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Short communication

Rapid detection of myrosinase-producing fungi: a plate method based on opaque barium sulphate formation

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Summary

A simple and rapid technique to assess the capability of fungi to produce myrosinase is reported. This was carried out by growing the tested fungi in sinigrin–barium agar plates. Strains capable of producing myrosinase were indicated by an opaque barium sulphate zone forming underneath and/or surrounding their colonies. This simple test has been confirmed by determination of myrosinase activity in liquid culture. In positive isolates, enzyme acitivity was detected in cell-free extracts, not in culture filtrates. In the case of non-myrosinase-producing strains, no opaque zone was observed and the enzyme was not detected either in cell-free extracts or in culture filtrates.

Introduction

Glucosinolate-degrading microorganisms, particularly fungi (Smits *et al*. 1993, 1994) have been considered to have a possible application in the nutritional improvement of materials containing glucosinolates e.g. rapeseed or mustard seed meals, prior to being utilized as animal feeds. Most strains were reported to produce myrosinase, the only known enzyme able to hydrolyse glucosinolates (Ohtsuru *et al*. 1969, 1973; Smits *et al*. 1993). Glucosinolate hydrolysis results in the production of D-glucose, sulphate and a series of pungent and/or goitrogenic compounds, nitrile, isothiocyanate or thiocyanate (Bones & Rossiter 1996). We reported that the occurrence of myrosinase activity within intact fungal cells of the *Aspergillus* sp. was closely related to its degradative potential for the glucosinolate sinigrin (Sakorn *et al*. 1999). Traditionally, liquid culture has been employed as the most reliable method to examine myrosinase-producing strains (Tani *et al*. 1971; Ohtsuru *et al*. 1973; Smits *et al*. 1993). However, this method is not convenient for screening purposes. This work presents herein a plate culture method to examine myrosinase-producing fungi via the detection of sulphate released from sinigrin hydrolysis.

Materials and methods

Fungal strains and growth in sinigrin–barium agar plates

A myrosinase-producing strain isolated in our laboratory, *Aspergillus* sp. NR-4201, and two non-glucosinolate-degrading strains from the NSTDA culture collection (Thailand), *A. flavus* 3040 and *A. oryzae* 3411 were used as positive and negative controls, respectively. Three *Aspergillus* sp., one *Rhizopus* sp. and one *Mucor* sp. isolated from decayed mustard seed meals were tested. Spores from a 2-week old culture grown on potato/dextrose/agar were point-inoculated at the centre of sinigrin–barium agar plates (5 mM sinigrin, 5 mM ammonium chloride, 2.5 mM barium chloride and 15 g agar/l in deionized water) and incubated at 28 °C. Growth and opaque zone formation were observed daily.

Liquid culture

Myrosinase-producing ability was assessed by inoculating 25 μl of spore suspensions (10^6 spores/ml) in 25-ml erlenmeyer flasks containing 2.5 ml sinigrin–glucose medium (5.5 mM sinigrin, 5.5 mM glucose and 5 mM ammonium chloride in 0.1 M sodium phosphate buffer, pH 6.5). The cultures were incubated at 28 °C under reciprocal shaking (150 rev/min). During cultivation, culture filtrate samples were taken periodically to assay for myrosinase activity, glucose and sinigrin contents (Wilkinson *et al*. 1984; Smits *et al*. 1993). After 48 h, the culture filtrate was filtered (Whatman filter paper no. 93) and washed with distilled water to separate the fungal mycelium. This was then suspended in 50 mM sodium phosphate buffer, pH 7.2 and disrupted with a mortar at 4 °C. Cell-free extracts obtained after centrifugation were subsequently assayed for myrosinase activity.

Enzyme assay

Myrosinase activity was determined using a coupled enzyme assay method (Wilkinson *et al.* 1984) with slight modifications. The assay was performed in 50 mM sodium phosphate buffer, pH 7.2 at 28 °C and ascorbic acid was omitted. One unit of myrosinase was defined as the amount of enzyme that catalysed the production of 1 μmol of glucose from sinigrin in 1 min.

Results and discussion

Aspergillus sp. NR-4201 and all fungi isolated from decayed mustard seed meals showed considerable growth on sinigrin–barium agar plates with colony growth rates between 7 and 10 mm/day. After 2 days, *Aspergillus* sp. NR-4201 and the other three *Aspergillus* strains exhibited an opaque zone, underneath and/or surrounding their colonies (Figure 1) while *Rhizopus* sp. and *Mucor* sp. did not. When incubations were prolonged up to 7 days, the opaque zone expanded proportionally to the colony size whereas with the *Rhizopus* and *Mucor* colonies, nothing was observed. No growth was observed for the two non-glucosinolate-degrading strains, *A. flavus* 3040 and *A. oryzae* 3411. In liquid culture, each of the four *Aspergillus* strains completely exhausted sinigrin within 48 h whereas none was consumed by the two non-glucosinolate-degrading strains. Myrosinase activity (0.22–0.28 U) was detected in each cell-free extract of the four strains but not in culture filtrates. It was therefore suggested that the myrosinase-catalysed sinigrin degradation by these *Aspergillus* strains was an intracellular process. These results agreed with the reports on *A. niger* AKU3302 (Ohtsuru *et al.* 1973) and *A. clavatus* II-9 (Smits *et al.* 1993). Liquid cultures of *Rhizopus* and *Mucor* produced the consumption of 12.7 and 14.5% of the sinigrin, respectively. It was surprising that the enzyme activity was not detected in either cell-free extracts or in culture filtrates. Even, when cultivations were extended for an additional 24 and 48 h, no enzyme activity was still detected. These results revealed that sinigrin consumption by the *Rhizopus* and *Mucor* would not be facilitated by myrosinase as demonstrated in *Fusarium oxysporum* @146 (Smits *et al.* 1993).

In plate cultures, except for the sulphate ion present in sinigrin, there was no other di- or tri-valent anions available in the agar medium. Therefore, the opaque barium salt would be deposited only when sinigrin released free sulphate ion to the medium. This, of course, takes place via myrosinase-catalysed hydrolysis. Barium chloride at a defined concentration showed no effect on fungal growth when comparing to that in barium-free medium. Experiments in test-tube agar slants showed the same performance as achieved in the agar plates. This method is thus, practical, rapid and convenient to be used as preliminary screening for myrosinase-producing fungi.

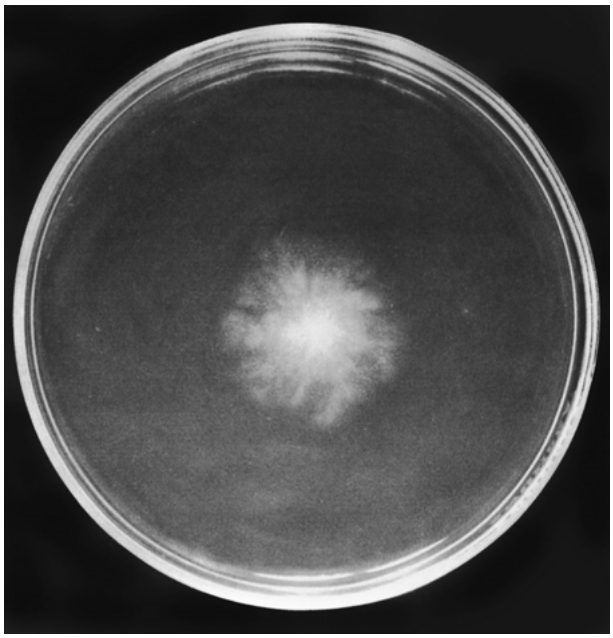

Figure 1. Growth of *Aspergillus* sp. NR-4201 in sinigrin–barium agar plate for 4 days. Whitening zone under colony indicated barium sulphate formation.

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