Agriculture and Natural Resources 50 (2016) 227–231

Contents lists available at ScienceDirect

Agriculture and Natural Resources

journal homepage: http://www.journals.elsevier.com/agriculture-and-natural-resources/

Original Article

Prebiotic synthesis from rice using *Aspergillus oryzae* with solid state fermentation

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ARTICLE INFO

Article history:
Received 1 May 2015
Accepted 23 February 2016
Available online 8 October 2016

Keywords:
Aspergillus oryzae
Pathogenic inhibition
Prebiotic
Probiotic
Solid state fermentation

ABSTRACT

Prebiotic synthesis from economical substrates (glutinous rice and rice bran) using *Aspergillus oryzae* TISTR 3102 and TISTR 3222 with solid state fermentation (SSF) was explored. The pH value, alpha-glucosidase activity and total reducing sugar (TRS) concentration were monitored during incubation with different substrate ratios of 1:1 and 1:2 weight per weight (w/w). After 7 d incubation under SSF in glutinous rice and rice bran at 1:2 w/w, the highest alpha-glucosidase activity (4.49 Unit/mL) and TRS concentration (16.84 g/L) were detected. The product mixtures consisted of several reducing sugars as prebiotic compounds, especially panose and isomaltotriose. The prebiotic properties were investigated for their growth stimulation on two probiotic strains (*Lactobacillus plantarum* and *Lactobacillus acidophilus*) and the inhibition of two pathogens (*Escherichia coli* and *Salmonella paratyphi*). These product mixtures under solid state fermentation (SSF) using *A. oryzae* in variable rice ratios displayed high efficiency with regard to prebiotic properties and were deemed appropriate for prebiotic production.

Introduction

There are many references of oligosaccharides with prebiotic properties (Barreteau et al., 2006; Swennen et al., 2006; Sangwan et al., 2011). The first common prebiotic property is stimulation of the probiotic strains *Lactobacillus* spp. and *Bifidobacterium* spp. (Su et al., 2007; Nazzaro et al., 2012; Olson and Aryana, 2012; Vitali et al., 2012). The most important prebiotic property is high efficiency in inhibiting intestinal pathogens such as *Escherichia coli*, *Salmonella* spp. and *Clostridium* spp. (Murry et al., 2004; de Moreno et al., 2010). Moreover, a prebiotic shows additional beneficial health effects in the host, such as the prevention of colon cancer (Reddy, 1998), mineral absorption improvement (Scholz-Ahrens et al., 2007), and regulation of lipid metabolism (Guo et al., 2012).

Solid state fermentation (SSF) is of considerable interest for prebiotic oligosaccharide production using the process of fungal fermentation and an economical substrate such as starch (rice bran, glutinous rice, wheat bran), sugar (maltose, sucrose and fructose) and corn flour (Mazutti et al., 2006; Ram and Rupinder, 2010). There are several prebiotics produced by fungal SSF using agroindustrial residues in order to add to their value (Suparp and Premsuda, 2010; Vimala et al., 2011).

The genus *Aspergillus* includes a large number of filamentous fungal species, most of which are very important for industrial applications because of their ability to produce amylolytic enzyme for degradation of plant cell wall polysaccharides (de Vries and Visser, 2001). Moreover, their products have obtained a GRAS (Generally Regarded As Safe) status. *Aspergillus* spp., in particular, is increasingly used for starch solid state fermentation in order to synthesize prebiotic oligosaccharides (Chandra et al., 2010; Saeid et al., 2011; Vera et al., 2012). Alpha-glucosidase is one of the amylolytic enzymes of interest produced by *A.* spp. This enzyme can catalyze many glucosides and oligosaccharides at the nonreducing end of the glucose structure and transfers glucosyl donors via transglucosylation activity in order to produce specific oligosaccharides that have potential prebiotic properties (Duan et al., 1994; Kato et al., 2002; Fernández-Arrojo et al., 2007).

The current study demonstrated the synthesis of prebiotic compounds using *Aspergillus oryzae* TISTR 3102 and TISTR 3222 with SSF and variable rice sources (glutinous rice and rice bran). The aim of this work was to observe the prebiotic synthesis efficiency from different microorganism strains and substrate ratios. The pH value, alpha-glucosidase activity, TRS concentration and components of the product mixtures during SSF were analyzed.

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Peer review under responsibility of Kasetsart University.

http://dx.doi.org/10.1016/j.anres.2016.02.004

Selected product mixtures were investigated for prebiotic properties by probiotic growth stimulation and pathogenic inhibition.

Materials and methods

A. oryzae TISTR 3102, TISTR 3222 and probiotic strains (*Lactobacillus plantarum* TISTR 1465 and *Lactobacillus acidophilus* TISTR 1338) were kindly provided from the Thailand Institute of Scientific and Technological Research. The intestinal pathogens (*E. coli* and *Salmonella paratyphi*) were obtained from the Department of Biology, Faculty of Science, Ramkhamhaeng University, Thailand. Glutinous rice and rice bran were purchased from the Department of Agricultural Technology, Faculty of Science, Ramkhamhaeng University and cleaned and prepared via an aseptic technique. All other chemicals used were analytical grade from the Sigma–Aldrich Company.

Solid state fermentation

The reaction mixtures containing glutinous rice and rice bran at 1:1 and 1:2 weight per weight (w/w) ratios, with 40% (w/v) moisture, were inoculated with spore suspensions of approximately 1×10^7 spore/mL of *A. oryzae* TISTR 3102 and TISTR 3222. After incubation at 37 °C for 7 d, 1 g of product mixture was collected every day for 7 d. The pH value, alpha-glucosidase activity and TRS concentration were determined.

Alpha-glucosidase activity determination

One gram of the product mixture was extracted by diluting with 5 mL distilled water and incubated at 150 revolutions per minute (rpm) and 37 °C for 1 h. The suspension was collected after further centrifugation at 8000 rpm at room temperature for 20 min. Alpha-glucosidase activity was determined using a modified method of McCue and Shetty (2003). The reaction mixture consisted of 0.1 mL of 9 mM p-nitrophenol alpha-D-glucopyranoside and 0.8 mL of 200 mM sodium acetate buffer at pH 4.6. The reaction mixture was incubated at 50 °C for 30 min and stopped. A sample of 1 mL of 100 mM sodium carbonate was added and the mixture was centrifuged at 13,500 rpm and room temperature for 5 min. The released p-nitrophenol was monitored spectrophotometrically at 400 nm and compared with a blank. A standard curve was established using p-nitrophenol dissolved in sodium acetate buffer. One unit of alpha-glucosidase activity corresponded to the release of 1 μmol of p-nitrophenol per min at pH 4.6 and 50 °C under assay conditions (Premsuda et al., 2012).

Total reducing sugar assay

The reaction mixture was determined for total reducing sugar using dinitrosalicylic acid (DNS) assay following the method of Miller (1959). After SSF, the product mixture was centrifuged at 13,500 rpm and room temperature for 5 min. Supernatant aliquot of 1 mL was mixed with 1 mL DNS reagent and incubated at 100 °C for 5 min. After cooling to room temperature, the reducing sugar was measured by monitoring absorbance at 540 nm. A standard and blank were prepared in the same way as the analyzed sample except for the addition of 1 mL standard solution and 1 mL buffer instead of the sample, respectively.

Thin-layer chromatography

Thin-layer chromatography (TLC) analysis was performed to compare the product mixtures from the SSF of *A. oryzae* TISTR 3102 and TISTR 3222 with glutinous rice and rice bran at the different ratios described above. After taking samples at appropriate times, the product mixture was centrifuged at 5000 rpm and room temperature for 15 min. The supernatant was dissolved with distilled water before being dotted on a silica TLC plate (6 μL of each supernatant). Glucose, maltose, isomaltose, maltotriose, panose and isomaltotriose were used as standard compounds. Using the mobile phase of n-propanol, with ethyl acetate and distilled water, a mixture in the ratio of 14:2:7 by volume was made. Each TLC plate was dried using incubation at 110 °C for 10 min. The product mixtures detected via visible spots were examined.

Probiotic growth stimulation

L. plantarum and *L. acidophilus* were grown at 37 °C for 48 h in an anaerobic chamber under anaerobic conditions in a culture medium of deMan, Rogosa and Sharpe (MRS) broth (used as the control) compared with the culture medium supplemented with 1 g/L of the selected product mixture from SSF. After incubation, the cultures were monitored by measuring the optical cell density at 620 nm using a spectrophotometer (double beam UV–Visible T110+, PG Instruments, Lutterworth, UK) at 620 nm (Siragusa et al., 2009).

Prebiotic effect on the inhibition of bacterial pathogens

Anaerobic cultivations of *L. plantarum* and *L. acidophilus* were performed in the anaerobic chamber at 37 °C for 48 h in MRS broth complemented with 1 g/L of product mixture from previous SSF. The product mixtures were centrifuged at 8000 rpm and 4 °C for 15 min and the supernatant was separately collected. Pathogenic strains (*E. coli* and *S. paratyphi*) were cultivated in nutrient broth at 37 °C for 24 h. An aliquot of pathogenic cultivation (50 μL) was inoculated on nutrient agar (NA) using the spread plate technique (Rousseau et al., 2005) and dried on an NA plate. Sterilized filter paper disks dipped in probiotic supernatant were placed on the pathogenic plate. After incubation at 37 °C for 24 h, the inhibition efficiency was illustrated based on the clear zone diameter and compared with the clear zone from the probiotic supernatant when cultivated without the SSF product mixture in the culture media used as a control.

Results and discussion

Product mixtures from solid state fermentation determination

The product mixtures from SSF at the different ratios of glutinous rice and rice bran with *A. oryzae* TISTR 3102 and TISTR 3222 showed similar trends in pH values (between 4.35 and 6.41) during 7 d of incubation. Noticeably, all pH values for the product mixtures showed no significant difference in either strain until after 3 d of incubation. The product mixtures from *A. oryzae* TISTR 3102 slightly decreased compared with *A. oryzae* TISTR 3222 (Fig. 1A). The product mixtures under SSF with *A. oryzae* TISTR 3102 demonstrated more alpha-glucosidase activity and TRS concentration than *A. oryzae* TISTR 3222 (Fig. 1B and C). The highest alpha-glucosidase activity and TRS concentration, 4.49 Unit/mL and 16.84 g/L, respectively were obtained from *A. oryzae* TISTR 3102 at the ratio of 1:2 (w/w) after 7 d of incubation. In contrast, *A. oryzae* TISTR 3222 showed very low alpha-glucosidase activity and TRS concentration during SSF incubation at both ratios. This provided evidence that a low pH value in the product mixture during the SSF incubation might affect *A. oryzae* TISTR 3222 to display very low alpha-glucosidase activity and TRS concentration. However, providing glutinous rice and rice bran at different ratios to the same microorganism did not have any significant effects on the alpha-glucosidase activity and TRS concentration in the product mixture. Remarkably, the alpha-glucosidase activity and TRS

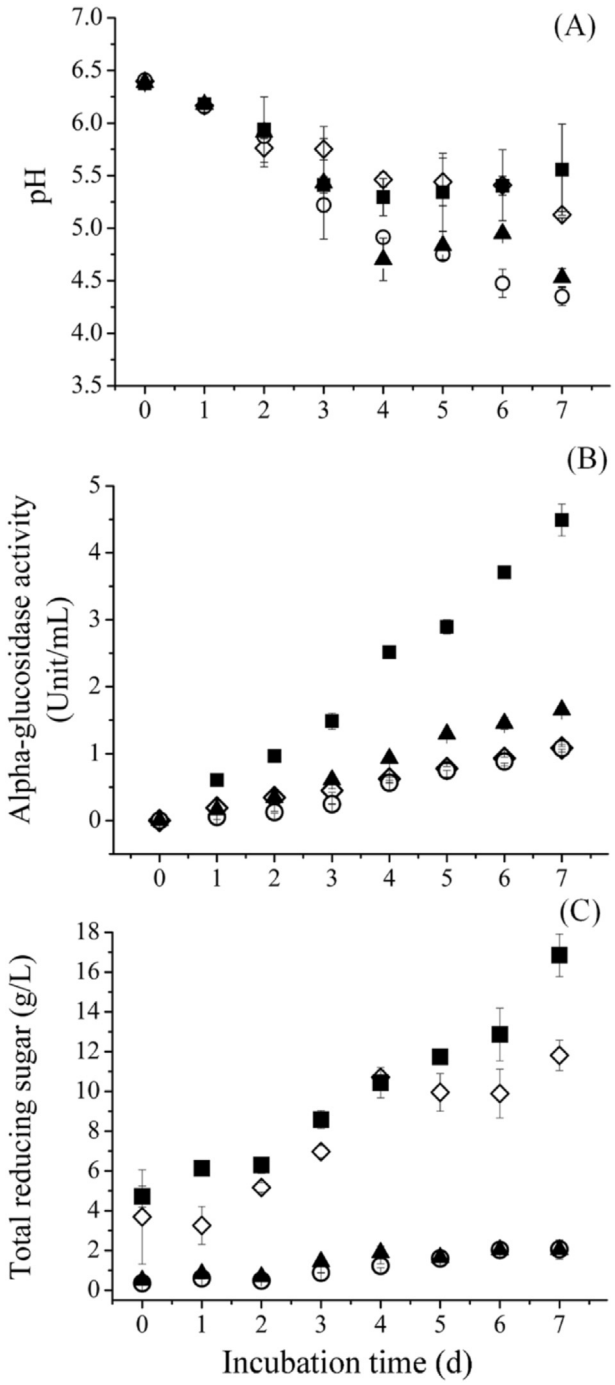

Fig. 1. pH value (A), alpha-glucosidase activity (B) and total reducing sugar concentration (C) during solid state fermentation (0–7 d) using *Aspergillus oryzae* TISTR 3102 and TISTR 3222 at different substrate ratios (weight per weight) of glutinous rice and rice bran. *A. oryzae* TISTR 3102 with 1:1 (◊), *A. oryzae* TISTR 3102 with 1:2 (■), *A. oryzae* TISTR 3222 with 1:1 (O) and *A. oryzae* TISTR 3222 with 1:2 (▲). Presented data are the average of triplicate analysis with error bars showing ± SD.

concentration significantly increased after 3 d incubation with both strains and substrate ratios, while the pH value decreased in the same incubation period.

Thin-layer chromatography analysis

As shown in the TLC results, the product mixtures from SSF with *A. oryzae* TISTR 3102 (Fig. 2) at different ratios (1:1 and 1:2 w/w) during 0–7 d incubation showed clear bands. These bands consisted of many reducing sugars compared to the standard compounds in band A (1:glucose, 2:maltose, 3:isomaltose, 4:maltotriose, 5:panose and 6:isomaltotriose), especially, panose and isomaltotriose which act as prebiotic compounds (Premsuda et al., 2008). In contrast, the product mixtures from *A. oryzae* TISTR 3222 displayed very low alpha glucosidase activity and TRS concentration as represented by the vague TLC bands (Fig. 3). Undoubtedly, the high activity of alpha-glucosidase during SSF incubation could synthesize more prebiotic compound in the product mixture.

Probiotic growth stimulation

The product mixture from SSF using *A. oryzae* TISTR 3102 and TISTR 3222 with different ratios of glutinous rice and rice bran—1:1 and 1:2 (w/w)—at 7 d incubation were selected to cultivate probiotic strains (*L. plantarum* and *L. acidophilus*) in MRS broth. After incubation at 37 °C for 48 h under anaerobic conditions, the probiotic growth was observed by monitoring the optical cell density in the spectrophometer at 620 nm. All the product mixtures from SSF showed higher cell density compared with the control sample (Table 1). Specifically for W2, the product mixture under SSF with *A. oryzae* TISTR 3102 at 1:2 (w/w) showed significantly higher optical density compared to the control in both probiotic cultures. This may provide evidence that prebiotics from the product mixture could stimulate probiotic growth. This could be further be explained by *Leuconostoc mesenteroides* fermentation which restricted the polymer size by the addition of maltose (Chung, 2002) and commercial prebiotics; fructooligosaccharide, galactooligosaccharide and inulin stimulating the growth of probiotic strains; *Lactobacilii* and *Bifidobacteria* (Huebner et al., 2007).

Pathogenic inhibition

The inhibition of intestinal bacterial pathogens (*E. coli* and *S. paratyphi*) was illustrated by the diameter of inhibition zone (Table 2). All product mixtures from SSF at the different substrate ratios using the two probiotic culture strains, *L. plantarum* and *L. acidophilus*, showed significantly higher pathogen inhibition than the MRS medium without the product mixture from SSF (control).

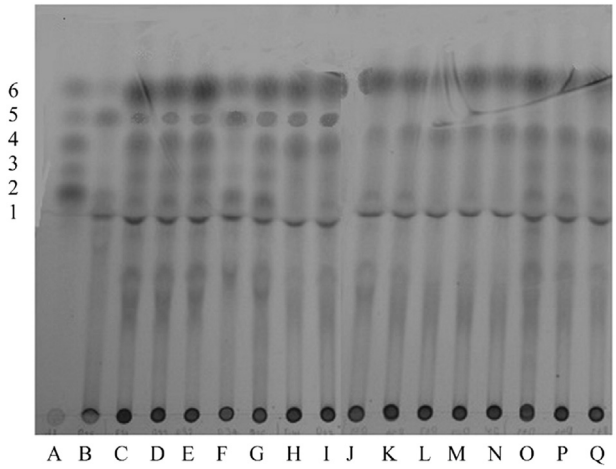

Fig. 2. Thin-layer chromatography analysis of solid state fermentation (SSF) using *Aspergillus oryzae* TISTR 3102 at different ratios of glutinous rice and rice bran during 0–7 d SSF incubation, all bands shown: A, standard compounds (1, glucose, 2, maltose, 3, isomaltose, 4, maltotriose, 5, panose and 6, isomaltotriose); B–I, ratio of 1:1 (weight per weight; w/w); and J–Q, ratio of 1:2 (w/w).

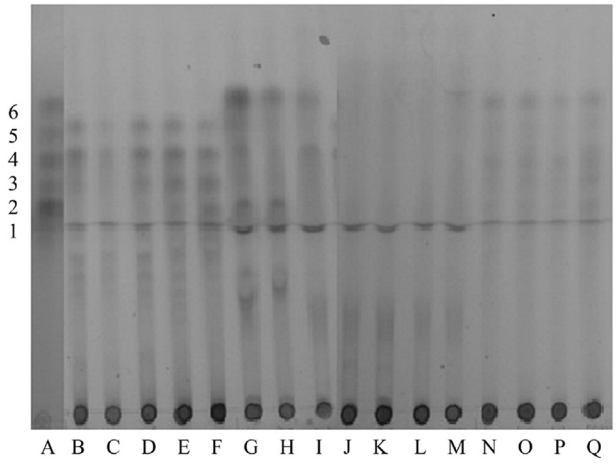

Fig. 3. Thin-layer chromatography analysis of solid state fermentation (SSF) using *Aspergillus oryzae* TISTR 3222 at the different ratios of glutinous rice and rice bran during 0–7 d SSF incubation, all bands shown: A; standard compounds (1, glucose, 2, maltose, 3, isomaltose, 4, maltotriose, 5, panose and 6, isomaltotriose); B–I, ratio of 1:1 (weight per weight; w/w) and J–Q, ratio of 1:2 (w/w).

Table 1
Optical cell density at 620 nm (OD620 nm) of probiotic strains (*Lactobacillus plantarum* and *Lactobacillus acidophilus*) incubated at 37 °C for 48 h under anaerobic conditions in media culture with 1 g/L of supernatant from solid state fermentation after 7 d at the different ratios of glutinous rice and rice bran (weight per weight) using: *Aspergillus oryzae* TISTR 3102 at 1:1 (W1); *A. oryzae* TISTR 3102 at 1:2 (W2); *A. oryzae* TISTR 3222 at 1:1 (W3); and *A. oryzae* TISTR 3222 at 1:2 (W4). Data represented as average ± SD with triplicate analysis.

Sample	OD_{620} nm	
	L. plantarum	*L. acidophilus*
deMan, Rogosa and Sharpe broth (MRS; control)	1.40 ± 0.02	1.35 ± 0.03
MRS + W1	1.50 ± 0.02	1.51 ± 0.01
MRS + W2	1.56 ± 0.01	1.52 ± 0.02
MRS + W3	1.54 ± 0.01	1.43 ± 0.03
MRS + W4	1.55 ± 0.02	1.40 ± 0.02

Interestingly, even at low pH values from SSF with *A. oryzae* TISTR 3222 after 7 d incubation in both substrate ratios (W3 and W4), a wider clear zone was not displayed compared to *A. oryzae* TISTR 3102 (W1 and W2). The inhibitory activity could be explained by antimicrobial compounds (bacteriocin and peroxide for example) and not merely lactic acid which is produced during fermentation (Kormin et al., 2001; Nilsang, 2010). These compounds influence pathogen adhesion in human intestinal mucus (Collado et al., 2006). Moreover, complementing media culture with prebiotic stimulates the probiotic growth and higher production of antimicrobial compounds. Since replacing chemicals with biological products has received more attention, probiotics and prebiotics have a promising future, especially as functional foods and for application in the pharmaceutical industry.

In conclusion, the synthesis of alpha-glucosidase and prebiotic compounds using SSF with *A. oryzae* TISTR 3102 and TISTR 3222 at different substrate ratios (1:1 and 1:2 w/w) of glutinous rice and rice bran, was analyzed. SSF using *A. oryzae* TISTR 3102 with a substrate ratio at 1:2 (w/w) for 7 d incubation was found to produce the maximum alpha-glucosidase activity (4.49 Unit/mL) and TRS concentration (16.84 g/L). The product mixtures from SSF incubation were confirmed to be prebiotic compounds (panose and isomalto oligosaccharides) from the TLC bands. The product mixtures from SSF using *A. oryzae* TISTR 3102 and TISTR 3222 at the different

Table 2
Diameter of clear zone from probiotic strains (*Lactobacillus plantarum* and *Lactobacillus acidophilus*) inhibited pathogens (*Escherichia coli* and *Salmonella paratyphi*) when cultured with 1 g/L of supernatant from solid state fermentation after 7 d at different ratios of glutinous rice and rice bran (weight per weight) with: *Aspergillus oryzae* TISTR 3102 at 1:1 (W1); *A. oryzae* TISTR 3102 at 1:2 (W2); *A. oryzae* TISTR 3222 at 1:1 (W3); and *A. oryzae* TISTR 3222 at 1:2 (W4). Data presented as average ± SD with triplicate analysis.

Sample	Clear zone diameter (cm)	
	E. coli	*S. paratyphi*
L. plantarum		
deMan, Rogosa and Sharpe MRS (MRS; control)	0.68 ± 0.08	0.85 ± 0.05
MRS + W1	0.92 ± 0.08	1.05 ± 0.13
MRS + W2	1.03 ± 0.08	1.22 ± 0.10
MRS + W3	0.73 ± 0.10	0.83 ± 0.13
MRS + W4	0.78 ± 0.08	0.88 ± 0.15
L. acidophilus		
MRS (control)	0.78 ± 0.03	0.90 ± 0.05
MRS + W1	0.85 ± 0.05	1.15 ± 0.15
MRS + W2	0.90 ± 0.10	1.18 ± 0.10
MRS + W3	0.73 ± 0.08	0.95 ± 0.10
MRS + W4	0.78 ± 0.08	1.05 ± 0.15

substrates ratios, after 7 d incubation were selected to cultivate probiotic strains (*L. plantarum* and *L. acidophilus*) and for determination of pathogenic inhibition (*E. coli* and *S. paratyphi*). The results showed that supplements of product mixtures from SSF using *Aspergillus oryze* TISTR 3102 with both substrate ratios in the media cultures would stimulate probiotic growth and inhibit pathogens compared to the control media. With this knowledge, the bioavailability of variable rice sources under SSF using *A. oryzae* may also be possible. Furthermore, using economical substrates and agroindustry residues not only adds to probiotic value, but also provides potential for future research in prebiotic production.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgements

The authors would like to thank Ms Wantana Wiraporn, Ms Kanyanee Pimairam and Ms Wanwipa Wansanit (Department of Biotechnology, Ramkhamhaeng University) and Ms Jutamad Sukcharoen (Thailand Institute of Scientific and Technological Research) for their experimental assistance. Financial support from Ramkhamhaeng University is gratefully acknowledged.

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