 30 min. After cooling and centrifuging, 2 ml of the supernatant was treated with 3 ml 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.

[1] All percentages are w/v unless otherwise specified

Protein. Approximately 3 g samples were boiled gently with 50 ml of 1 N NaOH. After cooling and centrifuging (4000 *g*, 15 min), the supernatant was diluted appropriately and the proteins were determined according to the method described by Lowry et al. (1951).

Carbohydrate. Approximately 3 g samples were boiled for 2 h with 50 ml water and 10 ml concentrated HCl. After centrifuging, an amount of the supernatant was appropriately diluted, supplemented with the anthrone reagent and boiled for 20 min. The absorbance was read at 625 nm against water treated similarly as the reference. Carbohydrate content was calculated from the standard curve prepared similarly using sucrose (AOAC 1975).

Biomass. A known volume of a sample from the inoculum culture was centrifuged at 12000 *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 h. The amount of biomass was expressed in milligrams dry weight per millilitre of culture broth.

pH. An approximately 2 g sample was stirred thoroughly in 20 ml distilled water and then the pH was measured using a Fisher Accumet (model 805 MP) pH meter (Fisher, Pittsburgh, Penn).

Enzyme preparation and assay procedures. Phytase activity was determined in the supernatant of liquid medium, and in the crude enzyme preparation obtained from the canola meal on which the mould was grown for 8 days. Crude enzyme was extracted from the meal using a 2% aqueous solution of $CaCl_2 \cdot 2H_2O$ and shaking the suspension for 1 h at 200 rpm. The meal to extractant ratio was 1:2 (w:v). The suspension was squeezed through a double layer of chesse cloth and centrifuged (4000 *g*, 15 min, 4° C). The clear supernatant was designated the crude enzyme preparation.

Phytase activity was assayed by measuring the inorganic phosphorus released from sodium phytate solution using the method described by Harland and Harland (1980). The reaction system contained 5 ml of 0.2 M acetate buffer (pH 4.7), 1.0 ml of 15 mM phytic acid solution, and 1.0 ml crude enzyme preparation or culture supernatant. The reactions were carried out at 50 or 60° C for 30 min and the reaction was stopped by adding 5 ml of 10% TCA.

One unit of enzyme activity is defined as the amount of enzyme preparation required to release 1 mg inorganic phosphorus from 2.0 mM phytic acid solution at the given temperature and pH.

Results and discussion

First, the composition of the canola meal used in this work was determined. The data obtained agree with those previously published (Table 1). The analysis shows that the meal used in this work contained 6.0% phytic acid. For reduction of the amount of this compound, *A. ficuum* was grown on the meal by a solid state technique.

The effect of the biomass amount in the inoculum on the reduction of phytic acid content is shown in Fig. 1A. 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 the processes differed. When the inoculum contained 101 mg biomass, 6 days incubation was required for complete hydrolysis of phytic acid in the meal. With an increase in the amount of biomass used for inoculation (proportionally larger volumes of inocula were

Table 1. Composition of canola meal

Components	This work (%)	Literature value (%)	References
Moisture	7.5	8.0–11.0	Alli and Houde (1987), Clandinin (1986); Graf (1986)
Crude protein	34.0	34.0–38.0	Alli and Houde (1987); Blair et al. (1987); Clandinin (1986)
Carbohydrate	26.0	28.0–32.0	Alli and Houde (1987); Blair et al. (1987); Clandinin (1986)
Crude fibre	15.0	11.0–15.0	Alli and Houde (1987); Blair et al. (1987); Clandinin (1986)
Ether extract	5.0	3.0– 6.0	Alli and Houde (1987); Blair et al. (1987)
Phytic acid	6.0	3.0– 7.0	Alli and Houde (1987); Blair et al. (1987); Clandinin (1986); Erdman (1979); Graf (1986); Harris (1988); Maga (1982); Stone et al. (1984)
Ash	6.5	6.0– 8.0	Alli and and Houde (1987); Harris (1988)

transferred), the time required for complete reduction of phytic acid content decreased. The phytic acid content was reduced to zero in 2 days in the system inoculated with 390 mg biomass.

Bearing in mind that various volumes of inocula were used and that their liquid phase contained some phytase, excreted from the biomass during its growth, it is obvious that the amount of the enzyme transferred to

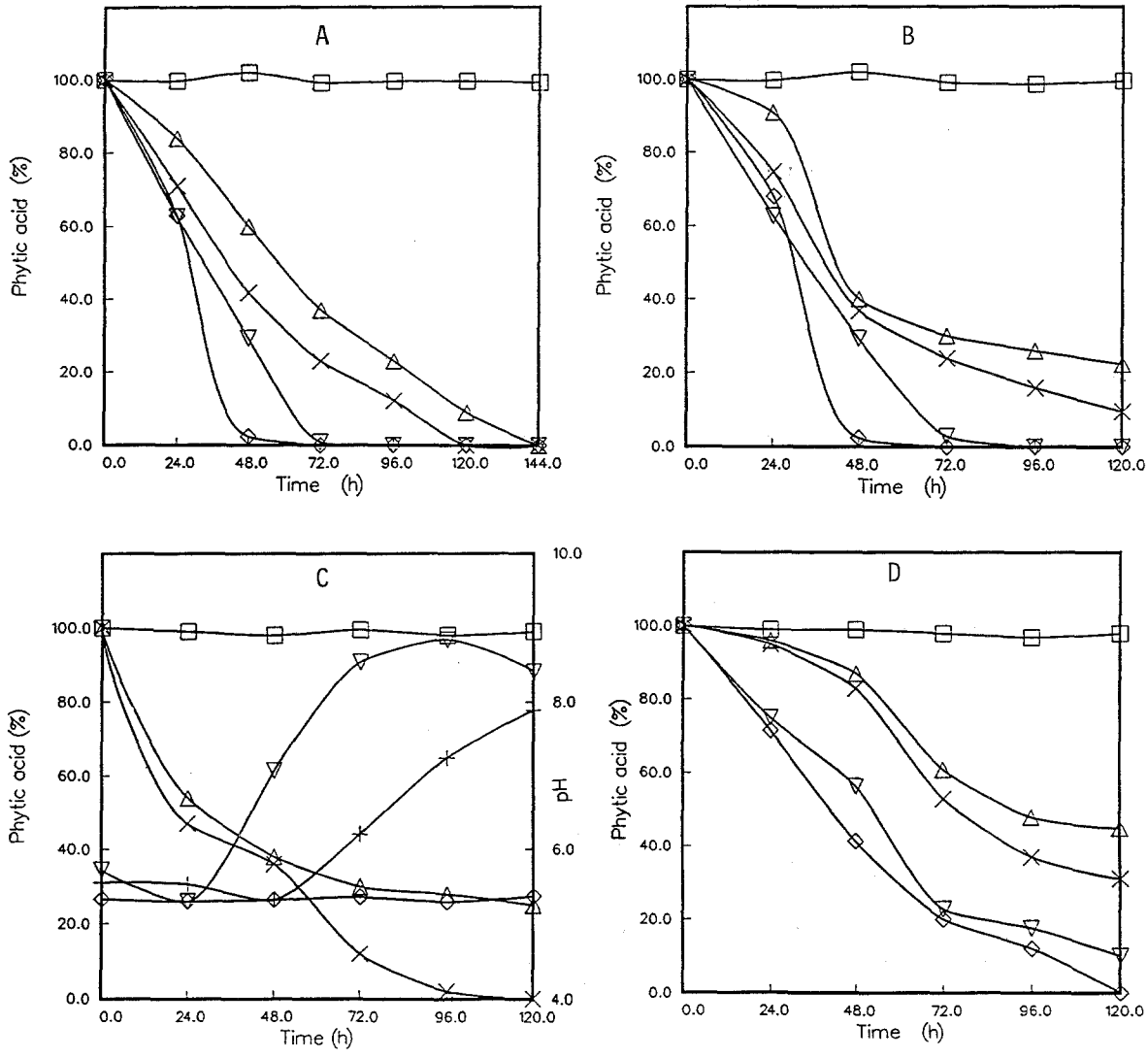

Fig. 1. A Effect of the amount of biomass in the inoculum on the rate of phytic acid content reduction in canola meal: □, 0.0 mg (control); △, 101 mg; ×, 202 mg; ▽, 330 mg; ◇, 390 mg. **B** Effect of age of inoculum on phytic acid content reduction: □, control (no inoculum); △, 2.0 days; ×, 3.0 days; ▽, 4.5 days; ◇, 6,0 days. **C** Effect of buffer on the reduction of phytic acid content. System without buffer: △, phytic acid; ▽, pH. System with buffer: ×, phytic acid; +, pH. Control (no inoculum): □, phytic acid; ◇, pH. **D** Effect of moisture content on the reduction of phytic acid content: □, 65%, control (no inoculum); △, 52%; ×, 60%; ◇, 67%; ▽, 78%

the canola meal medium was different in each of the above systems. Therefore, in addition to the biomass the transferred enzyme has also an 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 biomass also contained 1.2 units of the enzyme, and the inocula containing larger amount of biomass contained proportionally larger amounts of the enzyme. The largest amount of the enzyme that was transferred with the liquid phase of inoculum containing 390 mg of the biomass to 50 g of the meal was 4.7 units.

Observing the development of the mould, it was noticed that during the first 24 h of incubation growth was visually unnoticeable in all the systems, but after 48 h and especially after 64 h the medium was well interwoven with mycelial biomass. The apparent 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 inoculum age on the phytic acid content reduction, it was found that older inocula were more efficient (Fig. 1B). 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 volume of inoculum broth used and it ranged from 0.37 units to 3.6 units for inocula 2–6 days old respectively. The final moisture content in every batch was the same.

During the growth of *A. ficuum* on the meal, the pH value increased from about 5.6 to 8.2 in 5 days of incubation (Fig. 1C). Knowing that the pH optima for microbial phytases are between 4.8 and 5.8 (Graf 1986), the SSF of a canola meal medium supplemented with water was compared with one in which water was substituted with the same amount of 0.2 M acetate buffer (pH 4.7). The results show (Fig. 1C) that the pH of the systems without and with buffer remained constant for the first 24 h and 48 h 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. Almost complete reduction of phytic acid content occurred in the buffered system in 4 days, whereas in the unbuffered system, only 71% phytic acid was hydrolysed in the same period of time. It can also be seen from this figure that in the first 24 h 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 (Fig. 1C). This means that it would be beneficial to have good pH control, but it would be also necessary to observe the effect of pH on the production of phytase.

It is known that the moisture content of the medium in SSF is very important for the growth of microorganisms, production of enzymes and for enzyme activity. 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 amount on phytic acid content reduction, tests were carried out with meal containing between 52% and 78% moisture. The results show (Fig. 1D) 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 h of fermentation was noticed in the two systems with the lowest amount of moisture. A slower growth of the microorganism and subsequently lower amount of phytase produced may be the reason for this. 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. 2A). Canola meal does not contain phytase (Stone et al. 1984), and therefore growth of the microorganisms already present in the meal is the most likely reason for it. To test the effect of inoculation, a non-sterile medium was inoculated with *A. ficuum* and the results were compared with those obtained with an uninoculated non-sterile medium and an inoculated sterile medium. The results show (Fig. 2A) that the best results were obtained when the sterile medium was inoculated with *A. ficuum*. In non-sterile medium *A. ficuum* 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* phytase. Another possibility is that, by sterilization, some compounds in the canola meal became more accessible to the microorganism for its growth. All these assumptions may be the reasons for lower reduction of phytic acid content in the non-sterile inoculated medium than in the sterile inoculated one.

During the execution of the majority of the above tests, changes in carbohydrate and protein content of the meal were also measured (Fig. 2B). It was found that about 50% of the 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 h of growth while the amount of insoluble fraction remained the same.

It was noticed that the apparent protein content of canola meal increased by 25% after 48 to 72 h of growth of the microorganism (Fig. 2B). The amount of protein started to decrease after the third day of incubation. Taking this into account, it is necessary to design the process in such a way (for example, a larger amount of the older inoculum) that the phytic acid content is completely reduced during the first 2–3 days to avoid the loss of that valuable component.

To test the reduction of phytic acid content in canola meal with a phytase preparation, the enzyme was extracted from canola meal after treating it by an SSF

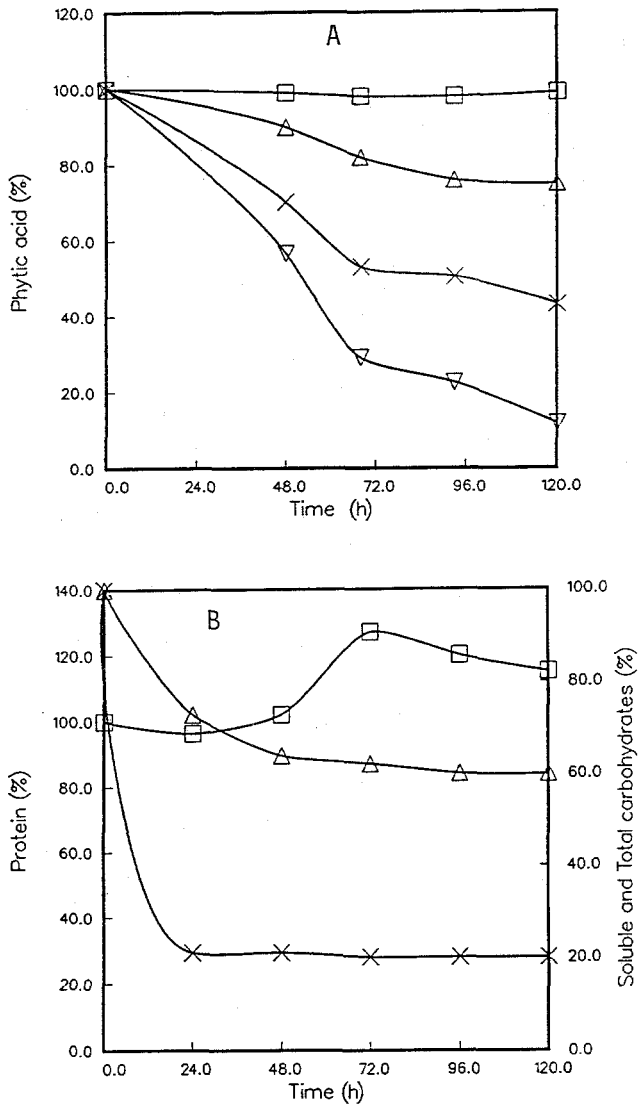

Fig. 2. **A** Reduction of phytic acid content in sterile and non-sterile media; □, sterile, uninoculated; △, non-sterile, uninoculated; ×, non-sterile, inoculated; ▽, sterile, inoculated. **B** Relative changes in protein and carbohydrate contents in canola meal during the solid state fermentation process: □, protein; △, total carbohydrate; ×, soluble carbohydrate

process. Two hundred grams of canola meal was inoculated with 1200 mg of 3-day-old *A. ficuum*, and was incubated at 30° C. The enzyme was extracted after 180 h of incubation. The results of three consecutive extractions are given in Table 2. It shows that 65% of the total enzyme was extracted in the first step.

Table 3. Reduction of phytic acid content in canola meal by an enzyme preparation in 20 h of incubation

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

Batches of fresh canola meal were treated with these enzyme preparations. After the addition of the enzyme preparation, the moisture content of the meal was about 62%. The concentration of enzyme was about 1.2 units/g canola meal in these tests. The process was carried out at different temperatures. The results in Table 3 show that the highest (58%) and the lowest (44%) reduction occurred at 45° C and 60° C respectively. Additional tests (the results of which are not given here) showed that faster enzyme denaturation at 60° C was the reason for the lower phytic acid content reduction at this temperature. In these tests 12 to 50 times larger amounts of phytase were used for the phytic acid hydrolysis compared to the phytase quantities transferred with inocula when the effects of the biomass amounts (Fig. 1A) and the inocula age (Fig. 1B) were studied. Thus, these tests suggest that the age and the amount of biomass, and not the relatively small quantities of the enzyme in the inocula, were a predominant factor influencing the rate of phytic acid content reduction in the meal.

Similar tests carried out at 30° C and 50° C with the enzyme preparation added to a suspension of canola meal (12% w/v) in acetate buffer resulted in a much faster reduction in phytic acid content (Fig. 3) than when the moisture content of the medium was only 62% (Table 3), although the same amount of enzyme preparation per gram of the meal was used. Ninety percent of the phytic acid content was reduced in 24 h of incubation at 30° C, and complete reduction was attained in 12 h at 50° C. A better mass transfer and a higher enzyme activity in the suspension of canola meal than in

Table 2. Extraction of the enzyme from canola meal treated in a solid state fermentation process

Parameters	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
Enzyme extracted in three extractions (%)	65	22	13

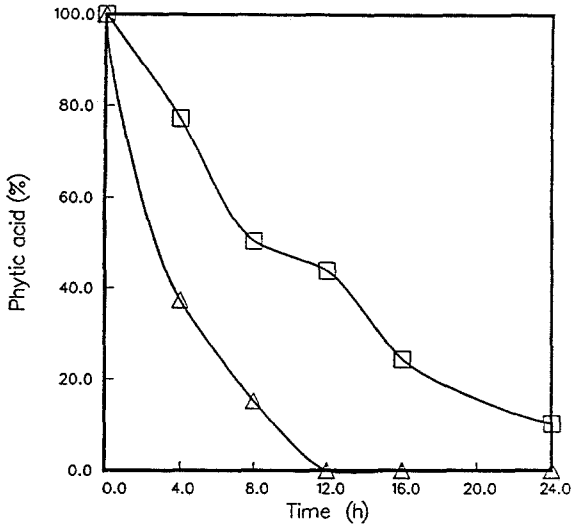

Fig. 3. Reduction of phytic acid content in a 12% canola meal suspension by the enzyme preparation. Temperature: □, 30° C; △, 50° C

the solid medium were probably the reason for the faster reduction of phytic acid content in that material.

In conclusion, solid state fermentation using *A. ficuum* has been found to be very effective in reducing the phytic acid content of canola meal. Complete reduction of phytic acid content was achieved within 48 h of fermentation in some tests. Both older inocula and a larger amount of biomass used for inoculation caused faster reduction of phytic acid content of canola meal. Substitution of water with buffer in the meal treated by the SSF increased the rate of phytic acid content reduction. Moisture level in the medium also affected phytic acid hydrolysis; a medium with 67% moisture was found to be the best for phytic acid hydrolysis. A relative increase in the amount of protein was noticed during the reduction of phytic acid content in canola meal by the SSF. The phytic acid content of canola meal can also be reduced using enzyme preparations extracted from the canola meal after its treatment with *A. ficuum* in an SSF process. The achieved reduction of the phytic acid content in canola meal and an apparent increase in the amount of protein, might lead to the conclusion that the meal after SSF treatment has a higher nutritional value than before. However, that should be confirmed by animal feeding study.

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