Integrated biocatalytic process for trehalose production and separation from rice hydrolysate using a bioreactor system

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**Abstract**

Rice starch can be hydrolyzed into maltose for trehalose bioconversion by enzymatic methods. In this study, we have successfully established an efficient production system for our recombinant PTTS in large scale. Three bio-treatments were developed to simplify the separation and purification of trehalose from complex rice saccharified liquid. The trehalose conversion rate of 64.63 ± 4.05% at 30°C can be reached using rice hydrolysate as the substrate in a 5 l fermentor system. By 1% of raw material koji fermentation, the highest concentration of bioethanol (3.61 ± 0.07%) was obtained at 30°C for 36 h. After 12 h of reaction time, the gluconic acid (24.47 ± 0.33 mM) was successfully produced by glucose oxidase (40 U/g rice) using residual glucose as a substrate. After the batch/continuous ionic exchange process, the trehalose can be successfully separated, crystallized and identified as 92.6 ± 0.02% purity and 94.2% of the recovery yield, respectively.

**Keywords:** Bio-treatment, Bioethanol, Bioreactor, Enzyme, Gluconic acid, Picrophilus torridus, Rice, Trehalose

**1. Introduction**

Rice, the most important cereal, is a major staple food with a wide cultivated area in many continents of the world (Asia, Africa, North America and the Middle East). For various industrial applications, the low-price byproduct of broken rice, which contains a great number of nutritive components such as starch, dietary fiber, protein, fatty acid and mineral, would be a more economical starting material during rice processing (Akoh, Chang, Lee, & Shaw, 2008). A variety of sweeteners, including sugar, sugar alcohols, maltose, glucose, fructose, trehalose, maltodextrins, cyclodextrins, and syrups can be produced by enzymatic hydrolysis using broken rice as a substrate in the food and pharmaceutical industries. Alternatively, they can be used as a natural culture for yeast fermentation to produce various fermented products such as wine and vinegar. The residual part of rice hydrolysate containing high protein and fibers are good for human health and can be reprocessed for pudding, gruel, instant milk, baby food and health food production.

Trehalose, consists of two subunits of glucose bound by an α, β-1,1-linkage, is a stable, colorless, odor-free, and biologically active disaccharide which is widespread in nature (Elbein, Pan, Pastuszak, & Carroll, 2003). In addition to serving as an energy and carbon source, trehalose is a functional component to help organisms acquire tolerance to cold, heat, desiccation, dehydration, and osmotic and oxidative stresses (Benaroudj, Lee, & Goldberg, 2001; Chen, Behar, Xu, Fan, & Haddad, 2003; Elbein et al., 2003; Kandror, DeLeon, & Goldberg, 2002; Purvis, Yomano, & Ingram, 2005). Its inertness property and the above mentioned ability to stabilize biomolecules imply a wide range of applications such as in the food, cosmetic, and pharmaceutical industries. For the food industry, trehalose can be used as an additive to improve the texture and extend the preservation period. For cosmetic applications, it is a functional moisturizer or liposome stabilizer. In medicine, trehalose can help to increase the preservation time of enzymes and protect mammalian cells from damage during the freeze-drying process. During the storage period, vaccines can be stabilized at room temperature and organs transplant can be protected by trehalose addition. Also, it can be used as a cryoprotectant in the cryopreservation of cells, sperms, tissues, or other materials to resist injury effect by frost.
Recently, several literatures have reported in regard to develop a general procedure for preparing syrups and fermentation products from starch or starch-rich materials by treating with multiple enzymes (Chang et al., 2010; Pandey, 1995; Schiraldi, Di Lernia, & De Rosa, 2002; Van der Veen, Veelaert, Van der Goot, & Boom, 2006; Wang, Tsai, Chen, Lee, & Shaw, 2007; Wang, Tsai, Lee, & Shaw, 2007). In particular, the starch component can be hydrolyzed into a soluble lower molecular weight oligosaccharide through liquefaction processes catalyzed by α-amylases. The liquefied starch hydrolysate which contains mainly oligosaccharides (mainly 8–12 glucose units) can be further hydrolyzed into lower molecular weight disaccharides (i.e. maltose syrup) by β-amylase or monosaccharides (i.e. glucose syrup) by glucoamylase (Pandey, 1995). Among which, the high maltose (glucosyl-α-1,4-glucopyranoside) syrup can be further converted into trehalose (glucosyl-α-1,1-glucoypyrano side) by a one step enzymatic process using trehalose synthase as a biocatalyst (Elbein et al., 2003; Nishimoto et al., 1996). Previously, we have cloned a novel recombinant Picrophilus torridus Tsase (PTTS) with a high catalytic efficiency of 60% trehalose bioconversion yield by using maltose as a substrate at 45 °C (Chen, Lee, & Shaw, 2006). Without any chemicals in the process, this enzyme can be combined with α-amylase, β-amylase, pullulanase and glucoamylase to simultaneously convert the low value, starch-rich agricultural produce (i.e. broken rice, sweet potato, etc.) into high value trehalose, bioethanol and high protein flour which can increase the farmer’s income and the nutritional value of low value agricultural produces (Chang et al., 2010; Shaw & Sheu, 1992; Wang, Tsai, Chen et al., 2007; Wang, Tsai, Lee et al., 2007). For further industrial applications, we expect such combined enzymatic process will be further simplified with a high conversion yield and lower production cost in a large scale fermentor system.

In this study, an efficient expression system for the recombinant PTTS production has been successfully expanded to a large scale. To simplify the production procedure, our recombinant PTTS is therefore used to combine with α-amylase, β-amylase, pullulanase, glucoamylase, raw koji material, Rhizopus crude enzyme, S. cerevisiae or glucose oxidase as multifunctional enzyme complex to produce high commercial value trehalose, high protein flour, bioethanol or gluconic acid from rice starch hydrolysate in one step (Fig. 1). Without any chemicals addition, we expect such efficient green process can be applied to other starch-rich of low-value agricultural produce, such as sweet potato, potato, cassava, and maize to increase the farmer’s income for widely industrial application.

2. Materials and methods

2.1. Materials

All mono- and disaccharides were purchased from Sigma Chemical Co. (St. Louis, MO, USA). α-Amylase (135 KIU/g), produced from Bacillus licheniformis and Pullulanase (1350 NPUN/g), produced from Bacillus subtilis were purchased from Novozyme Company Inc. (Denmark). Glucoamylase (400 ± 40 U/g), produced by B. licheniformis was purchased from Lyven Company Inc. (French). Glucose oxidase (125 KU/g), produced from Aspergillus niger was purchased from Sigma Chemical Co., Actonitrile was from TEDIA Company Inc. (USA). All other chemicals and reagents were of analytical grade.

2.2. Microorganisms

The transformant of the modified Escherichia coli strains Rosetta B (DE3) (Δref, ΔtreA) (Novagen), containing the PTTS gene, was kindly provided by Dr. Po Ting Chen (Southern Taiwan University of Technology, Taiwan). All E. coli strains were both cultured in Luria–Bertani (LB) broth/agar supplemented with 100 µg/ml ampicillin and 34 µg/ml chloramphenicol (LB-Amp-Cm) for gene cloning and protein expression at 30 °C overnight (16 h) in an orbital shaker with a speed of 150 rpm.

The commercial yeast Saccharomyces cerevisiae was kindly provided by Masahisa Machinery Co. Ltd. (Taiwan) and activated in Yeast Malt (YM) broth at 30 °C for 48 h. Rhizopus sp. NCH-1 was screened from raw koji material of commercially local fermented rice produced in Changhua (Taiwan) and activated in a potato dextrose agar (PDA) plate at 30 °C. Aspergillus oryzae BCRC 32279 was purchased from FIRD (Taiwan) and activated in a potato dextrose agar (PDA) plate at 30 °C.

2.3. Production of recombinant PTTS in fermentor system

Individual colonies harboring PTTS genes were cultivated and activated with LB broth containing 100 µg/ml ampicillin and 34 µg/ml chloramphenicol at 30 °C, in an orbital shaker at a speed of 150 rpm for 16 h. Cell growth of the transformant was monitored by measuring the absorbance at 600 nm using a spectrophotometer (HITACHI U-2001, Japan). After the OD_600 value of cell broth was increased from 0.1 to 0.5, the production level was up-scaled into a 5 l of fermentor system (working volume: 3 l) with 1vvm (air volume/culture volume/min) aeration and an agitation speed of 250 rpm at 30 °C for 0–16 h. During the large-scale fermentation process, the OD_600 value, dissolved oxygen and enzyme activity of the sampling culture broth were determined and recorded. The culture broth was centrifuged (1300g, 10 min) at 4 °C and the supernatant was removed. The residual cell pellet was re-suspended in 50 mM sodium phosphate buffer (pH 6.0) and lysed by a sonicator (Misonix, model XL-2020) with 5% of power for 1 min (15 s bursts and 15 s intermission/batch). After centrifugation at 12,000g for 30 min, the supernatant containing crude enzyme was collected for further analysis of trehalose synthetic activity and applications of multi-functional products production.

2.4. Sweet potato β-amylase preparation

The raw material, fresh sweet potato (Tainung No. 66), was peeled, shaved and pressed with 1.5-fold of ddH₂O to form a juice and filtered by a cheese cloth. Afterwards, the sweet potato juice kept at 4 °C for 2 days, and the residual supernatant was heated at 60 °C for 10 min to eliminate the maltose hydrolysis reaction caused by α-glucosidase. The solution was then kept at 4 °C for 2 days and centrifuged at 8000g for 30 min at 4 °C to obtain a β-amylase-rich supernatant. The enzyme solution was concentrated by ultrafiltration using a 100 kDa molecular weight cutoff membrane (Amicon Ultra, Millipore, Bedford, MA), and pooled as a partially purified β-amylase preparation which will be preserved at 4 °C for further maltose production.

2.5. Protein quantification

Protein concentration was measured by using a Bradford protein assay kit purchased from Bio-Rad Lab (Hercules, CA) and using bovine serum albumin (BSA) as a standard. The standard curve was completed by determining of the absorbance and BSA concentrations and the concentration of unknown protein was calculated according to this curve.

2.6. Enzyme characterization

The activity of sweet potato β-amylase was analyzed by a modified method according to a previous report (Miller, 1959). The reaction mixture containing 0.25 ml crude enzyme solution, 0.75 ml of 1% soluble starch solution and 0.5 ml of 0.1 M sodium...
acetate buffer (pH 4.6) was used to perform the reaction at 37 °C for 30 min. The reaction was stopped by addition of 1.5 ml of 3,5-dinitrosalicylic acid (DNS) reagents and boiled for 5 min until the color emerged. After addition of 5 ml of distilled water, the production amount of reduced sugar (maltose) was determined spectrophotometrically at 540 nm and calculated by a standard curve. An enzyme activity unit (1 U) was defined as the amount of enzyme liberating 1 mg maltose per minute under the assay conditions.

\[
\text{β-Amylase activity (U/ml)} = \frac{\text{Released maltose (U/ml)}}{\text{Reaction time (30 min)}} \times \frac{1}{\text{Dilution fold}} \times \frac{1}{\text{Diluted enzyme amount (ml)}}
\]

For recombinant PTTS activity, the analysis method was modified from our previous report (Chen et al., 2006). All enzyme activity was measured by the conversion amount of trehalose from maltose per unit time. The standard reaction was performed by adding 50 μL crude enzyme into 1000 μL reaction mixture containing 50 mM sodium phosphate (pH 6.0) and 150 mM maltose and incubating at 45 °C for 25 min. The reaction was terminated by heating the mixture at 100 °C 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 min.

The activity of glucose oxidase was measured by rapidly adding 10 μl of glucose oxidase into 950 μl of well mixed substrate solution containing 0.3 M sodium phosphate (pH 5.6), 1 M glucose, Triton stabilized o-dianisidine and horseradish peroxidase (500 U/ml) with 80:10:4:1 of volume ratio. All reactions will be carried out in a cuvette and analyzed at 460 nm for 2 min by a colorimeter. One unit of enzyme activity was defined as the amount of enzyme that catalyzes the formation of 1 μmol of H₂O₂ per min at 25 °C.

\[
\text{Glucose oxidase activity (U/ml)} = \frac{\text{Slope} \times A}{0.5 \times 11.5 \times 1 \text{ cm} \times 8 \text{ ml}}
\]

where Slope is the straight slope of the absorbance variation in 2 min. A is total reaction volume. B is glucose oxidase addition volume (ml) 11.6 is the OD₄₆₀ of o-dianisidine standard (cm²/μmol), 0.5 is a half reactive amino group of o-dianisidine. 1 cm is the distance passed through of cuvette.

2.7. High maltose syrup production in bioreactor system

All rice raw materials (broken rice of Tainung No. 67) were milled into rice powder and filtered by a sieve of 40 meshes. The
rice powder (150 g) was dissolved by adding hot distilled water (70–80 °C) to obtain the rice liquid with 10% of final concentration in a 5 l fermentor system. For liquefaction, the α-amylase (33.75 unit/g starch) was added and well mixed with 10% of rice liquid to start the enzymatic hydrolysis reaction at 200 rpm of agitation rate and 0.1vvm of aeration for 1 h. Since higher temperature would result in gelatinization of starch which is easier for a thermostable α-amylase reaction. The effect of different gelatinization temperatures (70–90 °C) on the maltose conversion rate was therefore determined and compared in this study.

For the further saccharification procedure, home-made sweet potato β-amylase (150 U/g starch) and pullulase (10 U/g starch) was added and mixed with a liquefied solution as the reaction temperature had been cooled down to 50 °C. All reactions were carried out in a 5 l fermentor system using 200 rpm of agitation rate and 0.1 vvm of aeration for 0–24 h to homogenize the reaction mixture for estimating the optimal reaction time of highest weight conversion rate (%) of maltose. The weight conversion (%) of maltose was calculated by using HPLC analysis and defined as (Fukushima, Sogo, Miura, & Kimura, 2004):

\[
S = \frac{P}{R \times 0.831} \times 100\%
\]

where S is weight % of conversion rate by starch saccharification, P is weight (g) of maltose amount contained in saccharified solution, R is addition amount (g) of rice powder, 0.831 is starch amount contained in rice (g).

2.8. Trehalose production from rice hydrolysate using bioreactor system

For further trehalose production, all experiments used rice hydrolysate as a substrate and carried out in a 5 l fermentor system. The bioconversion reaction was started by adding crude PTTS solution (3.5 U/g maltose) with a 100 rpm agitation rate at 30 °C for 0 to 24 h to optimize the weight % conversion of trehalose. The weight % of trehalose conversion rate was calculated by HPLC analysis.

The weight conversion of trehalose (%)

\[
= \frac{\text{Weight (g) of Trehalose}}{\text{Weight (g) of maltose contained in rice hydrolysate}}
\]

2.9. Three bio-treatment methods to simultaneous produce various useful products

Since the rice hydrolysate was used as major substrate for the enzymatic process, all final conversion solutions would be a complex mixture containing multiple sugars and substances such as trehalose, maltose, glucose, maltotriose, protein and mineral. Therefore, it would be more difficult to obtain high purity of trehalose for further industrial applications. For this reason, we attempted to develop various bio-treatment methods to simplify the refining process of trehalose by converting unwanted residues (i.e. glucose) into useful products in one step. Three bio-treatment methods were developed to hydrolyze all residual disaccharides and trisaccharides into monosaccharide (i.e. glucose) by addition of various biocatalysts including (i) raw koji material, (ii) combination of Rhizopus crude enzyme and S. cerevisiae, or (iii) glucose oxidase to simplify the following purification procedure of trehalose and produce other useful bioproducts at the same time.

2.9.1. Estimation for one-step raw-material koji reaction

The raw koji material, was a mixture abounds with glucoamylase and active dry yeast, trace amount of cellulase and acidic protease, which had been widely applied for wine manufacturing by using raw rice or raw sorghum as material. For trehalose applications, it could be used as a multifunctional biocatalyst for alcohol fermentation followed by hydrolysis of all residual disaccharide and trisaccharide into glucose in one-step. In this study, all trehalose conversion solution was therefore treated with 0.1%, 0.5% and 1% (w/v) of raw koji material at 30 °C and 40 °C for 0–48 h, respectively. The concentration of bioethanol was analyzed by GC as described by Nigam (2001) and carbohydrate content was calculated by HPLC analysis as described by our previous report (Chang et al., 2010).

2.9.2. Estimation for combined reaction of crude Rhizopus sp. amylase and S. cerevisiae

Similarly, bioethanol could be also produced by combined reaction with crude Rhizopus sp. amylase and S. cerevisiae for the combined bio-treatment reaction. All trehalose conversion solution was therefore used as substrate to be hydrolyzed and fermented by adding 10% or 15% of home-made crude Rhizopus amylase and 5%, 8% and 10% of S. cerevisiae at 30 °C for 0–60 h. All reactions were terminated by enzyme inactivation at 100 °C for 10 min before carbohydrate analysis by HPLC. The crude Rhizopus sp. amylase was produced by inoculating 1% active Rhizopus sp. spore suspension into 10% broken rice as substrate (1:10; v/v) at a shaking speed of 150 rpm and 30 °C for 3 days. The cultivation solution was filtrated through filter paper (No. 1, Advantec) to obtain the crude Rhizopus amylase for further fermentation applications.

2.9.3. Estimation for bioconversion of gluconic acid from rice hydrolysate

Instead of raw-material and co-reaction of crude Rhizopus sp. amylase and S. cerevisiae addition, we used glucoamylase to hydrolyze all residual maltose and maltotriose into glucose and added glucose oxidase for gluconic acid production to simplify the separation step of trehalose from the complex reaction mixture of rice hydrolysate. All hydrolysis reactions were started by adding gluco-amylose (3.33 U/g rice) at a shaking speed of 180 rpm and 50 °C for 2.5 h. After the hydrolysis reaction had completed, the remaining glucose was converted into gluconic acid by adding glucose oxidase (13, 26 and 40 U/g rice) at a shaking speed of 160 rpm and 35 °C for 0–60 h. All reactions were terminated by enzyme inactivation at 100 °C for 10 min before carbohydrate and organic acid analysis was performed by HPLC.

2.10. Bioethanol and organic acid analysis

After multiple enzymatic reactions, all samples were filtered by 0.22 μm membrane and 10 μl of filtrate was used for further analysis. The analysis method for bioethanol was according to our previous report (Chang et al., 2010). The amount of organic acid (i.e. gluconic acid) was measured using a high performance liquid chromatography (HPLC) (Hitachi, Model L-2130) system equipped with an Diode array detector (Hitachi, Model L-2455) at a flow rate of 0.5 ml/min. An organic acid analysis column (Mightysil, RP-18, 250 × 4.6 (mm), 5 μm) equilibrated with 0.02 M (NH4)2PO4 buffer (pH 2.3) was used. The retention time of gluconic acid was 7.05 min and the concentration was calculated by using an external standard calibration method.

2.11. Different refinements for crystalline trehalose production

After bioethanol/gluconic acid separation, the trehalose-rich solution was further decolorized by a two-layer filter containing diatomite and active carbon. The colorless filtrate was collected...
and stored at 4 °C for the following trehalose purification applications.

2.11.1. Lime method

The final filtrate was heated to 103–105 °C and then adjusted pH to 7.6–7.8 by adding 20 Brix° of lime solution. After mixing well and heating for 30 min, the trehalose-rich fluid was placed to make the sedimentation occur by itself at room temperature. All treatments were filtered to remove the residual precipitation and obtain a clean trehalose-rich solution for further conductivity analysis.

2.11.2. Batch ionic exchange method

For the initial regeneration stage, the cationic (DIAION PK216, Taiwan) and anionic (DIAION WA30LL, Taiwan) exchange resin was activated with 4-fold volume of 4% HCl and 8-fold volume of 2–4% NaOH, respectively. Both of them were then washed with deionized water until the liquid was neutral. All crude trehalose-rich solutions were mixed with cationic following anionic exchange resin (mixed ratio was 2:1; v/v). The solution was first passed through the cationic resin and then through the anionic resin at 2–3 ml/min flow rate to neutralize the H+ and OH− ions, which remained in the sugar fluid. After the sugar fluid was completely passed through the column, all residual resins were then washed by deionized water until no sugar was detectable (0 Brix°) existed within the liquid.

2.11.3. Continuous ionic exchange method

 Afterwards, both cationic and anionic exchange resins were regenerated by the batch ionic exchange method as previous described. All crude trehalose solutions were filled through a series connection column (column volume: 39.02 cm³), and combined with cationic and anionic exchange resin (sugar fluid: resin = 2:1; v/v). The solution was first passed through the cationic resin and then through the anionic resin at 2–3 ml/min flow rate to neutralize the H+ and OH− ions, which remained in the sugar fluid. After the sugar fluid was completely passed through the column, all residual resins were then washed by deionized water until no sugar was detectable (0 Brix°) in the liquid.

As the purified sugar fluid obtained, each sample was further concentrated at 50 °C in a reliever and crystallized by adding 4- to 10-fold volume of 99% ethanol at 4 °C overnight. The crystalline of trehalose was then obtained by filtration and washed by a small amount of warm ethanol simultaneously. Since the residual ethanol might remain on the surface of the crystalline trehalose, all collected samples were then dehydrated at 40 °C for 12 h for further yield calculations.

2.12. Yield and purity of crystalline trehalose

For the product yield calculation, the purification step of trehalose solution, including filtration, decolorisation, ionic exchange, sediment and crystalline, etc. was evaluated and the yield of product was defined as:

\[
\text{Yield of crystalline trehalose (\%)} = \frac{\text{Weight of crystal trehalose (g)}}{\text{Total trehalose amount in fermentation of ethanol}} \times 100\%
\]

For the purity of crystalline product evaluation, 0.5 M of trehalose standard was prepared to compare with the same concentration of our crystalline sample and calculated by HPLC analysis. The purity of crystalline trehalose was defined as:

\[
\text{Purity of crystalline trehalose (\%)} = \frac{\text{Concentration of crystalline sample (mM)}}{\text{Concentration of trehalose standard (mM) \times 0.99}} \times 100\%
\]

0.99 is the purity of trehalose standard.

3. Results and discussion

Broken rice, a residual product during rice processing in the mill, a starch-rich material at low price, is majorly used as animal feeds. To increase its economical value, we previously established that the trehalose and bioethanol could be simultaneous produced by a co-enzymatic reaction from rice in small flask (Chang et al., 2010). However, for a large scale system, the primary cost would be greatly affected by the enzyme production and trehalose purification. A simplified and efficient purification procedure to separate the trehalose from residual sugar mixtures with similar characteristics is another obstacle that needs to be overcome for higher market competition rate. More feasible bio-treatment methods to completely convert all of the residual disaccharide and tri saccharide into valuable products (i.e. bioethanol and gluconic acid) are also discussed in this study (Fig. 1).

3.1. Production of recombinant PTTS in bioreactor system

Even the expression of our recombinant PTTS production had been well estimated in small-scale flask (Chen et al., 2006), but it is more necessary and practical to develop a functional system for the large quantity production of our active recombinant enzyme for further industrial applications. For a 5 l fermentor system, the cells were stationary grown with the lowest dissolved oxygen amount at 6 h, which was similar to those obtained by small-scale flask as shown in Fig. 2. Before that, most of cells might be in a state of cell division by consumption of a high amount of oxygen in the culture solution. The constant-rate of dissolved oxygen amount were kept by simultaneous agitation and aeration and rose again since the equilibrium state of cell growth and death reached a steady state. That is why the dissolved oxygen ratio, another index available for cell growth estimation, was increased as the stationary phase of cell growth was reached after 6 h cultivation (Fig. 2). The highest activity (8.56 ± 1.14 U/ml) of the trehalose synthase was obtained at 30 °C for 12 h, which indicated that the expression of our recombinant PTTS was stable and workable in a 5 l fermentor system.

3.2. High maltose content of rice hydrolysate preparation using the bioreactor system

Generally, most of rice fluid could be gelatinized at a broad range of 55–79 °C. However, according to our preliminary experi-
ment, we found that the gelatinization of rice fluid did not complete at 70 °C, which might because of a higher content of amylose, smaller starch particles or sugar inhibition. That means, for our rice flour preparation, a higher reaction temperature was required to break the β-structure of raw starch and swell it into an α-form by entering of H₂O. To realize the effect of gelatinization temperature on maltose production yield in bioreactor, Fig. 3 showed that less glucose was produced while a higher temperature (>70 °C) was reached, suggesting it might because of the incomplete hydrolytic reaction of α-amylase to block the following maltose conversion reaction that is catalyzed by β-amylase and pullulanase. After 22 h of saccharification, the highest maltose conversion rate could be reached (77.02 ± 0.42%) at 80 °C of gelatinization temperature, which was at least a 17% higher maltose conversion rate than that obtained at 70 °C (data not shown). All experiments were carried out by a series of enzymatic reactions without any autoclave process to save energy and time for a large scale system. Also, we found that both the optimal saccharification time and maltose conversion rate achieved in a 5 l fermentor was similar to those obtained by the small-scale system (Chang et al., 2010). A moderate gelatinization temperature of 80 °C accompanied by 18–22 h of saccharification was therefore used as the standard pretreatment condition for following high maltose syrup preparation in the fermentor system.

3.3. Trehalose production from rice hydrolysate in the bioreactor system

Based on the small-scale flask production system, different reaction parameters had been well studied and the results showed that a higher reaction temperature might result in a lower trehalose conversion rate, whereas higher temperature might reduce the probability of the microbial contamination in a particular food for industrial applications (Chang et al., 2010; Schiraldi et al., 2002). For the large-scale fermentor system, we found that the trehalose production could reach 64.63 ± 4.05% conversion rate when at 30 °C for 22 h by using rice hydrolysate as a substrate (Fig. 4), suggesting that our recombinant PTTS could be stably expressed and activated in the large-scale system as well as in the small flask (Chang et al., 2010).

3.4. Three bio-treatment methods to simplify the separation and purification of trehalose

Since the trehalose conversion solution contained a number of carbohydrates and some proteins, pigments, amino acids and metal ions by using low price starch-rich agricultural produce as a substrate. The residual sugar mixture, including glucose, maltose and maltotriose, was the major obstacle for separation and purification of high trehalose purity. The development of various active enzymes with highly hydrolytic activity on raw starch or carbohydrates for conversion into glucose gives rise to the possibility of bioethanol fermentation would be one of the feasible strategies to improve the efficiency of trehalose separation and purification from the reaction mixture containing complex components. Without consumption of trehalose, valuable fermentation byproducts, such as bioethanol or gluconic acid, should be simultaneously obtained in one step to increase the returns for further industrial applications.

Two bio-treatment methods were therefore developed by using raw material koji or a combination of Rhizopus crude amylase and S. cerevisiae as biocatalysts to eliminate all residual saccharide for bioethanol fermentation applications. The results showed that all residual maltose and maltotriose could be simultaneously converted into glucose and fermented into bioethanol by adding 0.1–1% of raw koji material at 30 °C for 4–12 h, as shown in Fig. 5(A–C). After 36 h of fermentation, all residual saccharides except trehalose could be completely consumed to obtain the highest concentration of bioethanol (3.61 ± 0.07%) at either 30 °C or 40 °C by using 1% of raw koji material as biocatalyst as shown in Fig. 5(C). Fig. 6(A–F) showed that all residual maltose and glucose could be completely exhausted by using Rhizopus crude enzyme.

![Fig. 3](image3.png)

Fig. 3. The effect of different gelatinization temperature and reaction time on the soluble sugar content variation by using rice flour as substrate for 24 h. The gelatinization of rice flour solution was carried out at (A) 70 °C, (B) 80 °C and (C) 90 °C, respectively. The saccharified reactions were added to α-amylase (33.75 U/g starch), pullulanase (10 U/g starch) and β-amylase (150 U/g starch) at 50 °C for 24 h. Each value is expressed as the mean ± S.D. (n = 3).

![Fig. 4](image4.png)

Fig. 4. Effect of time variation on soluble sugar concentration/conversion ratio of fermented re-saccharified liquid catalyzed by recombinant PTTS at 30 °C. The conversion ratio of trehalose (%) was defined as trehalose (g)/maltose (g) × 100%. Each value is expressed as mean ± S.D. (n = 3).
concentration (%) were analyzed by HPLC and GC, and calculated by an external standard method. Each value is expressed as the mean ± S.D. (because of the lower hydrolytic activity of reaction time to convert all of residual glucose into bioethanol by consumption of trehalose, the combined reaction required a longer concentration at 30°C for 42 h and 36–42 h, respectively. However, during the saccharification and fermentation period, it was interesting to find that the consumption rate of glucose was faster than maltose, compared to those obtained by raw koji material (Fig. 5). Suggesting it might because of the lower hydrolytic activity of Rhizopus crude enzyme and/or a partial of glucose was utilized as carbon source for yeast cell growth. The highest bioethanol concentration, 1.65 ± 0.26%, and 2.08 ± 0.28%, was therefore obtained by using 10% and 15%, respectively, of Rhizopus crude enzyme solution with 8% of yeast concentration at 30°C for 42 h (data not shown). Without consumption of trehalose, the combined reaction required a longer reaction time to convert all of residual glucose into bioethanol by yeast fermentation, whereas the price of both Rhizopus crude enzyme solution and S. cerevisiae was lower than raw koji material. Therefore, based on the “natural” and “economical” reasons, the home-made raw koji material would be the most suitable choice with a shorter reaction time and lower energy cost for further industrial applications.

For gluconic acid production, the result showed that the conversion of all residual glucose to gluconic acid was stopped and its concentration remained constant after 12 h of reaction time. Similarly, regardless of whether 13, 26 or 40 U/g of glucose oxidase was used, the gluconic acid production was stopped and its concentration remained steady after 12 h of reaction time, whereas the gluconic acid production yield increased as the enzyme amount increased (data not shown). Such a drop of pH might be because of a higher amount of gluconic acid production and a lower pH of 3.0 would result in slower self-hydrolysis rate of glucono-lactone, a competitive inhibitor of glucose oxidase, to suppress the catalytic activity of the enzyme (Wilson & Turner, 1992). To remain at a steady pH for the reaction environment, the addition of neutralizing agents such as calcium hydroxide (Ca(OH)₂) seems to be a feasible strategy to precipitate the gluconic acid as calcium gluconate (Sumitra, Fontanille, Panday, & Larrochel, 2006). In this study, although all experiments represented a similar tendency for both glucose depletion and gluconic acid production, the highest enzyme amount (40 U/g rice) that could be achieved resulted in a 1.5-fold higher concentration of gluconic acid (24.47 ± 0.33 mM) than that obtained by 13 U/g enzyme rice (data not shown). Therefore, the enzymatic conversion of all residual glucose into gluconic acid without consuming any trehalose has been developed and would be another efficient bio-treatment method to simplify separation of trehalose from rice hydrolysate.

3.5. Different refinements for crystalline trehalose production

Traditionally, the mixed sugar solution could be purified by the lime, sulfitation, ionic exchange or a combined method. However, there was only limited information in regard to the trehalose purification process with timesaving and high purity advantages. Therefore, in this study, we monitored the conductivity variation of crude trehalose solution to compare the purification efficiency of different methods. Since lower conductivity means more ions were removed and a higher purity of sugar was achieved, the highest conductivity (5580 ± 59.57%) by the lime method might due to the calcium hydroxide [Ca(OH)₂] remaining in the trehalose preparation. On the contrary, the conductivity of the continuous method with a dual column was significantly reduced to the lowest conductivity (53.88 ± 0.18%), which was 6.5-fold lower than that of the batch method, whereas the batch system was 4.3-fold lower in conductivity (349.53 ± 0.87%) than that obtained by first cation exchange step (1518.67 ± 10.07%) of continuous method. Overall, in comparison to the lime method, it is clear that the ionic exchange column was the most efficient tool for purification of trehalose. The one step batch method seems to be the most economical way, however, for high quality demands, and the continuous one would be the most suitable choice for the manufacturing industry.

After filtration, decoloration, ionic exchange and sedimentation steps, the residual trehalose-rich solution could be easily crystallized by ethanol precipitation with a 92.6 ± 0.02% purity of trehalose and a 94.2% recovery of trehalose.

Overall, we found that the maltose yield (77.02 ± 0.64%) produced from the liquefied rice starch hydrolysate in a 5 l fermentor system was 5.4% lower than that obtained by the small scale flask method at 50°C for 18–22 h. At a lower reaction temperature (30°C), the trehalose conversion rate of our recombinant PTTS can be reached 64.63 ± 4.05% in a 5 l fermentor system which was ~30% higher than that of the small scale flask method (Chang et al., 2010), suggesting a higher reaction temperature would reduce the catalytic efficiency of the enzyme as described in previous reports (Chen et al., 2006; Schiraldi et al., 2002). The large quantity production of the recombinant PTTS to convert low value starch-rich of rice raw materials into value-added trehalose product in a 5 l fermentor system was therefore successfully established in this study.

Three bio-treatment methods to exhaust all residual maltose, maltotriose and glucose and convert them into bioethanol or gluconic acid for high efficiency trehalose separation and purification from the complex rice starch saccharified solution on a large scale was also developed in this study. Without adding any artificial cul-

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**Fig. 5.** Effect of different addition concentration of raw koji material on soluble sugar content and bioethanol production variation by simultaneous re-saccharification and bioethanol fermentation. (A)-(C) Indicate 0.1%, 0.5%, and 1% of raw koji material were added at 30°C, respectively. The soluble sugar concentration (mM) and bioethanol concentration (%) were analyzed by HPLC and GC, and calculated by an external standard method. Each value is expressed as the mean ± S.D. (n = 3).
ture solution and any consumption of trehalose, all residual glucose could be fermented by 1% of raw koji material with a ~10% higher bioethanol concentration (3.61 ± 0.07%) and a 28 h shorter fermentation time than those obtained by our previous report (Chang et al., 2010). Also, it is feasible to convert the residual glucose into another valuable product (glucconic acid) and the highest concentration of gluconic acid (24.47 ± 0.33 mM) was obtained by using 40 U/g enzyme rice after 12 h of reaction time. By using the batch/continuous ionic exchange process, the trehalose products could be successfully separated, crystallized and identified with a 92.6 ± 0.02% purity and 94.2% recovery yield, respectively.

In conclusion, we found that the production yield and quality of trehalose in a larger scale fermentor system was as good as that obtained by the small scale flask, indicating that the combined bioprocess for multifunctional trehalose production was pretty stable and the production scale has been successfully expanded in this study. Without any chemical addition, such a “green” bioprocess can be used to convert low price starch-rich crops (i.e. sweet potato, corn or potato) into a number of valuable products (i.e. trehalose, bioethanol or gluconic acid) in one step. The efficient bio-treatment process would benefit farmers by increasing the economical value of their agriculture produces for higher competitiveness in the future.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2012.03.065.

References


