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# Biodegradation of glucosinolates in brown mustard seed meal (*Brassica juncea*) by *Aspergillus* sp. NR-4201 in liquid and solid-state cultures

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**Abstract**

*Aspergillus* sp. NR-4201 was assessed by degrading glucosinolates in brown mustard seed meal (*Brassica juncea*). A liquid culture of the strain, in a medium derived from the meal, produced total degradation of glucosinolates at 32 h. Under these conditions, the glucosinolate-breakdown product, allylcyanide, was formed in culture filtrates. In a plate culture under sterile conditions, the growth of the strain in heat-treated meal media was shown to be effective at 30 °C with 51% moisture, as determined by the measurement of the colony growth rate. On the laboratory scale, solid-state culture under the same conditions gave rise to total glucosinolate degradation within 48 h. In comparison, under non-sterile conditions in either heat-treated or non heat-treated meal samples, the degradations were complete after 60 and 96 h, respectively. In these cases, growth was associated with some out-growths of contaminating fungi, mainly *Rhizopus* sp. and *Mucor* sp. The glucosinolate-breakdown product, allylcyanide, was not detected in the solid-state meal-media culture presumably due to evaporative loss from the fermentation matrix.

## Introduction

Oilseeds of the genus *Brassica* such as rape (*B. napus*) and mustard (*B. juncea*, *B. nigra* or *S. alba*) are some of the most significant oil-producing crops in the world (FAO 1981). In addition, by-products remaining after seed-oil pressing, traditionally known as rape seed or mustard seed meals are protein-enriched materials (Sosulski & Sarwar 1973; Maheswari et al. 1981). However, their utilisation as animal feeds is restricted due to their variable content of anti-nutritional substances, especially glucosinolates (1-thiol-$\beta$-S-D-glucosides). Exposure of the stored glucosinolates to endogenous degradative enzymes, myrosinase (thioglucoside glucohydrolase EC 3.2.3.1) which are contained in such seeds, causes the degradation of glucosinolates. This leads to the production of D-glucose, sulphate and a series of S and/or N containing metabolites, nitriles, thiocyanates or isothiocyanates (Bones & Rossiter 1996). Glucosinolate-breakdown metabolites have had their toxicities established in experimental animals (Fenwick et al. 1983; Hill 1979).

There have been attempts to improve the nutritional quality of these oilseed meals. Destruction of the endogenous myrosinase may be not reasonable, since some intestinal microflora can promote glucosinolate-degrading activity (Oginsky et al. 1965; Nugon-Baudon et al. 1988, 1990). Of these, several physico-chemical methods have been introduced to eliminate the undesirable glucosinolates or their breakdown products (Ballester et al. 1970; Rauchberger et al. 1979; Shahidi et al. 1988; Van Megen 1983). However, all these processes have major drawbacks in reducing nutritive enrichment from the meals. Biological processing is probably an example of an interesting method to be applied (Brabban & Edwards 1994; Palop et al. 1995; Smits et al. 1993, 1994). In this instance, fungi are ideal microorganisms, since most strains are capable of potential growth in solid materials which offers cost advantages in the drying process and decreases the risk of deterioration (Pandey

1992). In previous papers, it was demonstrated that *Aspergillus* sp. was highly capable of degrading the glucosinolate sinigrin in liquid culture associated with the expression of intracellular myrosinase (Sakorn et al. 1999, 2002). This current work describes an improved process for glucosinolate degradation by *Aspergillus* sp. NR-4201 in both liquid and solid-state cultures.

## Materials and methods

### *Fungal strain*

A laboratory isolate of *Aspergillus* sp. was used (Sakorn et al. 1999, 2002). The strain was maintained on potato dextrose agar slants, and sub-cultured monthly. Spore suspensions were made from two-week old cultures and adjusted to desired concentration with distilled water using a Neubauer haemocytometer.

### *Glucosinolate degradation in liquid culture*

Brown mustard seeds (*Brassica juncea* var. Forge) were processed to extract their seed-oil content using a IBG Monfort BB85 expellator. In order to inactivate seed myrosinase, the seed meal was heated at 120 °C for 30 min (heat-treated meal). After grinding in a mortar, 200 g of finely ground mustard powder was suspended in 2 litres of boiling water. The slurry was vigorously stirred for 2 h and then filtered. The yellow-brown mustard extract obtained after centrifugation (10,000 × g, 20 min) was diluted with an equal volume of 0.1 M sodium phosphate buffer, pH 6.5 before autoclaving (121 °C, 15 min). Liquid culture was performed by incubating mixtures of 400 $\mu$l-inoculum ($10^6$ spores/ml) and 40 ml-mustard extract medium which were contained in a 250-ml Erlenmeyer flask at 30 °C in a waterbath, shaken at 150 rev $min^{-1}$. At regular intervals of incubation, culture filtrates were taken to assay the content for glucose, glucosinolates and glucosinolate-breakdown products. Fungal mycelium, harvested by filtration through Whatman filter paper No. 93 and washing with distilled water, was placed on a pre-weighed watch glass and dried at 70 °C until constant weight was obtained.

### *Growth optimisation in mustard seed meal*

Preliminary studies of fungal growth in solid meal material samples were carried out as follows. Ten g aliquots of heat-treated meal were filled in each petri dish, prior to being sterilized by heating at 90 °C for 120 min. Then, sterile distilled water was added to reach the desired moisture levels (21–60%) and mixed thoroughly. Each meal sample was flattened evenly over the dish. Spores from the two-week culture were point-inoculated at the centre of the dish and incubated at desired temperatures (25–43 °C). Growth was observed daily by measuring the diameter of the colony formed.

### *Solid-state culture*

Laboratory-scale solid-state culture of *Aspergillus* sp. (sterile condition) was carried out in a 250-ml Erlenmeyer flask containing 10 g of heat-treated meal. Optimum moisture contents and incubation temperature obtained from the preceding experiments were used. In this case, inoculation was carried out by means of aseptic-mixing technique using varying concentrations of inoculum ($10^5$–$10^7$ spores/g meal). At regular intervals of incubation, samples were taken twice from the culture flasks and milled with a coffee-blender for 30 sec. Each 0.2 g of the milled samples was then placed into a screw-capped vial. One portion was extracted with hexane, prior to being analysed for the content of glucosinolate-breakdown product(s). The other portion was extracted with boiling water (3 ml). After cooling and centrifuging, the supernatant was then determined for glucose and glucosinolate content. All experiments were done in duplicate.

To test non-sterile differences, experiments were achieved according to the procedure described above, but heat-treated and non heat-treated meals were used (i.e. with no sterilization).

### *Moisture content and protein nitrogen analysis*

Moisture content was determined by drying the meal samples at 90 °C for 24 h. While the content of protein nitrogen was analysed by Kjeldahl method (AOAC 1990).

### *Aflatoxin analysis*

Individual meal samples (inoculum-free or solid-state culture) obtained after autoclaving were dried at 90 °C for 12 h before grinding in a mortar. Meal powder was then extracted with chloroform prior to being analysed for aflatoxins by thin-layer chromatography (Pons & Goldblatt 1965).

*Glucosinolate analysis*

The determination of glucosinolate and glucose content was achieved by a method based on the coupled-enzyme assay. (Smits et al. 1993; Wilkinson et al. 1984).

*Glucosinolate-breakdown metabolites*

Analysis of glucosinolate-breakdown products was conducted using a Hewlett-Packard series II 5890 gas chromatograph, equipped with a flame ionisation detector (FID) and a carbowax column (Chrompack; 50 m × 0.3 mm ID), connected to the injector port. The carrier gas (helium) was constantly operated at a flow rate of 0.5 $cm^3$ $min^{-1}$ and a pressure of 50 kPa. Temperature of the column was initially maintained at 80 °C for 6 min and then was programmed to increase 10 °C $min^{-1}$ for a further 6 min, while those of the injector and detector were maintained at 230 °C and 280 °C, respectively. Samples were extracted with an equal volume of cold hexane (GC grade). After adding approximately 2 g of solid ammonium sulphate, capping, shaking and centrifuging (3,000 × g, 10 min), 1 $\mu$l of the hexane layer was injected.

## Results and discussion

*Growth and glucosinolate degradation in liquid culture*

*Aspergillus* sp. exhibited considerable growth in the mustard extract medium (*Brassica juncea*). The growth profiles are illustrated in Figure 1. At an early cultivation period, glucose was produced in culture filtrates and rose to a maximum level at 24 h. Glucose and glucosinolates were totally consumed at 32 h. The behavior of glucose and glucosinolate consumption resembled the results of our previous report in sinigrin-glucose medium (Sakorn et al. 1999). In this case, glucose should be processed from some other substrates, not the glucosinolates. This suggestion was supported by the liquid culture results of this *Aspergillus* strain in a sinigrin medium which showed that no liberation of glucose was observed in the culture filtrates (Sakorn et al. 1999). *Brassica* oilseeds have been reported to contain variable amounts of polysaccharides (Ohlson 1972), and it was confirmed by our report that mustard seed meal of this study contained 21.3% carbohydrates (Rakariyatham 2000). These polysaccharides have been confirmed to serve as substrates for glucoamylase that released glucose into culture medium (data not shown). In addition to the glucosinolate degradation, the product allylcyanide showed a delayed accumulation in culture filtrates. Its maximum level (about 60% of initial glucosinolate concentration) was reached at 44 h before declining (Figure 1). The result agreed with our previous report (Sakorn et al. 1999) and with liquid culture results of *Aspergillus clavatus* II-9 (Smits et al. 1993) using sinigrin as a substrate. Surprisingly, only trace amounts of allylcyanide (or none in some cases), were detectable in cell-free extracts of the *Aspergillus* (data not shown). Presuming that the compound was generated intracellularly, the fungal cells were metabolically active in eliminating the toxic allylcyanide.

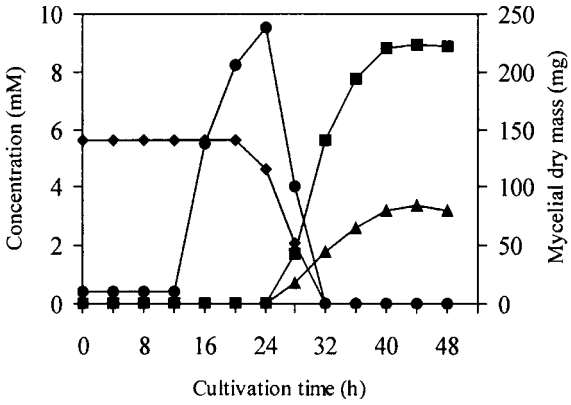


*Figure 1.* Growth profiles of *Aspergillus* sp. NR-4201 in mustard extract medium (*Brassica juncea* seeds). Production and consumption of glucose (●), degradation of glucosinolates (♦), production of allylcyanide (▲), and formation of fungal biomass (■) are shown.

*Growth optimisation in mustard seed meal*

Growth of *Aspergillus* sp. in mustard seed meal was observed at initial moisture contents between 35 and 60%, and at temperatures between 25 and 37 °C. At the moistures below 35% and temperatures above 40 °C, no growth was observed. However, the strongest growth rate (colony growth rates of 0.9 mm/h) was demonstrated at 51% moisture and 30 °C.

*Laboratory-scale solid-state culture*

Growth of the *Aspergillus* in sterile heat-treated meal was re-investigated at moisture levels of 40–60%. It was found that poor growth was observed at 40 and 46% moisture levels. In this case, glucosinolate degradation at 40% moisture was not complete at

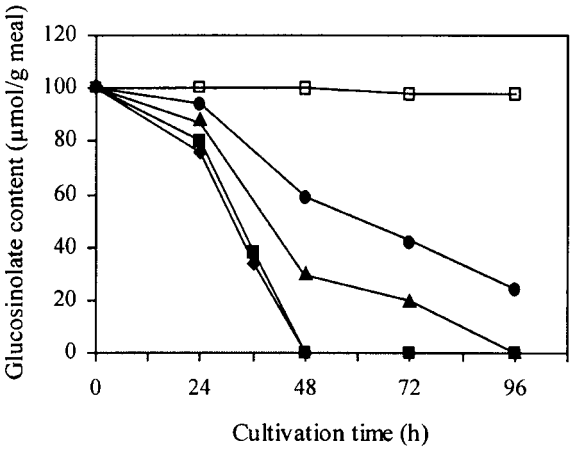


*Figure 2.* Effect of initial moisture content on glucosinolate degradation in heat-treated mustard seed meal by *Aspergillus* sp. NR-4201 (sterile condition). Experiments were carried out at 30 °C, inoculum density of $10^6$ spores/g meal and varying moisture levels at 40% (●), 46% (▲), 51% (■) and 60% (♦). Inocumum-free culture at 51% moisture (□) is shown.

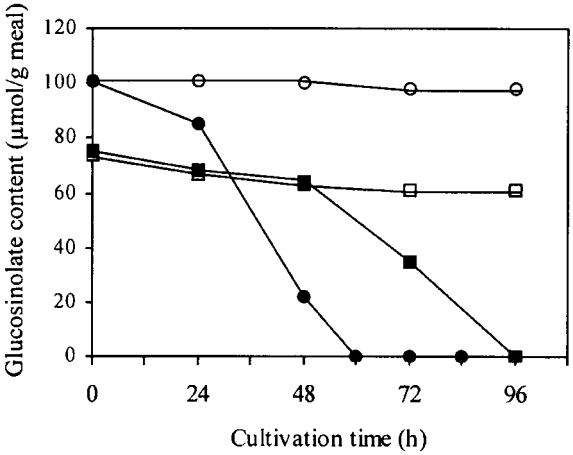


*Figure 3.* Glucosinolate degradation in heat-treated (●) and non heat-treated (■) mustard seed meals by *Aspergillus* sp. NR-4201 (non-sterile condition). Experiments were carried out at 30 °C, 51% moisture and inoculum density of $10^6$ spores/g meal. Each inoculum-free culture is represented with the corresponding transparent symbol.

96 h, while the complete degradation at 46% moisture was at 96 h (Figure 2). At 51 and 60% moisture levels, growth and sporulation were well-developed and caused the degradation of total glucosinolates at 48 h (Figure 2). Inoculum density of $10^6$spores/g meal was sufficient, while lower inoculum levels prolonged the degradation times (data not shown). None of the glucose was detected in any meal samples, determined during the cultivation period at 24–96 h. The lack of glucose production suggests that enzymes, which enable the generation of glucose did not function with a very limited availability of water. All hydrolase enzymes require adequate water for enzymatic catalysis and substrate solubility (Pandey 1992). It was not surprising that allylcyanide did not remain in any of the meal samples, since the compound was simply evaporated from the fermentation matrix. This was confirmed by the result using *Aspergillus clavatus* II-9 in brown mustard seed meal (Smits et al. 1993). In addition, protein nitrogen content of the culture meals was increased from 36.8% (non-culture, heat-treated meal) to 43.7 and 48.2%, respectively, for the 2- and 4-day cultures while protease was found in the culture meals. Therefore, most of the protease accounted for the protein nitrogen accumulation in the fermentation matrix. It was shown that no aflatoxins were detected in the 4-day culture, as determined by thin-layer chromatography.

In case of non-sterile experiments (no sterilization of the meal media at 90 °C for 120 min), the cultivation time used for degrading the total glucosinolates of heat-treated meal samples was extended up to 60 h (Figure 3). It was observed that the culture was associated with some out-growths of contaminating fungi in which major strains were identified as *Rhizopus* sp. and *Mucor* sp.. These undesirable out-growths might have an affect on the growth of the *Aspergillus*, prolonging the degradation-time for glucosinolates. When non heat-treated samples were used, no growth of the *Aspergillus* and other contaminating microbes were observed within the first two-days. After that time, growth was observed with the association of contaminating fungi (see above observations). Completion of glucosinolate degradation was observed at 96 h (Figure 3). Even after moistening the non heat-treated meals, glucosinolate levels were deficient by 25%. This was influenced by seed myrosinase-catalysed glucosinolate degradation. Such an effect generated the pungent-smelling compound, allylisothiocyanate which was detected in those meal samples. Allylisothiocyanate was established as a potent anti-microbial substance (Isshiki et al. 1992). Fungal growth was enabled after the volatile allylisothiocyanate disappeared (after approximately 2 days).

The improvement of mustard meal free from glucosinolates accomplished by solid fermentation of *Aspergillus* sp. is a simple and available method for industrial use. However, the fact that allylcyanide was present in mustard meal should be a greater concern.

## Acknowledgements

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