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Effects of Hom Mali Brown Rice Flour Extract on *Aspergillus niger* Growth

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ABSTRACT

The objectives of this study were to determine the optimum extraction process for Hom Mali brown rice flour (HMBRF) and to investigate the growth of *Aspergillus niger* using the extract as a culture medium. A completely randomized design (CRD) factorial experiment was used to assess the effects of α-amylase, BAN® 480L, concentrations (0.025, 0.050, 0.075 and 0.100 % v/w) and digestion time (15, 20, 25, 30 and 35 min). The optimum conditions for HMBRF extraction involved mixing HMBRF with distilled water (1:3.7), adding α-amylase at 0.05 % v/w of HMBRF, digesting for 30 min at 75°C in a water bath, then cooling down rapidly in an ice bath, boiling for 10 min to stop enzyme activity and separating the supernatant by centrifuging at 5,000 rpm for 30 min. HMBRF extracted solution contained total soluble solids (TSS) with 16.80°Brix and 6.78% reducing sugar. The growth of *A. niger* in the extract at 6°Brix was not significantly different ($p>0.05$) from growth in the control medium (PDB, commercial). There was high correlation between TSS and the growth of *A. niger*. This study revealed that HMBRF extract contained nutrients suitable for growing *A. niger*. Therefore, processing of the culture medium in dry form should be subjected to further study.

Key words: HMBRF, α-amylase, *Aspergillus niger*, extract, growth

INTRODUCTION

The information on recent studies of media formulation using locally available materials is scanty (Poopathi *et al.*, 2002). A preliminary study by Petchaboon *et al.* (2007) found that *Phytophthora infestans* could be cultivated on culture media prepared from either glutinous rice or japonica rice. *Aspergillus niger*, *Fusarium moniliforme*, *Penicillium* sp. and *Curvularia palescens*, isolated from onion, carrot, orange and maize, respectively, were cultured on agar medium from corn meal dextrose agar (Adesemoye and Adedire, 2005). *Aspergillus niger* is used in the industrial preparation of citric acid and gluconic acid and has been assessed as acceptable for daily intake by the World Health Organization (Marin *et al.*, 2004). Potato dextrose agar (PDA) and potato dextrose broth (PDB) are recommended for isolation and enumeration of yeasts and molds from dairy and other food products (David and Mary, 2003). There are two major types of PDA and PDB based on the preparation method, either ready-to-use medium

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or culture medium prepared from fresh potatoes (Alexopoulos and Beneke, 1961). PDA and PDB are prepared from fresh potatoes starting with boiling 200g potato in 500 ml distilled water for 30 min on a hot plate, filtering with a filter cloth, adjusting the volume to 1 L with distilled water, adding 2.0% dextrose and adding 1.5% agar for a solid medium (Alexopoulos and Beneke, 1961; Booth, 1971; Harrigan and McCance, 1976). In Thailand, ready-to-use PDA and PDB are fully imported at high cost and making fresh media that use potato as a main raw material is problematic due to the cost and shortage of fresh potatoes, as well as the inconvenient preparation method. Therefore, it is necessary to investigate new nutritional sources for media preparation, which are based on locally available materials that are less expensive than potato. Broken Hom Mali brown rice (HMBR), a by-product from the rice milling process, is available in large quantities and has few uses. Previous study has found that rice flour is a source of important nutrients, such as protein, carbohydrate, phosphorus and calcium. Thus, rice flour could be suitable as a raw material for culture media preparation, especially if this use results in the conversion of raw agricultural materials into value-added products. However, in fresh potato extract preparation, after the potato has been boiled for 30 min and filtered, the extracted solution was a clear liquid and could be used in culture media. By contrast, boiling HMBRF in the same manner as fresh potato produces an HMBRF extract that is too thick to filter. Thus, a digestion step with an enzyme needs to be added to the HMBRF extraction process. The enzyme, α-amylase, was chosen for the HMBRF digestion step as it was possible to readily obtain the purified product and it could reduce the thickness of the HMBRF extract (Srisoth and Piyachomkwan, 2003). Therefore, the objectives of this study were to determine the optimum extraction process for HMBRF and to investigate the growth of *Aspergillus niger* using HMBRF extract as the culture medium.

MATERIALS AND METHODS

Hom Mali brown rice flour preparation

Broken Hom Mali brown rice (HMBR) was obtained from the Royal Chitralada Project. The preparation commenced with drying 10 kg of broken HMBR at 55°C, then milling and sieving with a particle size 100 mesh (Nukit, 2006). Proximate analysis of the HMBRF was performed as described by AOAC (2000).

Microorganisms and inoculums

Aspergillus niger TISTR 3089 obtained from the Thailand Institute of Scientific and Technological Research (TISTR) was used throughout this study. Lyophilized *A. niger* was activated in potato dextrose broth (PDB, Difco), incubated in a rotary shaker at 150 rpm and 30±2° C for 48 h, before being transferred using a sterile loop onto three locations per plate of potato dextrose agar (PDA, Difco) and incubated at 30±2°C for 7 d. Mycelium of *A. niger* were transferred to PDA slant using a sterile needle, incubated for 7 d and stored at 4°C until used. Prior to use in the experiment, *A. niger* was propagated at the center of a PDA plate for 7 d, then a sterile cork borer (0.4 cm) was used to cut a plug from the tip of the *A. niger* mycelia, which was subsequently transferred onto the formulated media (Sriswadskulmee, 2002).

Effect of the extraction process on quality of HMBRF extract

A completely randomized design (CRD) factorial experiment was used to assess the effects of α-amylase (BAN® 480L, Novo Nordisk, Denmark) concentration (0.025, 0.050, 0.075 and 0.100 % v/w) and digestion time (15, 20, 25, 30 and 35 min) on TSS of extracted solutions. The extraction process was prepared according to the procedure of Pradistpong (1996) with some modifications (Figure1).

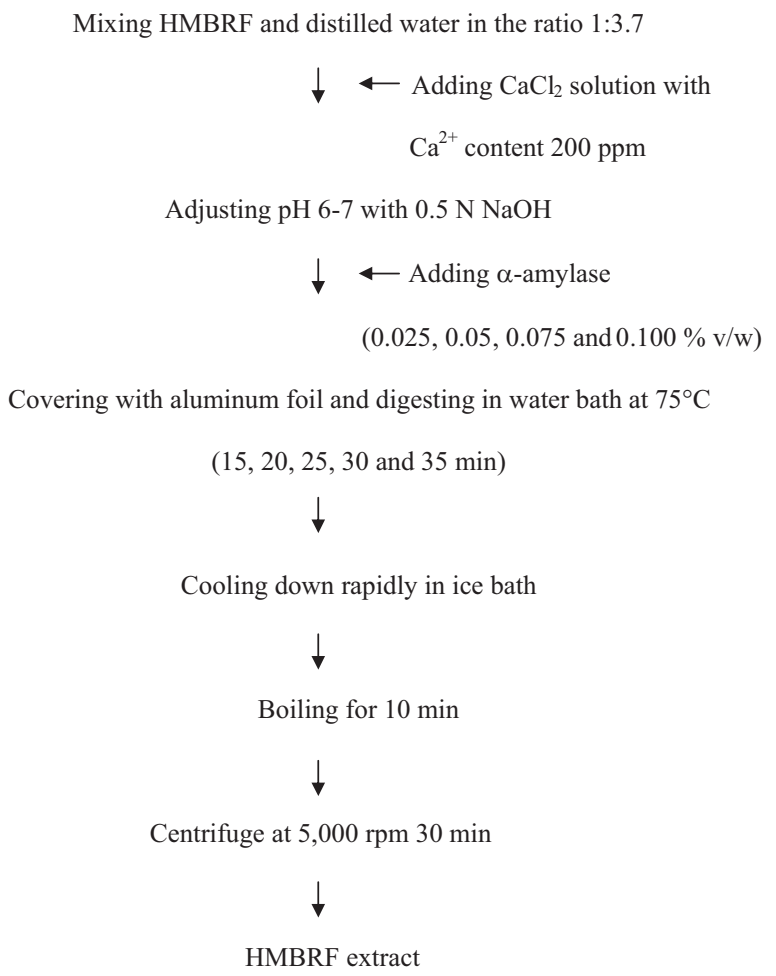

Figure 1 HMBRF extraction process.
Source: Modified from Pradistpong (1996)

Components of the extracted solutions were measured and analyzed as follows: total soluble solids (TSS) by a hand refractometer (ATAGO, Japan); color (L* a* b*) by a Lovibond Reflectance Tintometer (RT100, PFX, UK); pH by a pH meter (WTW, Japan); and total solids (TS) AOAC (2000) and reducing sugars (RS) according to Somogyi (1952), using a spectrophotometer (UV 9200, China). Measurements were made in triplicate and average values were reported. Analysis of variance (ANOVA) was used to determine the effect of quality characteristics. The differences between treatments were determined using Duncan's multiple range test (DMRT) comparisons.

Effect of HMBRF extract on growth of *Aspergillus niger*

HMBRF extract was dissolved in distilled water at 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10° Brix, stirred until homogenous and TSS measured using a hand refractometer. The HMBRF extract was used as a liquid medium with and without 2% dextrose with unadjusted pH. Then, 50 ml of each concentration was placed in separate 250-ml Erlenmeyer flasks and sterilized in an autoclave

at 121°C for 15 min. Sterile media were inoculated with a cork borer of *A. niger* culture and incubated in a rotary shaker at 150 rpm and 30±2°C. After 4 d of incubation, cells of *A. niger* were filtrated through a Whatman No.1 filter paper with a vacuum pump, washed with 50 ml distilled water twice, dried in a hot-air oven at 80°C overnight and put into desiccators until a constant weight was recorded (Junlakun, 2002). A control medium, PDB (commercial) was prepared using the instructions by suspending 24.0 g of the powder in 1 L of purified water, mixing thoroughly, heating with frequent agitation and boiling for 1 min to completely dissolve the powder, followed by autoclaving at 121°C for 15 min.

Analysis of variance (ANOVA) was used to determine the optimum concentration of HMBRF extract in culturing *A. niger* compared with the control medium (PDB, commercial).

RESULTS AND DISCUSSION

The composition of HMBRF

HMBRF was prepared from 10 kg of broken HMBR by grading, drying, milling and sieving (through a 100 mesh). The yield of HMBRF was 68% and its composition was 86.10% carbohydrate, 10.61% moisture content, 7.80% protein, 3.18% fat, 1.30% ash and 1.62% crude fiber. Fresh potato, which is usually used as a substrate for making media for growing fungi, was composed of 82.0% moisture content, 15.3% carbohydrate, 2.5% protein and 0.2% fat, while ash and crude fiber were not found (Puwastien *et al*., 1999). The results from the preliminary study found that carbohydrate and protein had an effect on the growth and metabolism of fungi as carbon and nitrogen sources (Suwanpinit and Suwanpinit, 2005). HMBRF proximate showed that the composition was potentially providing enough nutrient for the growth of fungi, especially carbohydrate and protein content. Therefore, HMBRF should be used as a local material for making culture media because it has sufficient nutrients for *A. niger* growth.

Extraction process of HMBRF

Effect of extraction process of HMBRF

For fresh potato extract preparation, potato was boiled for 30 min and filtered. In this case, the extracted solution was a clear liquid and able to be used as culture medium. However, if boiling HMBRF to prepare it in the same way as potato, the resultant HMBRF extract would be too thick to filter. Thus, a digestion step needs to be added in the HMBRF extraction process. Starch can be digested in three ways: using an acid, such as hydrochloric acid (HCl), using an enzyme, such as α-amylase and by using both an acid and an enzyme (Srisoth and Piyachomkwan, 2003). The enzyme, α-amylase, was chosen for use in the HMBRF digestion step because it was easy to obtain the purified product and it had a short digestion time. α-amylase is an endo-glucosidase that cleaves the α-1, 4-glucosidic bond of the substrate at internal positions to yield dextrins and oligosaccharides. The activity of α-amylase is increased by increasing its content and the digestion time. The optimum pH and temperature for activity varies depending on the enzyme source (Wong, 1995). Commercially, α-amylase, that is used in particular for starch liquefaction is most often obtained from *Bacillus licheniformis*, in the form of α-amylase, BAN® 480L (Wong, 1995).

The effect of various initial volumes of α-amylase in the range of 0.025-0.100 % v/w of HMBRF on TSS production was investigated. The initial volumes of α-amylase and digestion time affected TSS as shown in Table 1. Furthermore, an interaction effect on TSS between the initial volume of α-amylase and the digestion time was also found. TSS in treatments using initial volumes of α-amylase at 0.025 % v/w of HMBRF and 35 min digestion time, and at 0.05, 0.075 and 0.100% v/w of HMBRF and 30 and 35 min digestion time

were higher and significantly different ($p \leq 0.05$) from other treatments. However, treatment using initial volumes of α-amylase at 0.05 % v/w of HMBRF and 30 min digestion time had the highest TSS with the shortest digestion time. Thus, an initial volume of α-amylase at 0.05 % v/w of HMBRF was selected for confirming digestion time.

Effect of digestion time on quality properties of extract solutions

Results from Table 2 showed that treatment using initial volumes of α-amylase at 0.05 % v/w of HMBRF and 30 min digestion time had the highest TSS and RS with a significant difference ($p \leq 0.05$) from other treatments. TSS and RS were significantly ($p \leq 0.05$) decreased and were constant after 35 min digestion time. These results confirmed results from a previous experiment that also showed 30 min was the most suitable digestion time for α-amylase at 0.05 % v/w of HMBRF. Color (L*, a*, b*) and TS of extracted solution using 0.05 % v/w of HMBRF and 30 min digestion time were highest and significantly different ($p \leq 0.05$), whereas pH was not significantly different ($p > 0.05$) in all treatments. Therefore, the optimum conditions for the HMBRF extraction process involved using α-amylase at 0.05 % v/w of HMBRF and digesting for 30 min in a water bath at 75°C.

Effect of HMBRF extract on growth of *Aspergillus niger*

Growth of *A. niger*, as measured by cell dry weight is shown in Table 3. The results showed that *A. niger* could grow in all treatments (1-10° Brix, with/without 2% dextrose). The cell dry weight of *A. niger* in extracted solutions increased when the concentration of the extracted solution

Table 1 Total soluble solids (TSS) of HMBRF extracted solutions.

Initial volume of enzyme (% v/w)	Digestion time (min)				
	15	20	25	30	35
0.025	B9.5±0.38e	C11.8±0.19d	B12.5±0.06c	B14.3±0.03b	B15.8±0.12a
0.050	B9.5±0.21c	B12.4±0.07b	B12.5±0.16b	A16.4±0.47a	A16.4±0.10a
0.075	B9.5±0.32d	B12.5±0.13c	A14.5±0.04b	A16.2±0.07a	A16.4±0.16a
0.100	A13.2±0.11c	A14.5±0.05b	A14.5±0.08b	A16.4±0.13a	A16.4±0.02a

Note: a-e = Mean ± standard deviation from three replications. Means values within a row followed by a different lower case letter were significantly different ($p \leq 0.05$).
A-C = Mean ± standard deviation from three replications. Means values within a column followed by the different upper case letters were significantly different ($p \leq 0.05$).

Table 2 The properties of HMBRF extract using 0.05 % v/w α-amylase at various digestion times.

Time (min)	Color			TSS(°Brix)	RS(%)	TS(%)	pH ^{ns}
	L*	a*	b*				
15	45.64d	1.23d	6.56d	9.6±0.00e	4.83±0.07d	10.99±0.26d	5.82
20	50.44c	1.33c	7.46c	13.2±0.00d	5.33±0.28c	14.50±0.30c	5.82
25	58.12b	1.43b	8.12b	14.5±0.00c	5.76±0.09bc	15.80±0.36c	5.82
30	61.44a	1.83a	8.48a	16.8±0.00a	6.78±0.04a	17.58±0.15a	5.85
35	59.14b	1.58b	8.02b	15.6±0.00b	5.93±0.09bc	16.63±0.47b	5.85
40	57.12b	1.48b	8.28b	15.2±0.00b	5.77±0.07bc	16.28±0.60b	5.85
45	58.42b	1.46b	8.24b	15.2±0.00b	5.81±0.01bc	16.43±0.80b	5.85

Note $^{a-d}$ = Mean ± standard deviation from three replications. Means values within a column followed by a different lower case letter were significantly different ($p \leq 0.05$).
^{ns} = Non significant difference ($p > 0.05$).

Table 3 Cell dry weight of *Aspergillus niger* using HMBRF extract as culture medium.

TSS (°Brix)	Cell dry weight (g/L)	
	Without 2% dextrose	With 2% dextrose
Control	7.22 ± 0.65 e	7.12 ± 0.45 f
1	1.58 ± 0.07 j	1.61 ± 0.07 j
2	3.00 ± 0.18 i	3.02 ± 0.15 i
3	4.16 ± 0.05 h	4.22 ± 0.26 h
4	5.51 ± 0.34 g	5.17 ± 0.16 g
5	6.24 ± 0.27 f	6.96 ± 0.39 f
6	7.56 ± 0.43 e	13.06 ± 0.42 e
7	8.38 ± 0.52 d	18.24 ± 0.41 d
8	10.02 ± 0.72 c	20.67 ± 0.73 c
9	10.80 ± 0.71 b	24.11 ± 0.58 b
10	12.36 ± 0.70 a	33.90 ± 0.69 a

Note $^{a\text{-}j}$ = Mean ± standard deviation from three replications. Means values within a column followed by a different lower case letter were significantly different ($p \leq 0.05$).
Control = PDB, commercial.

increased. However, there was no significantly different growth ($p>0.05$) in extracted solution at 6°Brix (without 2% dextrose) and 5°Brix (with 2% dextrose), when compared with the control medium (PDB, commercial). This indicated that there was no need to add 2% dextrose in the 6° Brix HMBRF extract solution to promote *A. niger* growth because there was enough sugar in the end products of the α-amylase digested starch and flour (Pandey *et al.*, 2000).

Furthermore, in order to prepare the same amount of medium (1 L), the cost using HMBRF extract at 6°Brix (liquid medium) was 25.25 baht / L, which was lower than the cost of the commercial ready-to-use medium (PDB), which was more than 200 baht/L (Difco).

CONCLUSION

The high content of carbohydrate and protein in HMBRF had the potential to grow *A. niger*. The optimum conditions for extracted HMBRF were mixing HMBRF with distilled water (1:3.7), adding α-amylase at 0.05 % v/w of HMBRF, digesting for 30 min in a water bath at 75°C, cooling down rapidly in an ice bath, boiling for 10 min and separating the supernatant by centrifuging at 5,000 rpm for 30 min. The HMBRF extract contained total soluble solids with 16.80° Brix and 6.78 % reducing sugar. The growth of *A. niger* in HMBRF extract at 6°Brix was the same as in the control medium (PDB, commercial). Furthermore, there was high correlation between TSS in the HMBRF extract and the growth of *A. niger*. Thus, HMBRF digested by α-amylase potentially provided enough nutrients for fungal growth. However, the culture medium from HMBRF extract should be tested with other fungi to assess its efficiency.

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