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Bioconversion of soybean residue for use as alternative nutrient source for ethanol fermentation

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ABSTRACT

Soybean residue (SR) was transformed, through solid-state fermentation by *Aspergillus oryzae* TISTR 3087 followed by hydrolysis, to a solution rich in free amino nitrogen (FAN), and used as the nitrogen source in an ethanol fermentation. Supplementing SR with waste cooked rice (CR) at a 75:25 mass ratio increased dramatically the production of protease compared to a fermentation with SR as the sole substrate (2822 U g^{-1} substrate against 33 U g^{-1}). Subsequent hydrolysis of the solid-state fermented solids produced as much as 1.8 g-FAN L^{-1}. The SR-derived hydrolysate was mixed with sugarcane molasses, to an initial sugar concentration of 110 g L^{-1}, and used for ethanol production by *Saccharomyces cerevisiae* TISTR 5339. The yeast produced 40.7 g ethanol L^{-1} with a yield of 0.42 g g-sugar^{-1} and a productivity of 0.62 g L^{-1} h^{-1}. This study demonstrates clearly that co-fermentation of SR and CR enhances protease production, and that SR is a feasible and low cost source of nitrogen for ethanol fermentation.

1. Introduction

Ethanol is an alternative fuel that has gained much attention as a gasoline additive because of its cleaner combustion compared to gasoline, and its renewability. Sugarcane molasses is a widely used substrate for fermentative ethanol production, due to its ready availability at a low cost and its readiness for bioconversion [1–3]. However, despite its high sugar content and small amounts of other elements, molasses has a low nitrogen content [2]; for this reason, additional nutrients, such as yeast extract and ammonium sulfate, are often included in molasses-based media [2,4–8]. Organic nitrogen sources like yeast extract give better results than inorganic ones [8], but they are usually expensive [9]. So, finding an alternative nitrogen source that is inexpensive and readily available is crucial for economic ethanol production.

Soybean residue (SR), also known as okara, is a protein-rich waste from the soy milk and tofu production processes. Its protein content ranges from 25% to 35% [10–12]. For every cubic meter of soy milk that is produced, about 250 kg of fresh soybean residue are obtained, with around 14 million tonnes of the residue being generated annually [13], posing a significant disposal problem. With a view to avoiding this problem, studies have investigated the use of SR as food, as a source for recovery of protein, oil and bioactive compounds (isoflavones), as animal feed and as a medium for mushroom cultivation [13–15]. However, based on its high protein content, SR could be hydrolyzed to yield a solution rich in amino acids and peptides that would be suitable as an alternative nitrogen source for fermentation processes. Despite this, information on the use of SR hydrolysates in ethanol production is scarce. Most studies have focused on utilizing its polysaccharide, not its protein [13,16]. The current study focused on the use of SR as a nitrogen source, with waste cooked rice (CR), which is carbon-rich, being added to the SR to improve the carbon to nitrogen (C:N) ratio of the mixed substrate for solid-state fermentations.

A sequential process of solid-state fermentation (SSF) followed by hydrolysis of the fermented solids has successfully been used with various substrates to produce hydrolysates rich in nutrients for microbial growth and product formation. Examples of products produced from such hydrolysates are succinic acid, lipids, polyhydroxybutyrate (PHB), and hydrogen [17–21]. The process involves the fermentative production of necessary enzymes for hydrolyzing

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macromolecules, *e.g.* amylase for starch, and protease for proteins, in the solid substrate. The fermented solids thus obtained are further hydrolyzed at temperatures ranging from 40 °C to 55 °C [22,23], allowing these enzymes to hydrolyze the residual substrate. Depending on the solid substrate and enzymes produced during the fermentation, the major components in the hydrolysate are fermentable sugars or assimilable nitrogenous substances. Fungal autolysis also occurs during the further hydrolysis process. This results in the release of microbial nutrients, for instance amino acids, peptides, nucleotides, phosphorus, vitamins and various trace elements, into the surrounding liquid [17]. This allows a reduction in the amount of nutrients that need to be added when the hydrolysate is used as a component of media in subsequent submerged fermentations.

In this study, SR was used as a main substrate for the production of protease by *Aspergillus oryzae* TISTR 3087 under SSF. The fermented solids were subsequently hydrolyzed to produce a solution rich in free amino nitrogen (FAN), which was then mixed with sugarcane molasses and used to produce ethanol by *Saccharomyces cerevisiae* TISTR 5339. The results obtained from this study demonstrate that it is feasible to use SR as an alternative nitrogen source for ethanol fermentation. Bioconversion processes of SR to ethanol, with mass balances, are also proposed in this study.

2. Material and methods

2.1. Microorganisms and culture condition

SSF was carried out with *Aspergillus oryzae* TISTR 3087 and ethanol production with *Saccharomyces cerevisiae* TISTR 5339. Both strains were purchased from the Thailand Institute of Scientific and Technological Research (TISTR), Pathum Thani. *A. oryzae* was grown on potato dextrose agar (PDA) at 30 °C for 3–4 days. Then, the spores were scraped and suspended in sterile distilled water and stored at −20 °C. *S. cerevisiae* was cultured in yeast malt extract (YM) broth at 30 °C until entering mid-exponential phase. The yeast was then harvested by centrifugation and re-suspended in the YM broth containing 30% (v/v) glycerol and stored at −20 °C. *S. cerevisiae* inoculum was prepared from the thawed stock culture by growing the yeast on YM agar. Then, a colony was subcultured in 100 mL of the YM broth at 30 °C for 48 h. The culture (10% v/v) was subcultured once in the YM broth and incubated at 30 °C for 18 h before use.

2.2. Substrates

Soybean residue (SR) was kindly provided by soymilk vendors around Khon Kaen University (KKU), Khon Kaen, Thailand. Waste cooked rice (CR) was collected from cooked-to-order restaurants around KKU. Contaminants in the CR (bones, vegetables, prawn shells) were removed by hand. The substrates were washed with tap water twice, sun dried, and dried in a hot air oven at 60 °C until their moisture contents decreased to below 10% (w/w). The dry substrates were ground using a pinned-disc mill, and then sieved to obtain particles of 0.5–1 mm. The ground substrates were packed in plastic bags and stored at room temperature in air-tight plastic containers to avoid changes in physical and chemical characteristics. Preliminary determination of reducing sugar (RS) and free amino nitrogen (FAN) concentrations revealed that CR contained 1.1 mg-RS g^{-1} and 0.3 mg-FAN g^{-1}, and SR contained 18.5 mg-RS g^{-1} and 0.4 mg-FAN g^{-1}. The sugarcane molasses was of a commercial grade, normally used as a plant fertilizer. It contained approximately 871 g-total-sugar L^{-1} and 0.27 g-FAN L^{-1}.

2.3. Solid-state fermentation for the production of protease

Although SR is rich in carbohydrate (45–53% on a dry basis, db) [13,14,24], it also contains lignin [13,14]. The production of sugar from SR is therefore difficult and thus the SR requires pretreatment to expose its polysaccharides. Also, due to the low saccharification yield, possibly as a result of lignin inhibiting enzymatic hydrolysis [25], it may be necessary to have an additional step to concentrate the sugar in the hydrolysate to reach a suitable level for fermentation [13]. For these reasons, in this study, SR was used as the source of nitrogen rather than as the carbon source, so as to reduce the complication in substrate preparation. The mixing ratio between SR and CR was firstly studied in order to ensure a suitable balance for maximizing protease production. To this aim, SR and CR were autoclaved separately at 121 °C for 15 min and mixed at different ratios, *i.e.* 100:0, 75:25, 50:50, 25:75, and 0:100, before the initial moisture contents were adjusted to 60% (w/w) on a wet basis (wb) with sterile distilled water. After being inoculated with 5.5×10^5 *A. oryzae* spores·g-substrate^{-1}, lots containing around 10 g (wb) of the inoculated mixture were transferred to Petri dishes and incubated at 30 °C for 3 days. Samples were taken periodically by taking the whole content of each dish. The fermented solids were stirred using a glass rod to increase the homogeneity before 3 g (wb) of this were used for moisture content determination. Five grams (wb) of the solids were suspended in 25–30 mL distilled water using a vortex mixer and then centrifuged at $2000 \times g$ for 15 min to separate the liquid portion from the solids. All the experiments were conducted in triplicate. Average values and standard deviations (SD) of the mean are reported. The liquid samples were stored at −20 °C for analyses of protease and amylase activities. The ratio that gave highest protease activities was selected for the subsequent hydrolysis experiment. A control experiment (the substrates with no fungal inoculation) was also carried out in parallel.

Growth of the fungus was monitored by conducting SSF at different ratios of SR and CR, as mentioned above. Experimental and uninoculated control sets were carried out simultaneously in triplicate. The weights of the substrates were measured every 6 h using a 4-decimal-place balance and were used to calculate the weight reduction ratio (WRR), as described by Wang et al. [22].

2.4. Further hydrolysis of fermented solids for hydrolysate production

Using the chosen SR:CR ratio, after the termination of the SSF, the fermented solids were further hydrolyzed by homogenizing them in sterile distilled water using a kitchen blender (pre-sanitized using 70% ethanol) followed by incubating at 50 °C for 72 h at uncontrolled pH. The initial solids concentration was varied from 10 to 100 g/L (db) with a total volume of 500 mL. Liquid samples (5 mL) were taken at 12-h intervals. They were centrifuged at $2000 \times g$ for 15 min. The supernatant was collected and stored at −20 °C for determination of RS and FAN. After the hydrolysis, the mixture was filtered through Whatman No. 1 filter paper and the filtrate was centrifuged at $22{,}000 \times g$ for 15 min. The clear supernatant was transferred into screw-capped bottles and stored at −20 °C for use in the ethanol fermentation. All experiments were done in duplicate and the average values are reported. The initial solids concentration that gave the highest FAN concentration was used to produce hydrolysate for the subsequent ethanol fermentation.

2.5. Bioethanol fermentation

A required volume of the hydrolysate was mixed with sugarcane molasses to make up a total volume of 1 L with an initial sugar concentration of 110 g L^{-1}. After transferring the hydrolysate