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ARTICLE

Simultaneous Production of Trehalose, Bioethanol, and High-Protein Product from Rice by an Enzymatic Process

Shu-Wei Chang,[§] Wei-Hsin Chang,[†] Maw-Rong Lee,[#] Tzung-Jie Yang,[#] Nu-Yi Yu,[†] Chin-Shuh Chen,*[,†] and Jei-Fu Shaw*[,†]

[†]Department of Food Science and Biotechnology, National Chung Hsing University, Taichung 402, Taiwan, [§]Department of Medicinal Botanicals and Health Care, Dayeh University, Dacun, Changhua 51591, Taiwan, and [#]Department of Chemistry, National Chung Hsing University, Taichung 402, Taiwan

Rice is a starch-rich raw material that can be used for trehalose production. It can be hydrolyzed with α-amylase, β-amylase, and pullulanase to produce high-maltose content of rice saccharified solution for bioconversion of maltose into trehalose by trehalose synthase (TSase). For this purpose, an efficient enzymatic procedure has been successfully developed to simultaneously produce value-added trehalose, bioethanol, and high-protein product from rice as substrate. The highest maltose yield produced from the liquefied rice starch hydrolysate was 82.4 ± 2.8% at 50 °C and pH 5.0 for 21−22 h. The trehalose conversion rate can reach at least 50% at 50 °C and pH 5.0 for 20−24 h by a novel thermostable recombinant *Picrophilus torridus* trehalose synthase (PTTS). All residual sugar, except trehalose, can be fully hydrolyzed by glucoamylase into glucose for further bioethanol production. The insoluble byproduct containing high yields of protein (75.99%) and dietary fiber (14.01%) can be processed as breakfast cereal product, health food, animal forage, etc. The conversion yield of bioethanol was about 98% after 64 h of fermentation time by *Saccharomyces cerevisiae* without any artificial culture solution addition. Ethanol can easily be separated from trehalose by distillation with a high recovery yield and purity of crystalline trehalose of 92.5 ± 8.7% and 92.3%, respectively.

KEYWORDS: β-Amylase; bioethanol; crystallization; *Picrophilus torridus*; rice; trehalose; trehalose synthase; starch; sweet potato

INTRODUCTION

Rice is a major staple food with a wide cultivated area in many continents of the world (Asia, Africa, North America, and Middle East). Such agricultural produce is rich in protein and starch, which can be used to chemically or enzymatically produce high-value products including sugar, fermentation goods, pharmaceutical ingredients, and high-protein flour (*1*). Among these, a variety of sugars that can be prepared from starch have been used in the food and pharmaceutical industries. Examples include sugar, sugar alcohols, maltose, glucose, fructose, trehalose, maltodextrins, cyclodextrins, and syrups. Fermentation goods such as wine and vinegar also have great commercial value. High-protein products have high nutritional value and are useful for producing pudding, gruel, instant milk, and baby food.

Trehalose is a disaccharide that consists of two subunits of glucose bound by an α,α-1,1-linkage and is widespread in nature and biologically active (*2*). It is a stable, colorless, and odor-free disaccharide. Due to its inertness property and ability to stabilize biomolecules, trehalose can be applied in fields such as the food, cosmetic, and pharmaceutical industries. In the food industry, trehalose can be used in the preparation of food subjected to drying processes or concentration and in cosmetics as a moisturizer or liposome stabilizer. In medicine, trehalose can help to preserve enzymes and protect mammalian cells from damage during freeze-drying; it can also be used as an additive to stabilize vaccines during storage at room temperature and to protect organs for transplantation. Trehalose can also be used as a cryoprotectant in the cryopreservation of cells, sperms, tissues, or other materials. The wide range of applications of this sugar has stimulated research to develop novel and economically feasible production systems (*3*−*5*).

Traditionally, starch or starch-rich material can be used as a substrate for fermentable sugars conversion by microorganisms, whereas the conversion rate is slow and usually takes a long fermentation time. Thus, there exists a need to develop better processes for preparing high-value starch-derived products and high-protein products from low cost agricultural produce such as rice.

Recently, a general procedure for preparing syrups and fermentation products by treating starch or starch-rich materials with enzyme has been developed (*6*, *7*). For example, the starch can be hydrolyzed by α-amylases through liquefaction processes. The aim of liquefaction is to convert insoluble granular starch into soluble lower molecular weight oligosaccharide. Usually, a thermostable α-amylase is added and the starch slurry heated to

*Authors to whom correspondence should be addressed [(J.-F.S.) telephone +886-4-2284-0201, fax +886-4-2285-3813, e-mail boplshaw@gate.sinica.edu.tw; (C.-S.C.) telephone +886-4-2284-0385, ext. 3140; fax +886-4-2285-4189, e-mail cschen@mail.nchu.edu.tw].

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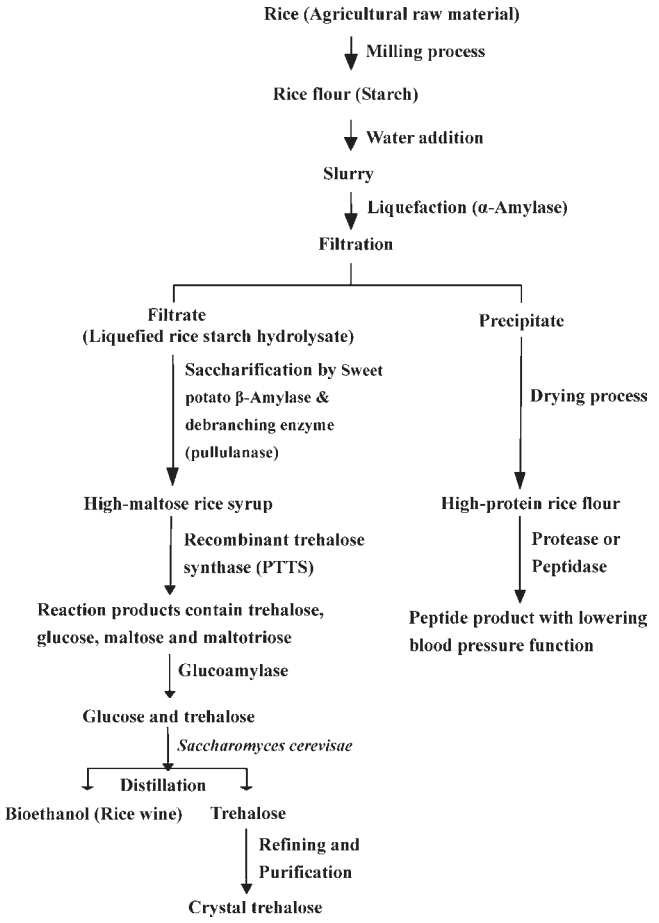

Figure 1. Schematics for the simultaneous production of bioethanol and purification of trehalose from reaction product mixture containing trehalose, maltose, glucose, and maltotriose by enzymatic method.

105−110 °C for 5−7 min, then cooled to 95 °C, and held for 1−2 h to complete the liquefaction process (*6*). The liquefied starch hydrolysate, which contains mostly oligosaccharides (mainly 8−12 glucose units), can be further converted into maltose syrup by β-amylase or glucose syrup by glucoamylase (*7*).

The high-maltose (glucosyl-α-1,4-glucopyranoside) syrup can be further converted into trehalose (glucosyl-α-1,1-glucopyranoside) by trehalose synthase (TSase) in a one-step enzymatic process (*2*, *8*). This enzymatic process has the advantages of simple reaction, high substrate specificity, high conversion yield, and low cost. We have cloned and purified a novel recombinant *Picrophilus torridus* TSase (PTTS), which can efficiently convert maltose (even at high maltose concentration) into trehalose at 45 °C with 60% yield (*9*).

In this study, we have developed a combinatorial biocatalysis process including α-amylase, β-amylase, pullulanase, and trehalose synthase to simultaneously produce high-value trehalose, bioethanol, and high-protein flour from rice (Tainung No. 67) produced in Taiwan (**Figure 1**). Without any chemicals in the process, we expect the efficient green process can be applied to other starch-rich agricultural produce, such as broken rice, sweet potato, cassava, or maize, to produce high-value industrial products and functional foods, which can increase the farmer's income and the nutritional value of low-value agricultural produces.

MATERIALS AND METHODS

Materials. All mono- and disaccharides were purchased from Sigma Chemical Co. α-Amylase (135 KNU/g) produced from *Bacillus licheniformis* and pullulanase (1350 NPUN/g) produced from *Bacillus subtilis* were purchased from Novozyme Co. Inc. (Denmark). Glucoamylase (400 ± 40 U/g) produced by *B. licheniformis* was purchased from Lyven Co. Inc. (France). Acetonitrile was from TEDIA Co. Inc. (USA). All other chemicals and reagents were of analytical grade.

Bacterial Strains and Plasmids. The expression vector pET-23a(+) (Novagen, Madison, WI) was used and transformed into the *Escherihcia coli* strains DH5α and Rosetta-gami B (DE3) (Novagen) for cloning and expression, respectively. *E. coli* strains were cultured in Luria−Bertani (LB) broth and on LB agar supplemented with 100 μg/mL ampicillin (LB-Amp) for cloning host or in combination with 15 μg/mL kanamycin, 12.5 μg/mL tetracycline, and 34 μg/mL chloramphenicol (LB-Amp-Kan-Tet-Chl) for expression host. All *E. coli* strains were cultured at 37 °C in an orbital shaker at a speed of 225 rpm unless otherwise mentioned.

β-Amylase Preparation from Sweet Potato. Fresh sweet potato (Tainung No. 10) was pressed and filtered by cheesecloth. The sweet potato juice was heated at 60 °C for 10 min to inactivate the α-glucosidase, which possesses maltose hydrolysis ability. The solution was then centrifuged at 8000 rpm for 20 min at 4 °C to obtain the supernatant, which contained abundant β-amylase. The enzyme solution was concentrated by ultrafiltration using a 100,000 molecular weight cutoff membrane (Amicrometer Ultra, Millipore, Bedford, MA) and pooled as a partially purified β-amylase preparation for the production of maltose.

Protein Quantification. Protein concentration was measured according to the method of Bradford using a protein assay kit purchased from Bio-Rad Laboratories (Hercules, CA) with bovine serum albumin (BSA) as standard. A standard curve was prepared according to the absorbance and BSA concentrations, and the concentration of unknown protein was calculated using this curve.

Analysis for Ash Content. All containers were washed and dried at 105 °C and cooled several times until the weight was constant. All samples were weighed and dried at 550−600 °C for 10 h until the weight was constant, indicating the sample incineration had been finished (*10*).

Crude Protein Analysis. All experiments were according to the Kjeldahl method (*10*), and the crude protein content (%) was calculated as total nitrogen × 5.95. The total nitrogen (%) was defined as

$$\text{total nitrogen (\%)} = [(b-a) \times 0.1 \times F \times 0.014 \times 100]/\text{weight of sample (g)}$$

where a = consumption volume of 1 N NaOH for sample (mL), b = consumption volume of 1 N NaOH for blank test (mL), and F = factor of 1 N NaOH.

Total Dietary Fiber Determination. All experiments were analyzed by Sigma total dietary fiber assay kit (Sigma TDF-100A). Fifty milliliters of 0.08 M phosphate buffer (pH 6.0) was well mixed with 1 g of sample and followed by the addition of 0.1 mL of heat-stable α-amylase solution and then shaking at 95 °C for 15 min. After the pH had been adjusted to 7.5, 100 μL of protease solution was added and reacted at 60 °C for 30 min. A 4-fold volume of 95% ethanol was added to precipitate completely for overnight. The precipitate was then washed three times by using 78% ethanol (20 mL/time), twice by using 95% ethanol (10 mL/time), and twice by using acetone (10 mL/time) to finish the filtration procedure. Finally, all samples were dried at 105 °C and weighed for further crude protein, ash, and total dietary fiber calculation. The formula of total dietary fiber (TDF) content (%) was

$$\text{TDF (\%)} = [R_{\text{sample}} - P_{\text{sample}} - A_{\text{sample}} - B]/\text{SW} \times 100$$

where $B = R_{\text{blank}} - P_{\text{blank}} - A_{\text{blank}}$, R = average weight of residue, P = average of protein content, and A = average of ash content.

Carbohydrate Analysis. The amounts of trehalose, glucose, and maltose after each enzymatic reaction were measured using a high-performance liquid chromatography (HPLC) (SFD 2100) system equipped with an RI detector (Schambeck SFD, RI 2000) at a flow rate of 0.9 mL/min. A carbohydrate analysis column (Hypersil-100 Amino, Thermo Hypersil-Keystone) equilibrated with 75% acetonitrile, 24% Milli-Q water, and 1% formic acid was used. The retention times of glucose, maltose, and trehalose were 8.0, 11.2, and 12.5 min, respectively. The percentage yield (percent weight conversion) was defined as grams of trehalose per gram of rice starch or initial maltose × 100% by using an external standard calibration method.

Analysis for Bioethanol Concentration. Chromatographic analysis was performed on a Hitachi G-3000 (Japan) gas chromatograph equipped with a flame ionization detector. Separation was carried out on a Carbowax 1500 (200 cm × 3 mm i.d.) stainless column. The operation conditions were the following: the temperatures of the injector and detector were set at 150 °C, the temperature of the column was maintained at 120 °C, and the carrier gas was nitrogen.

Enzyme Characterization. The activity of sweet potato β-amylase was analyzed according to a modified method give in a previous paper (*11*). The reaction mixture contained 0.25 mL of crude enzyme solution, 1% soluble starch solution (0.75 mL), and 0.5 mL of 0.1 M sodium acetate buffer (pH 4.6), followed by reaction at 37 °C for 30 min. The reaction was stopped by the addition of 1.5 mL of 3,5-dinitrosalicylic acid (DNS) reagents. The incubation was performed in a boiling water bath for 5 min. After dilution with 5 mL of distilled water, the production amount of reduced sugar (maltose) was determined spectrophotometrically at 540 nm. In each set of experiments, a standard curve was prepared with maltose solutions of different concentrations. An enzyme activity unit (1 U) was defined as the amount of enzyme liberating 1 mg of maltose per minute under the assay conditions.

$$\beta\text{-amylase activity (U/mL)} = \frac{\text{released maltose (mg/mL)}}{\text{released time (30 min)}} \times \text{dilution fold} \times \frac{1\ \text{mL}}{\text{diluted enzyme amount (mL)}}$$

The activity of PTTS was assayed by measuring the amount of trehalose produced from maltose. The standard reaction was performed by adding 1.5 μL of purified enzyme into a 50 μL reaction solution containing 50 mM sodium phosphate (pH 6.0) and 150 mM maltose and incubating in a 45 °C water bath for 25 min. The reaction was terminated by heating the mixture in boiling water for 15 min. One unit of enzyme activity was defined as the amount of enzyme that catalyzes the formation of 1 μmol of trehalose per minute.

Rice Hydrolysate Preparation. All rice raw materials (Tainung No. 67) were pretreated with dry milling and wet grinding, respectively. For dry milling, the rice powder was filtered by a sieve of 40 mesh. For wet grinding, the rice was ground into rice liquid by adding a 10-fold volume of water. All materials pretreated with both methods will be liquefied and saccharified into rice hydrolysate to compare their soluble sugar contents and maltose conversion rates, respectively.

For liquefaction, 3 g of rice powder and 181.5 μL of α-amylase (7.08 units/g of starch) were well mixed with 30 mL of distilled water at 60 °C for 2 h. All of the reaction mixture was transferred to an autoclave, and the temperature was raised gradually to 121 °C over 20 min. During heating duration, the α-amylase will be activated to liquefy the rice starch, whereas it will be inactivated with material sterilization as the temperature goes above 100 °C.

For further saccharification procedure, the liquefied solution was adjusted to pH 5.0, and 78 μL (7.5 unit/g starch) of self-made sweet potato β-amylase and 39 μL (10 unit/g starch) of pullulanase were added as reaction mixture. All reactions were carried out at 50 and 60 °C, for various reaction times (0−24 h), and stopped at 100 °C for 15 min. The weight conversion (%) of maltose was calculated by using HPLC analysis and defined as weight (g) of maltose/weight (g) of rice starch × 100% by using an external standard calibration method (*12*). For insoluble content determination, all residual precipitates were dried at 50 °C overnight and weighed for further calculation. The insoluble sugar content (%) was defined as weight (g) of insoluble solid content/weight (g) of rice × 100%.

Trehalose Production from Rice Hydrolysate. For further trehalose production, all experiments used rice hydrolysate as substrate, which is catalyzed by PTTS (15.35 ± 1.2 U/mL) at various temperatures and reaction times to optimize the weight percent conversion of trehalose.

$$\text{weight conversion of trehalose (\%)} = \frac{\text{weight (g) of trehalose}}{\text{weight (g) of maltose}} \times 100\%$$

Separation and Purification of Trehalose. The enzymatic conversion solution is a mixture containing multiple sugars and substances such as trehalose, maltose, glucose, maltotrise, protein, and mineral, which have to be treated by various steps to refine trehalose with high purity for further industrial applications. In this stage, 100 mL (10% rice raw material) of saccharified solution was used as substrate for the hydrolysis of residual disaccharides and trisaccharides into glucose by commercial glucoamlyase (3.33, 6.67, and 13.33 μL/g of rice), and its hydrolytic efficiency was estimated by HPLC analysis. All reactions were carried out at 50 or 60 °C for 0−12 h and stopped at 100 °C for 10 min, respectively.

The glucose-rich resaccharified solution was further inoculated and fermented with 3, 5, and 8% (v/v) of *S. cerevisiae* at 25 °C for 0−72 h. The concentration of bioethanol was analyzed by GC, and carbohydrate content was calculated by HPLC analysis as described above. Bioethanol concentration was calculated by using an external standard calibration method, and bioethanol conversion efficiency (yield) was calculated as described by Nigam (*13*).

Bioethanol was separated from the trehalose solution by distillation, and trehalose-rich solution was further decolored by a two-layer filter containing diatomite and active carbon. The final fluent was collected and stored at 4 °C for further separation and purification of trehalose.

The ionic exchange method was utilized to remove residual mineral and color from the trehalose-rich solution by a series connection with both cationic (DIAION PK216, Taiwan) and anionic (DIAION WA30LL, Taiwan) exchange resin. Initially, the resin was washed by 4-fold volume of 4% HCl, an 8-fold volume of 2−4% NaOH, and deionized water until the effluent was neutral. All crude trehalose solutions were filled through a series connection columns and washed by deionized water until no sugar was detectable at 2−3 mL/min of flow rate. Each filtrate was concentrated at 60 °C in a reliever and crystallized by adding a 4-fold volume of 99% ethanol at 4 °C overnight. The crystalline trehalose was then obtained by filtration and washed by a small amount of warm ethanol simultaneously. Because the residual ethanol might remain on the crystalline surface, all collected samples have to dry at 40 °C for 12 h for further recovery calculation.

Product Recovery. For product recovery calculation, each purification step of trehalose solution including filtration, decolor, ionic exchange, sediment, and crystalline, etc., will be evaluated, and the recovery of product was defined as

$$\text{recovery of crystalline trehalose (\%)} = \frac{\text{weight of crystal trehalose (g)}}{\text{total trehalose amount in fermentation of ethanol}} \times 100\%$$

Structure Identification of Trehalose by Liquid Chromatography−Mass Spectrometry (LC-MS). The LC-MS method was carried out using an Agilent 1100 HPLC system (Agilent Technologies, Waldbronn, Germany) coupled to a linear ion trap mass spectrometer (LTQ, Thermo-Electron, San Jose, CA), equipped with an electrospray ionization interface. A Hibar Clia-Merck NH_2 column (4.6 × 250 mm, 5 μm) was used for chromatographic separation. The mobile phase was composed of water with 0.1% acetic acid/acetonitrile (20:80, v/v). The mobile phase was delivered at a flow rate of 1 mL/min. The sample volume injected in the HPLC system was 5 μL. Analysis run time was 20 min. The mass spectrometer was operated in the negative ionization mode under the following conditions: spray voltage, 4 kV; capillary temperature, 275 °C; sheath gas, 30 (arbitrary units); auxiliary gas, 10 (arbitrary units); and sweep gas, 1(arbitrary units). For MS^n experiments, helium was used as collisional gas and the normalized collisional energy was set at the range of 15−20%. Precursor ion was selected with an isolation width of 1.5 Th and activated for 30 ms. In quantitative analysis, consecutive reaction monitoring (CRM) mode was utilized.

Analysis of Product Purity. The analysis of trehalose by HPLC and LC-MS was performed to verify and calculate its crystalline purity (%); the equation was defined as

$$\text{crystalline purity of trehalose (\%)} = \frac{\text{trehalose concentration of samples (mM)}}{\text{concentration of trehalose standard (mM)} \times 0.99} \times 100\%$$

where 0.99 indicated the purity of trehalose standard.

The crystal sample was analyzed by HPLC and determined by using trehalose as standard. The result shows that the retention times (RT) of trehalose standard and crystal sample are the same.

Table 1. Analysis of Monosaccharide and Disaccharide Contents in the Saccharified Liquid Pretreated by Different Processes

		liquefaction		saccharification		
rice type	pretreatment process	maltose[a] (%)	glucose[b] (%)	maltose[a] (%)	glucose[b] (%)	conversion rate of maltose[c] (%)
Tainung No. 67	wet grinding	17.18 ± 1.75 d	8.49 ± 0.70 d	44.72 ± 1.41 d	9.96 ± 1.64 d	55.87 ± 4.61 d
	dry milling	10.44 ± 0.21 d	3.20 ± 0.39 e	45.40 ± 0.22 d	7.20 ± 1.34 d	54.64 ± 0.27 d

[a] Maltose yield (%) = maltose (g)/rice (g) × 100%. Means with the same letter are not different significantly ($P < 0.05$). [b] Glucose yield (%) = glucose (g)/rice (g) × 100%. Means with the same letter are not different significantly ($P < 0.05$). [c] Conversion rate of maltose (%) = maltose (g)/rice starch (g) × 100% Means with the same letter are not different significantly ($P < 0.05$).

For further structure identification, full-scan and MS^2 mass spectra were obtained by infusing 1 μg/mL of trehalose dissolved in mobile phase and detected in the negative mode. The characteristic ions m/z 401 and m/z 743 were adduct ions of $[M + CH_3COOH - H]^-$ and $[2M + CH_3COOH - H]^-$ in negative electrospray ionization mass spectra. The ion of $[M + CH_3COOH - H]^-$, m/z 401, was selected as precursor ion and proceeded in MS^n experiment. The result indicated that the greatest intensity of product ion was generated characteristically through the loss of one molecule of acetic acid. To identify the fragment pathway and increase the selectivity, an MS^3 mass spectrum of trehalose was obtained by selecting the base peak from the MS^2 mass spectrum. The purity of trehalose was proved.

Statistical Analysis. All experimental results were analyzed by SPSS software (SPSS Institute, Chicage, IL) to establish the significant difference of various treatments by using Duncan's multivariate analysis. The data will be considered as significant difference with $P < 0.05$.

RESULTS AND DISCUSSION

Many foods, pharmaceutical ingredients, and functional foods can be derived from starch. In this study, we have demonstrated the rice material at a low price can be used to produce high-value trehalose, bioethanol, and high-protein functional food by enzymatic method.

Procedure for Simultaneous Production. As shown in **Figure 1**, starch-rich rice raw material can be used as a major substrate for the simultaneous production of high-value trehalose, bioethanol, and high-protein flour or bioactive peptide products by enzymatic method. The final products such as bioethanol and trehalose can be separated easily by distillation to significantly reduce the production cost and increase the yield in this procedure. After a drying process, the high-protein rice flour or its bioactive peptide product was therefore obtained to reach the complete utilization of rice raw material in this study. In the development of a most efficient procedure for the simultaneous production of multiple functional products, different influence parameters will be analyzed to maximize their production yield for further industrial applications.

***β*-Amylase Extraction from Sweet Potato.** The recovery of sweet potato starch was calculated as 11.17%, and the activity of the concentrated β-amylase solution was determined as 239.1 U/mL (65.95 U/g of sweet potato) by using soluble starch as substrate at 37 °C.

Effect of Different Pretreatment Methods on Rice Liquefaction and Saccharification. Different pretreatments for rice raw material might cause different probabilities of broken rice, which might benefit the catalytic efficiency of amylase. In this experiment, we attempt to compare the effect of two pretreatment methods, wet grinding and dry milling, on the maltose conversion rate of rice liquefaction and saccharification. The result shows that there is no significant difference ($P > 0.05$) on maltose conversion rate (~50%) of rice liquefaction and saccharification by using wet grinding or dry milling as pretreatment method (**Table 1**), even though the glucose content obtained by wet grinding was higher than that observed with the dry milling method, but it does not tally with our aims in this study. For further experimental procedure simplification, the dry milling was therefore chosen as a suitable approach to break the rice raw material and sieve the powder (40 meshes) as substrate in the following experiments.

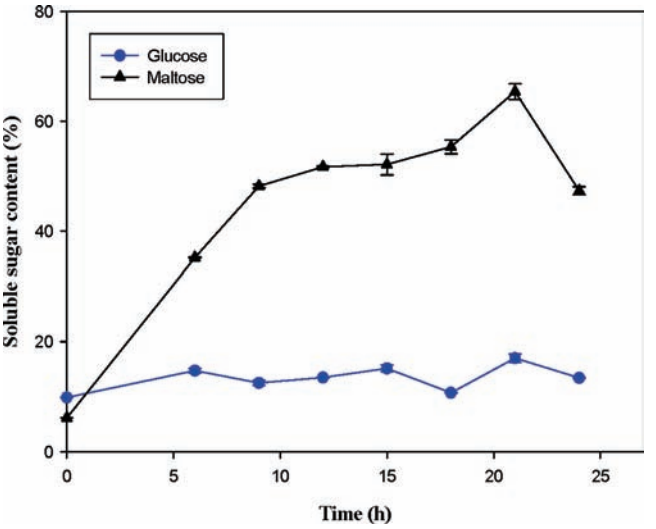

Figure 2. Effect of different saccharification times on soluble sugar content variation by using 10% (w/v) of rice starch as substrate at 50 °C. The soluble sugar content (%) was defined as maltose or glucose (g)/rice (g) × 100%, and all experiments were carried out in triplicates.

Effect of Different Reaction Times on Rice Saccharification. Rice starch is a macromolecule, which can be hydrolyzed into short-chain starch by α-amylase, except the branch α-1,6 linkage. During the saccharification period, pullulanase was added to hydrolyze the branch α-1,6 linkage of rice starch and to benefit the catalytic efficiency of β-amylase. Because higher dextrose equivalent (DE) values of starch hydrolysate would cause higher glucose production amount and lower maltose production yield, it would be very important to control a low DE value with a suitable reaction time for α-amylase (*14*, *15*). For different reaction time tests (0−24 h), the maltose production catalyzed by sweet potato β-amylase was increased as the reaction time was prolonged. As the reaction time was extended to 24 h, the maltose production yield was decreasing instead, which might be caused by the heat hydrolysis of maltose and microbial contamination with glucose consumption simultaneously. Therefore, the highest production yield of maltose was 78.7 ± 1.7% at 50 °C for 21 h, and all following experiments will be held at the same reaction conditions (**Figure 2**).

For the rice saccharification, longer reaction time might increase the glucose production and result in lower production of maltose. The oligosaccharides can be hydrolyzed into maltose with 78.71 ± 1.71% of conversion rate by sweet potato β-amylase at 50 °C for 21 h. However, as the reaction was extended (> 21 h), the conversion rate of maltose tended to drop, which might be because the residual sugars (i.e., maltotriose, maltose, etc.) have been hydrolyzed by heating or contaminated by microorganisms (**Figure 2**).

Table 2. Analysis of the Saccharified Liquid Composition Treated by β-Amylase and Pullulanase Using Rice Starch Hydrolysate as Substrate at Different Temperatures

		soluble sugar content[a]				
rice type	saccharification temp (°C)	glucose (%)	maltose (%)	maltotriose (%)	insoluble solid content[b] (%)	conversion rate of maltose[c] (%)
Tainung No. 67	50	9.19 ± 0.75	68.48 ± 2.36	0.51 ± 0.03	7.01	82.40 ± 2.84 d
	60	7.50 ± 1.10	66.74 ± 3.55	0.68 ± 0.06	7.04	80.31 ± 4.27 d

[a] Souble sugar content (%) = sugar (g)/rice (g) × 100%. [b] Insoluble solid content of saccharified liquid (%) = residue (g)/rice (g) × 100%. [c] Conversion rate of maltose (%) = maltose (g)/starch (g) × 100%. Means with the same letter are not different significantly ($P < 0.05$).

Table 3. Analysis of the Soluble and Insoluble Solid Contents for Trehalose Conversion by Using Starch Saccharified Liquid as Substrate at 50 °C

	soluble sugar content[a]				insoluble solid content		
rice type	glucose (%)	maltose (%)	maltotriose (%)	trehalose (%)	protein (%)	dietary fiber (%)	conversion rate of trehalose[b] (%)
Tainung No. 67	14.89 ± 0.28	25.53 ± 3.25	0.51 ± 0.03	37.39 ± 1.83	75.99	14.01	54.60 ± 2.45 c

[a] Souble sugar content (%) = sugar (g)/rice starch (g) × 100%. [b] Conversion rate of trehalose (%) = trehalose (g)/maltose (g) × 100%. Means in the same column with the same letter are not different significantly ($P < 0.05$).

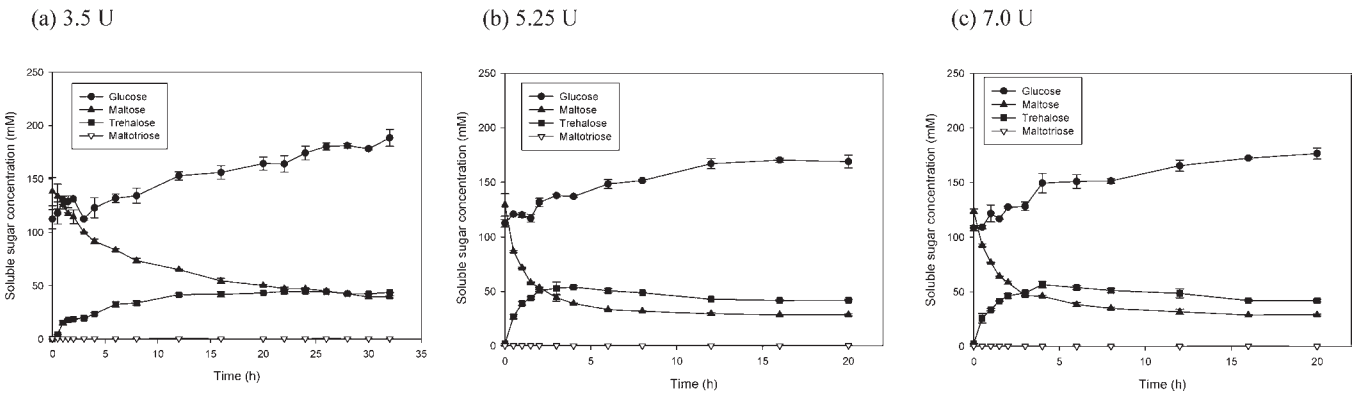

Figure 3. Effects of different PTTS amounts and reaction times on soluble sugar content variation by using rice saccharified liquid as substrate at 50 °C: (**a**) 3.5 U of PTTS addition; (**b**) 5.25 U of PTTS addition; (**c**) 7.0 U of PTTS addition. The soluble sugar concentration (mM) was analyzed by HPLC and calculated by an external standard method. All experiments were carried out in triplicates.

Effect of Different Temperatures on Rice Saccharification. Because amylases derived from different sources might possess different temperature preferences, for various industrial applications, it is better to utilize a thermostable enzyme to avoid the probability of harmful microbial contamination. For this purpose, we have evaluated the effect of high reaction temperature (50 and 60 °C) on the variation of soluble and insoluble solid content (%) production by sweet potato β-amylase-catalyzed saccharification from rice raw material. The result showed that the β-amylase can convert at least 80% of starch into maltose at both 50 and 60 °C, whereas the maltose conversion rate at 50 °C (82.4 ± 2.8%) was slightly higher than that obtained at 60 °C (80.3 ± 4.3%), indicating the optimal reaction temperature of sweet potato β-amylase was 50 °C for saccharification applications. All data were considered as having no significant difference with $P > 0.05$ (**Table 2**).

For the insoluble solid content analysis, we found that there is no significant difference in insoluble solid content (~7%) between 50 and 60 °C (**Table 2**). After the drying process, the total dietary fiber amount and protein amount of rice residue were analyzed and resulted in about 14.01 and 75.99%, respectively (**Table 3**).

To avoid any possible microbial contamination, higher reaction temperature would be the most favorable environment for industrial applications, whereas enzymes might be unstable as temperatures > 50 °C (*16*, *17*). Also, the catalytic efficiency and reaction rate of rice saccharification catalyzed by β-amylase could be further increased at high temperature. The sweet potato β-amylase prepared in our laboratory was quite stable at either 50 or 60 °C with a maltose conversion rate of at least 80% by using rice starch hydrolysate as substrate, whereas the maltose yield (82.40 ± 2.84%) at 50 °C was slightly higher than that obtained at 60 °C (80.31 ± 4.27%) (**Table 2**). This suggested our enzyme was more stable at 50 °C, and a similar result was also obtained by Ueda and Ohba (*18*).

Table 3 shows there was about 7% of insoluble solid contained in rice raw materials, and the insoluble solid content was not significantly influenced by place of origin or reaction temperature. All rice residues contained 75.99% of protein and 14.01% of dietary fiber, which can be used for breakfast cereal products and health foods or other industrial applications.

Effect of Different PTTS Amounts and Reaction Times on Trehalose Conversion from Rice Starch Hydrolysate Derived Maltose Syrup. After rice saccharification by α- and β-amylase, 3.5, 5.25, or 7.0 U/maltose (g) of PTTS was added into the rice hydrolysate derived maltose syrup to evaluate the production yield of trehalose at 50 °C for 0−32 h, respectively. The result shows the lower PTTS amount (3.5 U) took longer (22−24 h) to reach > 50% of trehalose yield than the higher PTTS amount (7.0 U) at 4 h (**Figure 3**). Because the higher enzyme amount increased the production cost, a lower PTTS amount (3.5 U) with a longer reaction time (22 h) was therefore chosen for all of the following experiments. Consequently, the highest trehalose conversion rate (54.6 ± 2.5%), catalyzed by sweet potato β-amylase and PTTS from rice starch hydrolysate in one step at 50 °C for 22 h, has been successfully established in this study (**Table 3**).

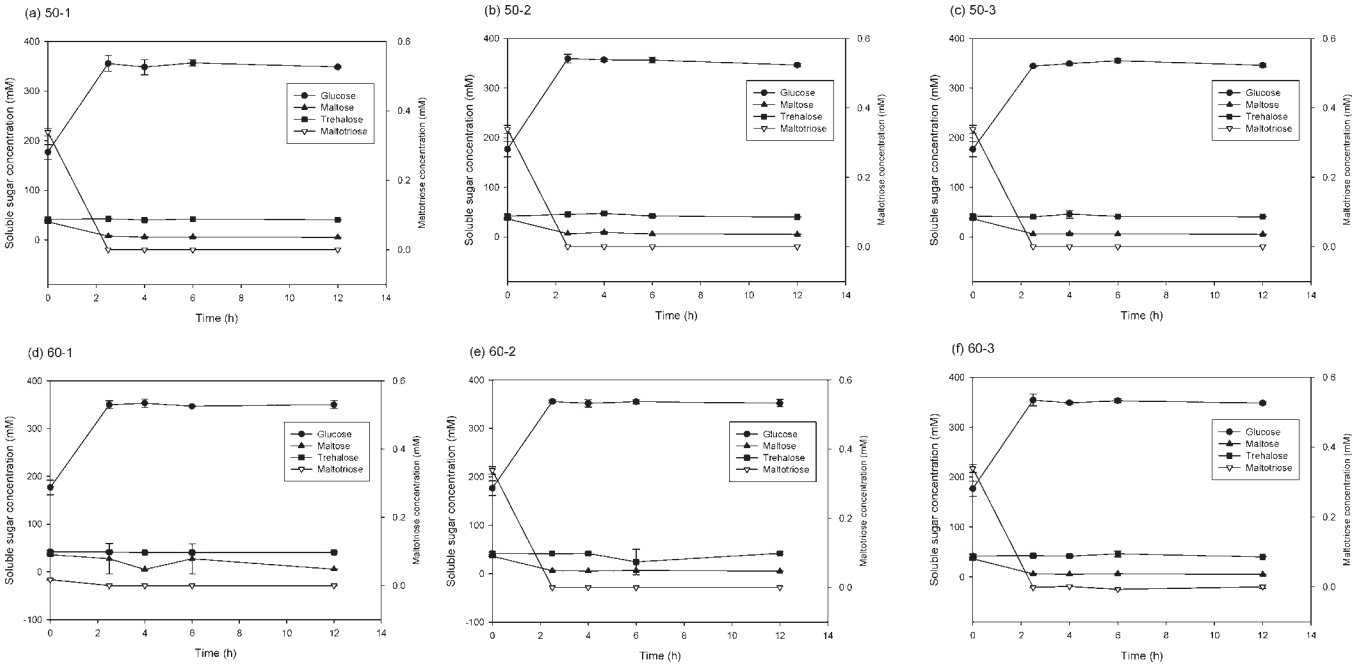

Figure 4. Effect of different glucoamylase amount additions and reaction times on the soluble sugar content variation by using rice saccharified liquid as substrate at 50/60 °C: (**a**−**c**) all reactions with added 3.33, 6.67, and 13.33 μL/g of rice of glucoamylase amount at 50 °C, respectively; (**d**−**f**) all reactions with added 3.33, 6.67, and 13.33 μL/g of rice of glucoamylase amount at 60 °C, respectively. The soluble sugar concentration (mM) was analyzed by HPLC and calculated by an external standard method. All experiments were carried out in triplicates.

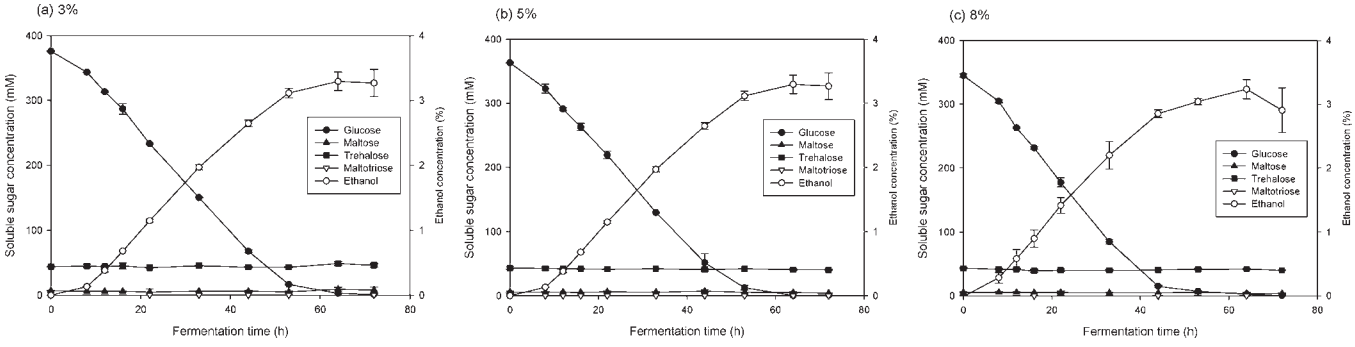

Figure 5. Effect of time variation on soluble sugar content and bioethanol content of fermented resaccharified liquid by inoculating 3, 5, and 8% (v/v) of *Saccharomyces cerevisiae* at 25 °C, respectively. The soluble sugar concentration (mM) was analyzed by HPLC and GC and calculated by an external standard method. All experiments were carried out in triplicates.

Simultaneous Purification of Trehalose and Production of Bioethanol from Reaction Products. The reaction products, 54.6% of trehalose and residual sugars (i.e., glucose, maltose, and maltotriose), were used as substrate for further simultaneous production of bioethanol and purification of trehalose as shown in **Figure 4**. For this purpose, all residual sugars (i.e., maltose, maltotriose, etc.) were hydrolyzed by glucoamylase into glucose, which can be converted to bioethanol by yeast fermentation and easily removed by distillation to simplify the trehalose purification steps. The result showed that after 2.5 h of reaction time, the residual maltose and maltotriose can be almost completely hydrolyzed into glucose by glucoamylase without affecting trehalose content as shown in **Figure 4**.

To obtain high-purity trehalose, the residual mixtures including glucose, maltose, maltotriose, limit dextrin, protein, amino acid, pigments, or metal ion, etc., have to be separated and removed from all reaction products. For monosaccharide and disaccharide purification, it is easy to separate by active carbon adsorption and ion exchange chromatography, whereas the cost was fairly high. It is even more difficult to separate maltose and trehalose, which have similar physical and chemical properties by adsorption or ion exchange approaches. For these reasons, we therefore designed a one-step procedure that combined glucoamylase-catalyzed resaccharification and anaerobic fermentation. All residual glucose produced from resaccharification can be converted into bioethanol and CO_2 without any trehalose consumption. The result showed that a ~98% of highest bioethanol conversion yield was obtained with 3−8% of *S. cerevisiae* fermentation at 25 °C for 64 h. The produced bioethanol can be simply separated from trehalose by distillation, and it is an additional product for industrial applications.

Effect of Different Concentrations of *S. cerevisiae* on Bioethanol Production. The yeast concentration and fermentation time were the most important factors for bioethanol production. The effect of different *S. cerevisiae* concentrations (3, 5, and 8%) on bioethanol production at different fermentation time was therefore investigated in this study as shown in **Figure 5**. The result showed that highest bioethanol concentrations 3.3% (equivalent to 98% of bioethanol conversion yield) were obtained by inoculating 3−8% of yeast concentration for 64 h without any

trehalose consumption (**Figure 5**). After separation of bioethanol by distillation, the trehalose-rich solution can be easily purified and crystallized by ethanol precipitation with 54.6% trehalose yield.

Conclusion. Low-value starch-rich rice raw materials can be converted into value-added products such as trehalose, bioethanol, and functional food in one step by a combinatorial enzymatic process of α-amylase, β-amylase, trehalose synthase, and glucoamylase. The optimal conversion rate of maltose syrup catalyzed by sweet potato β-amylase from rice was calculated as 82.40 ± 2.84% at 50 °C for 21−22 h. The highest trehalose yield (54.60 ± 2.45%) can be obtained by 3.5 U/maltose (g) of PTTS from the maltose syrup at 50 °C for 20−24 h. The residue of rice after the removal of starch by α-amylase hydrolysis contained high protein (75.99%) and dietary fiber (14.01%), which can be used as functional food (*14*, *15*). A simple purification method of trehalose has been successfully developed in combination with the resaccharification and fermentation processes. The highest bioethanol conversion yield of about 98% was obtained for 64 h of fermentation time. All nutritional sources required for *S. cerecvisiae* growth were provided from natural rice saccharified liquid without the addition of any artificial culture solution. The trehalose products have been successfully separated, crystallized, and identified with 92.3% purity and 92.5% recovery yield by LC-MS analysis.

In conclusion, each kilogram of rice (75% starch) can produce about 375 g of trehalose (U.S. $5/kg) and 368 g of bioethanol (U.S.$0.6/kg) through this enzymatic process. The residual precipitate can be used as a natural high-protein−high-dietary-fiber health food without any chemical additive. Alternatively, the residual rice protein can be hydrolyzed into small peptides and used as ACE inhibitor for lowering blood pressure applications (unpublished result). Protein engineering and enzyme immobilization are currently underway to further improve the catalytic efficiency of PTTS to increase trehalose yield. This work demonstrated the feasibility of the simultaneous production of various value-added products such as maltose, trehalose, and bioethanol by multiple enzymes from low-price crops in one step. A similar process could be applied to other starch-rich agricultural produces (i.e., sweet potato, corn, potato, etc.) to benefit the farmers and the development of agriculture.

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