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# A low-cost production, characterization, and application of raw starch degrading enzyme from the thermophilic filamentous bacterium, *Laceyella sacchari* P43

Wanthanee Khetkorn[1], Ausawadee Phonlamai[1], Chatsuda Sakdapetsiri[2], Vichien Kitpreechavanich[3], Thanasak Lomthong[1]*

[1]Division of Biology, Faculty of Science and Technology, Rajamangala University of Technology Thanyaburi, Pathumthani 12110, Thailand.
[2]Department of Plant Pathology, Faculty of Agriculture at Kamphaengsaen, Kasetsart University Kamphaengsaen Campus, Nakhon Pathom 73140, Thailand.
[3]Department of Microbiology, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand.

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**ABSTRACT**

Raw starch degrading enzyme (RSDE) is a group of enzymes that can directly degrade starch granules at low temperatures without the gelatinization process resulting in reduced cost and energy consumption. This work aimed to optimize the enzyme production, purification, and characterization of RSDE from *Laceyella sacchari* P43. The highest enzyme production was found at 171.8 ± 6.53 U/mL when using the low-cost agricultural crop and by-products from the brewing industry, including broken rice powder and dried brewer's yeast at 8.0 and 4.7 g/L, respectively. The optimized medium resulted in a 5.03-fold improvement in RSDE production and an 8.5-fold reduction in cost compared to the non-optimized medium. The optimum physical factors enhancing RSDE production were pH at 6.5 and 50°C. The fed-batch fermentation was carried out in a 3.0 L airlift fermenter, which showed the highest enzyme production at 201 ± 11.53 U/mL. The purified enzyme has a molecular weight of 50 kDa and 35.2 purification folds with optimum activity at pH 6.0 and 55°C. The enzyme stimulated the activity by $Co^{2+}$, $Mn^{2+}$, and $Ca^{2+}$ and was strongly inhibited by N-Bromosuccinimide, which confirmed that it was α-amylase. The crude enzyme could hydrolyze the low-grade broken rice powder, yielding 40.31 ± 2.21 g/L after incubating for 9 h. This study developed the enzyme production process using low-cost substrates and enzyme application for sugar syrup production, which could be applied for further industrial application.

## 1. INTRODUCTION

Starch is one of the most abundant carbohydrates found in higher plant biomass, second only to cellulose, and is extensively utilized as a substrate for sugar syrup manufacture for various purposes, such as bioethanol, lactic acid, and fermentation foods [1-3]. The two main steps in most starch-based bioprocesses are (i) starch liquefaction, which typically involves thermostable α-amylase gelatinizing the starch substrate at 80–105°C**.** And (ii) the liquefied starch was saccharified by glucoamylase after cooling down the reaction to 60°C, then converting the glucose released from the starch to a target product. From the conventional process, high energy consumption and operation costs were required at an industrial level, which estimated that the energy consumption of the pre-cooking step accounts for 30–40% of total production expenses [4-6]. To address this issue, the raw starch degrading enzyme (RSDE) is interested in lowering energy consumption in various starch conversion processes [7].

RSDE refers to a class of enzymes that hydrolyze granular untreated starch at temperatures below the liquefaction temperature, resulting in significant energy savings compared to conventional procedures [6,8]. RSDE contained a raw starch binding site that could contact the starch granule without the gelatinization process (starch liquefaction) [9]. The degradation of RSDE at a lower temperature than the gelatinization step could reduce the viscosity problem from starch gelatinization at high temperatures [6]. RSDE has been used in a variety of applications, such as bioethanol production [2,7], bacterial cellulose production [3], and bakery production [10]. From the previous studies, RSDE can produce from various microorganisms such as *Aspergillus* sp. [7] *Penicillium oxalicum* [8], and *Laceyella sacchari* [9]. *L. sacchari* was promised as a candidate for starch-digesting enzyme production [11-13]. Recently, RSDE was isolated from *L. sacchari* LP175, which is α-amylase, and showed the optimum activity against raw starch at pH 6.5 and 50°C [11]. The *L. sacchari*

*Corresponding Author:
*Thanasak Lomthong,*
*Division of Biology, Faculty of Science and Technology, Rajamangala University of Technology Thanyaburi, Pathumthani 12110, Thailand.*
*E-mail: thanasak_l@rmutt.ac.th*

P43 was an alternative strain previously identified. It could grow at a high temperature and use agricultural crops as the substrate, which is interesting for use as a candidate to produce thermostable RSDE at the industrial level [14].

Broken rice is rice that has broken during the milling process and has fragments that are less than three-quarters the length of the whole intact grain [15]. The process yielded 14% of the entire amount of broken rice, usually considered low-value waste and mainly utilized for animal feed [16-17]. The application of RSDE for syrup manufacturing is of great interest to take advantage of the low-cost value of broken rice [15]. Dried brewer's yeast is a by-product of the brewing industry, rich in vitamins, amino acids, and microminerals [18-19]. The price of dried brewer's yeast was lower than the commercial yeast extract powder by about 25 times, which has the potential to be used as a substrate for microbial growth and enzyme synthesis.

Therefore, this work aimed to optimize the RSDE production from the *L. sacchari* P43, using the low-cost agricultural crop as substrate. The enzyme was purified, characterized, and applied to produce sugar syrup from the low-grade broken rice powder.

## 2. MATERIALS AND METHODS

### 2.1. Substrate Preparation and Chemical Composition Analysis

The broken rice was obtained from the rice mill village in Nong Don District, Saraburi Province, Thailand. The obtained broken rice was then ground to a powder using an electric grinder, put through a sieve with a mesh size of 60 (or 0.25 mm), and kept in a dry place until it was used. The total starch assay (Megazyme International Ireland, Wicklow, Ireland) was used to determine starch content, whereas the AOAC methods [20] were used to determine protein, fat, and fiber content, and amylose content was determined using amperometric titration with potassium iodate solution [21,22] at Kasetsart University. In the case of enzyme production, raw broken rice powder was wrapped in alumina foil and sterilized in an autoclave to separate it from the solution medium.

Dried brewer's yeast was purchased from the local feed market in Nakhon Ratchasima Province, Thailand. Starch content, protein, fat, and fiber were analyzed as described above.

### 2.2. Microorganisms and Cultivation

A potent bacterial strain of RSDE production, *L. sacchari* P43, isolated from Phetchaburi province in Thailand, was obtained from Kasetsart University's Department of Microbiology [14]. The P43 strain was grown on the nutrient broth (100 mL) (3.0 g/L beef extract and 5.0 g/L peptone) in a 250 mL Erlenmeyer flask at 50°C for 24 h and used as inoculum at 10% (v/v) to obtain an optical density of 0.7 at a wavelength of 600 nm [11].

### 2.3. Optimizing Parameters for Enzyme Production using a Low-cost Media

For RSDE production by P43 strain, the central composite design (CCD)-based response surface methodology (RSM) was used to determine each parameter's individual and combined effects for enzyme production under batch fermentation in a shaking flask. The broken rice powder and dried brewer's yeast concentrations were used as carbon and nitrogen sources, respectively, as indicated in detail in Table 1. There are five levels of CCD, namely 0, +1, −1, −α, and +α [10]. The enzyme production medium was prepared in 250 mL of Erlenmeyer flask following the CCD design with 50 mL of the medium volume with an initial pH of 6.5. All the flasks were sterile by autoclaving at 121°C for 15 min. The 10% (v/v) of P43 inoculum was fixed with all experimental runs and incubated in a rotary shaker (150 rpm) at 45°C for 48 h. At the end of fermentation, the culture supernatant was centrifuged (5000 rpm) at 4°C for 10 min, and the cell-free supernatant of each experimental run was used to determine the RSDE activity. STATISTICA 12 for Windows™ was used to analyze the data statistically [15]. The optimum concentrations of broken rice powder and dried brewer's yeast were used to validate enzyme production [23].

The physical factors' effect on enzyme production was investigated using the optimum concentrations of broken rice powder and dried brewer's yeast, including pH and temperature [24]. The effects of pHs on the RSDE production were varied at 5.0–8.0 by adjusting the medium's pH using 0.1 N HCl and NaOH. The temperatures varied at 40–60°C using the optimum pH described above.

### 2.4. Fed-Batch Fermentation

A 3.0 L airlift fermenter was used for fed-batch fermentation to increase the volume of enzyme produced per fermentation period using 2.0 L of the optimum medium at optimum pH equipped with oxygen and pH electrodes to scale up enzyme production with 10% (v/v) of P43 inoculum. The fermentation was cultivated at the optimum temperature with 0.5 vvm of aeration rate for 36 h [24]. The sterile feed medium (50 mL) containing 10 g of broken rice powder in distilled water was added to the reactor, and cultivation was continued for 48 h. The samples were taken every 12 h to determine RSDE activity and growth. The lyophilizer (Operon, Korea) concentrated the crude RSDE obtained from *L. sacchari* P43 after centrifuging (5000 rpm) at 4°C for 10 min. The enzyme powder was dissolved in 0.1 M phosphate buffer (pH 6.0) and used for enzyme purification and application [9].

### 2.5. Purification of RSDE Produced from *L. sacchari* P43

The purification procedure was followed by Lomthong *et al*. [9] and was carried out at 4°C throughout. The enzyme was purified using an anion exchange column (5.0 mL, HiTrap Q HP) pre-equilibrated with 50 mm Tris-HCl at pH 8.0, which connected to the FPLC system Akta purifier (GE Healthcare, Amersham Pharmacia Biotech). The enzyme was then eluted with a linear gradient NaCl (0-1.0 M) and exchanged the pH to 6.0 using Ultra-15 Centrifugal Filter Units with a 30 kDa cutoff (Merk Millipore, Bedford, MA, USA). The concentrated enzyme was then second purified by raw starch absorption by mixed with 10.0% (w/v) of raw cassava starch, as Lomthong *et al*. [9] reported.

All samples obtained from each purification step were visualized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and zymogram using a Bio-Rad™ system [9]. Precision Plus Protein™ was used as a protein standard to calculate the molecular mass of purified RSDE [25].

**Table 1:** Five levels of central composite design for RSDE optimization by P43 strain between Broken rice powder ($X_1$) and Dried brewer's yeast ($X_2$).

| Independent variables | Level | | | | |
|---|---|---|---|---|---|
| | −1.414 | −1 | 0 | 1 | 1.414 |
| $X_1$ Broken rice powder concentration (g/L) | 2.34 | 4.0 | 8.0 | 12.0 | 13.66 |
| $X_2$ Dried brewer's yeast concentration (g/L) | 1.47 | 2.5 | 5.0 | 7.5 | 8.54 |

RSDE: Raw starch degrading enzyme.

### 2.6. Characterization of the RSDE

#### *2.6.1. Effects of pH and temperature*

The optimum pH of the purified enzyme was determined by measuring the enzyme activity at standard conditions with different pHs (3.0–12.0) of the reaction: pH 3.0–5.5 (acetate buffer), pH 6.0–7.5 (phosphate buffer), pH 8.0–10.0 (Tris-HCl buffer), and pH 10.0–12.0 (glycine-NaOH buffer) [11,26].

The optimal temperature was obtained by measuring RSDE activity at various incubation temperatures (30–80°C) while maintaining the optimal pH. Relative enzyme activity was used to express enzyme activity [11,26].

#### *2.6.2. Effect of metal ions, detergents, and inhibitor*

The effects of metal ions, detergents, and inhibitors were determined by assaying the RSDE activity at standard assay conditions with the presence of 1 mM of each metal ion ($Zn^{2+}$, $Co^{2+}$, $Mg^{2+}$, $Mn^{2+}$, $K^{+}$, $Na^{+}$, $Ca^{2+}$, $Fe^{3+}$, $Hg^{2+}$, and $Cu^{2+}$), 1.0%, (v/v) detergents (Triton X-100, Tween 20, Tween 80, and SDS), and 5.0 mM inhibitors N-Bromosuccinimide (N-BS) and ethylenediaminetetraacetate (EDTA) using optimum pH buffer and temperature. The reference value (100%) was considered using the enzyme's activity without metal ions, detergents, and inhibitors at standard assay conditions [9].

#### *2.6.3. Raw starch digestibility*

Raw starch digestibility was determined by assaying the RSDE activity of the purified enzyme with 2% (w/v) of various kinds of starch and flour, including potato starch was obtained from Thai Found Industry Co., Ltd. (Bangkok, Thailand), cassava starch was obtained from Tongchan Co., Ltd. (Bangkok, Thailand), rice flour was obtained from Choheng rice vermicelli factory Co., Ltd. (Nakhon Pathom, Thailand), corn starch obtained from Charoenworrakit Co., Ltd. (Samutprakarn, Thailand) and wheat flour was obtained from United flour mill public Co., Ltd. (Samutprakarn, Thailand). The reaction was incubated at the optimum pH and temperature for 1.0 h. A relative of enzyme activity was used to express the raw starch digestibility of each substrate [11]. Scanning electron microscopy (SEM) was used to examine the structure of native and digested starches and flours with RSDE. Starch granules from the reaction were washed in distilled water, dried at 50°C for 12 h, and then examined by SEM at 10.0 kV (model SU8020; Hitachi, Tokyo, Japan). [15].

### 2.7. Production of Sugar Syrup from Low-grade Broken Rice

The low-grade broken rice was used as a substrate for sugar syrup production by RSDE from *L. sacchari* P43. The reaction was operated in 250 mL of Erlenmeyer flask containing 50 mL of crude enzyme dissolved in 0.1 M phosphate buffer pH 6.0, which adjusted the final enzyme activity at 300 U/mL with broken rice powder at 100 g/L. The reaction was incubated at 55°C for 9 h. Samples were obtained regularly to determine reducing sugars using the dinitrosalicylic acid technique [27]. Thin-layer chromatography was used to qualitatively detect liberated sugars from the hydrolysis, as described by Sassaki *et al*. [28]. The residue of broken rice powder following hydrolysis with RSDE from the P43 strain was examined under SEM as described above.

### 2.8. Analysis

#### *2.8.1. Enzyme activity*

The RSDE activity was calculated from the amount of liberated reducing sugars released from the hydrolysis of cassava starch (2.0% [w/v]) at optimum conditions. The quantity of enzyme releasing 1 µg of glucose equivalent per minute was defined as one unit of RSDE activity [11,29].

#### *2.8.2. Growth determination*

The spread plate method was used to count total plate counts to determine the growth of the P43 strain. The viable cell was estimated as log CFU/mL [30].

#### 2.8.3. Statistical analysis

Values from several treatments were analyzed using the one-way analysis of variance at a 95% confidence level**.** The Duncan multiple range tests were used to evaluate mean differences.

## 3. RESULTS AND DISCUSSION

### 3.1. Chemical Composition of Broken Rice Powder and Dried Brewer's Yeast

The chemical compositions of broken rice powder and dried brewer's yeast are shown in Table 2. Broken rice powder was rich in starch content (86.53 ± 0.25%) with 23.53 ± 0.44% amylose content, indicating the appropriate substrate for inducing RSDE production by P43 strain. In comparison, dried brewer's yeast contained a high amount of protein (48.80 ± 0.06%), which can be used as a low-cost nitrogen source for bacterial growth and enzyme production. Dried brewer's yeast is a by-product of the brewing industry, usually used as a low-cost functional food ingredient, fermentation substrate, and feed additive [19,31]. Pejin *et al*. [32] reported that brewer's yeast could be used as a substrate for bacterial growth and lactic acid production. The previous study produced RSDE from *L. sacchari* LP175 using cassava starch and yeast extract as substrates [11]. Therefore, this study will provide a low-cost medium for RSDE production using broken rice powder and dried brewer's yeast by *L. sacchari* P43.

### 3.2. Optimizing Parameters for Enzyme Production Using a Low-cost Media

According to the experimental RSM with a CCD mentioned above, the combined effects of broken rice powder and dried brewer's yeast concentrations on RSDE production by the P43 strain were determined. The results of each experimental run are shown in Table 3. The highest enzyme production was found in runs 9–11. The results of 11 runs were subjected to construct the response and contour plots which are shown in Figure 1. The response showed that the enzyme was found at high concentration when using broken rice powder in the 6.0–10.0 g/L range and dried brewer's yeast in the 4.0–6.0 g/L [Figure 1]. The predicted equation ($Y$) was provided from the model shown below, which can be used to predict the enzyme production at the optimum concentration of two substrates.

$$Y = -53.1322 + 35.5343X_1 + 37.2761X_2 - 2.1293X_1^2 - 3.7166X_2^2 - 0.3176\,X_1X_2$$

Where $Y$ is the predicted value of RSDE production by *L. sacchari* P43, $X_1$ and $X_2$ are broken rice powder and dried brewer's yeast concentrations, respectively.

The coefficient of determination ($R^2$) was 0.9603 for RSDE production, which could be acceptable for application as the $R^2$ value was 0.7–0.99 [33]. The corresponding $p$ values of broken rice powder ($X_1$) and dried brewer's yeast ($X_2$) were 0.0003 and 0.002, which showed the significance at the $P < 0.05$ for RSDE production by *L. sacchari* P43 [Supplementary Table S1].

**Table 2:** Chemical compositions of broken rice powder and dried brewer's yeast.

| Component (%) | Analysis (%) | |
|---|---|---|
| | Broken rice powder | Dried brewer's yeast |
| Starch | 86.53±0.25 | 12.39±0.39 |
| Protein | 8.54±0.01 | 48.80±0.06 |
| Fat | 0.64±0.01 | 0.12±0.01 |
| Fiber | 0.21±0.00 | 0.11±0.01 |
| Amylose | 23.53±0.44 | ND |

Values are averages of three determinations. ND: Not detected.

**Table 3:** Experimental design based on CCD with two parameters for RSDE production by P43 strain, including broken rice powder concentration ($X_1$) and dried brewer's yeast concentration ($X_2$).

| Run | Level | | Actual level | | RSDE (U/mL) | |
|---|---|---|---|---|---|---|
| | $X_1$ | $X_2$ | $X_1$ | $X_2$ | Observed | Predicted |
| 1 | 0 | −1.414 | 8 | 1.47 | 140.40±4.18 | 137.90 |
| 2 | 1 | −1 | 12 | 15 | 120.83±4.08 | 127.10 |
| 3 | −1 | −1 | 4 | 2.5 | 125.70±3.44 | 121.72 |
| 4 | 1.414 | 0 | 13.66 | 5 | 111.87±9.76 | 106.73 |
| 5 | −1.414 | 0 | 2.34 | 5 | 98.77±9.46 | 108.11 |
| 6 | 1 | 1 | 12 | 7.5 | 108.83±7.54 | 108.60 |
| 7 | −1 | 1 | 4 | 7.5 | 126.40±8.62 | 115.92 |
| 8 | 0 | 1.414 | 8 | 8.54 | 113.77±10.39 | 120.45 |
| 9 | 0 | 0 | 8 | 5 | 175.77±11.47 | 175.63 |
| 10 | 0 | 0 | 8 | 5 | 175.03±5.06 | 175.63 |
| 11 | 0 | 0 | 8 | 5 | 176.07±9.88 | 175.63 |

Values are averages of three determinations. CCD: Central composite design, RSDE: Raw starch degrading enzyme.

From the predicted equation, the highest enzyme production was calculated at 176.02 U/mL when using broken rice powder and dried brewer's yeast at 8.0 and 4.70 g/L, respectively. The optimum concentrations of broken rice powder and dried brewer's yeast were used to validate the model at the same conditions, which found that the P43 strain could produce an enzyme at 171.8 ± 6.53 U/mL, which is close to the predicted activity from the model. From the predicted and actual values of the enzyme production, we could conclude that the model was accurate and appropriate for enzyme production by *L. sacchari* P43.

Broken rice powder has been described as a low-cost source of starch that may be used to induce RSDE synthesis [15]. In contrast, brewer's yeast is a rich source of nitrogen, amino acids, and vitamins that can support the growth of bacterial strains [19]. From the previous study, Sankeerthana *et al*. [34] utilized broken rice waste as a substrate for protease production by *Aspergillus flavus* under solid-state fermentation. Ofongo *et al*. [35] also applied broken rice substrate for cellulase enzyme production by *Aspergillus niger*, *Trichoderma viride,* and *Rhizopus oryzae* under submerged fermentation, which revealed that broken rice can be used as a low-cost substrate for various enzymes production.

The optimization process in this study could improve the enzyme production up to 5.03 folds compared to the un-optimized medium (34.16 ± 3.11 U/mL) when using 2.80 g/L yeast extract and 4.93 g/L cassava starch as substrates [11]. The production cost per liter of the optimal medium obtained through the RSM approach (broken rice powder and dried brewer's yeast) was 0.023 USD/L. In comparison, the non-optimized medium cost (cassava starch and yeast extract) was 0.195 USD/L, which showed an 8.5-fold reduction in cost compared with the non-optimized medium, as shown in Table 4.

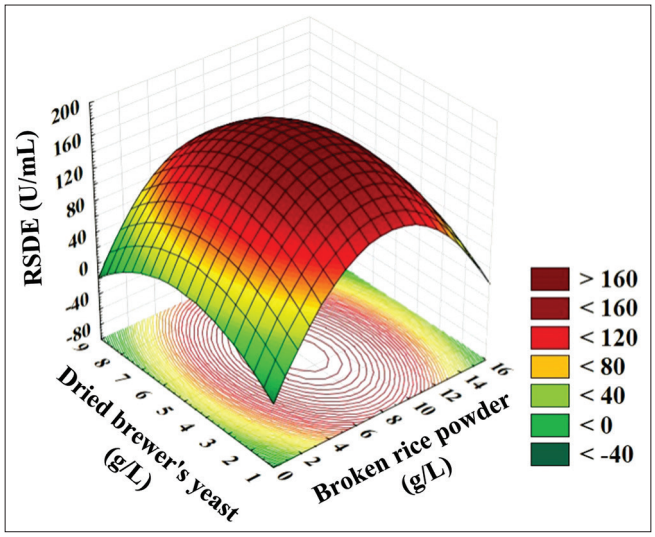


**Figure 1:** Response and contour plots of combined effects between broken rice powder ($X_1$) and dried brewer's yeast ($X_2$) concentrations for raw starch degrading enzyme production by *Laceyella sacchari* P43.

The optimum physical factors for RSDE production by *L. sacchari* P43 were pH at 6.5 and temperature of 50°C, which yielded 188.6 ± 6.21 U/mL, as shown in Figure 2. The pH of the medium was the main effect on microbial metabolism, which could support the enzymes in the cell and balance the cell protein structure. *L. sacchari* was reported as a thermophilic filamentous bacterium that preferred to grow at high temperatures (45–55°C) since it contained some heat shock protein genes that may be expressed and function at high temperatures as reported by Kaur *et al*. [36]. From the previous reports, *L. sacchari* TSI-2, isolated from a hot spring in India, could grow at the optimum pH and temperature of 7.0 and 50°C, respectively [12]. Then the optimum pH of 6.5 and temperature of 50°C were used to scale up under fed-batch culture fermentation.

### 3.3. Fed-Batch Fermentation

As described above, fed-batch culture fermentation was performed in a 3.0 L airlift fermenter to enhance the volume of enzyme production per fermentation period. Scaling up of enzyme production could reduce the fermentation time and operation process and may improve enzyme production [15,30]. After 36 h of culture, the broken rice powder was added to the reactor to stimulate the production of enzymes. The maximum enzyme production was increased to 201 ± 11.53 U/mL, as shown in Figure 3. Broken rice powder contains high starch content, which could induce RSDE production by the P43 strain. From the previous study, *L. sacchari* LP175 was successful for PLA degrading enzyme and RSDE production under a fed-batch fermentation process by adding PLA powder at 0.52 g/L and raw cassava starch at 3.34 g/L at 30 h of cultivation [30]. Li *et al*. [37] also used the fed-batch strategy for raw starch-degrading α-amylase production in *Bacillus subtilis* to obtain high cell density and enzyme activity. Then, in this work, fed-batch culture could increase enzyme production up to 5.88 times over un-optimized medium and conditions, and the produced enzyme was

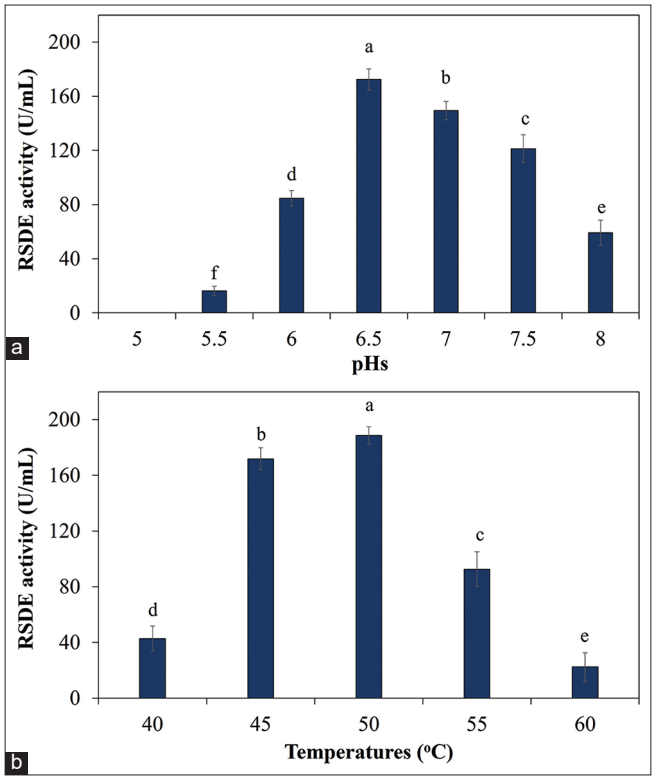


**Figure 2:** Effects of physical factors on raw starch degrading enzyme production by P43 strain. (a) pH and (b) temperature. Where error bars = ±SD, different lowercase letters above the bar indicate significant ($P < 0.05$) differences among means.

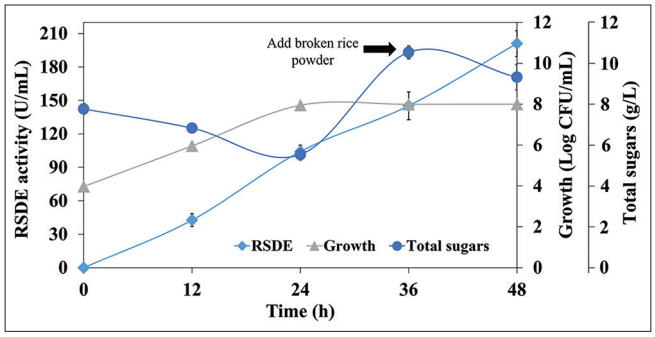


**Figure 3:** Fed-batch culture of raw starch degrading enzyme production by *Laceyella sacchari* P43 without pH control at 50°C for 48 h.

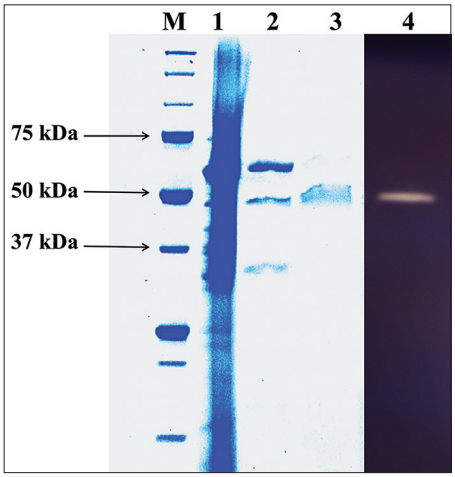


**Figure 4:** The sodium dodecyl sulfate polyacrylamide gel electrophoresis chromatogram of raw starch degrading enzyme produced from P43 strain: 1: crude enzyme; 2: partially purified enzyme after HiTrap Q; and 3: purified enzyme by raw starch adsorption.

utilized for further characterization and application.

### 3.4. Purification of RSDE Produced from *L. sacchari* P43

The crude RSDE produced from *L. sacchari* P43 was purified by anion exchange column (HiTrap Q) and adsorption on raw cassava starch powder, as shown in the results at each step in Supplementary Table S2. The specificity was increased to 2973.8 U/mg with 35.2 folds of purification and 62.3% enzyme yields from the purification process [Supplementary Table S2]. The anion exchange column could separate three bands of the proteins from several proteins in the crude enzyme [Figure 4]. Raw starch adsorption could separate the RSDE band from the contaminated protein, as shown in the SDS-PAGE gel chromatogram [Figure 4]. The zymogram also confirmed the activity of the single-band protein purified from the processes, which could estimate the molecular weight of 50 kDa.

### 3.5. Characterization of the RSDE

The optimum pH and temperature of RSDE produced from *L. sacchari* P43 showed an optimum condition at pH 6.0 and 55°C [Figure 5]. The purified enzyme also increased activity when metal ions were added, including $Co^{2+}$, $Mn^{2+}$, and $Ca^{2+}$ [Table 5], compared to the control without metal ions. While $Zn^{2+}$, $Fe^{3+}$, $Hg^{2+}$, and $Cu^{2+}$ strongly inhibited the RSDE activity. The RSDE produced from *L. sacchari* P43 showed resistance in the presence of Triton X-100, Tween 20, and Tween 80, slightly inhibited by these detergents [Table 5]. SDS inhibited the RSDE activity by more than 50%, similar to EDTA. N-BS also strongly inhibited RSDE activity [Table 5] which could be confirmed that RSDE produced from *L. sacchari* P43 was α-amylase [9].

**Table 4:** Cost of media for RSDE production by *Laceyella sacchari* P43.

| Medium | Production cost per L (USD) | RSDE production (U/mL) |
|---|---|---|
| Cassava starch+yeast extract | 0.195 | 34.16±3.11 |
| Optimized medium in this study | 0.023 | 171.8±6.53 |

RSDE: Raw starch degrading enzyme.

In the case of raw starch digestibility, the RSDE produced from *L. sacchari* P43 could digest all kinds of starch and flour, as shown in Table 6. The difference in digestion ability may result from the different starch granules' shape and size, affecting the enzyme's binding ability to substrate. The scanning electron micrographs of all starches and flours are shown in Figure 6, which form pits and holes on the surface and cause changes in the structure of the starch granules after hydrolyzing by RSDE for 9 h. These results confirmed the ability and potential for application of RSDE produced from *L. sacchari* P43 in starch digestion with various proposes at an industrial in the future.

### 3.6. Production of Sugar Syrup from the Low-Grade Broken Rice

The low-grade broken rice was used as a substrate for sugar syrup production by RSDE produced from *L. sacchari* P43, which obtained

reducing sugar at 40.31 ± 2.21 g/L after incubating at 55°C and pH 6.0 for 9 h [Figure 7a]. RSDE from *L. sacchari* P43 generated glucose, maltose, maltotriose, and oligosaccharide from the hydrolysis of broken rice powder [Figure 7b], which were slightly increased after 3 h of incubation and increased exponentially after 6 h of hydrolysis from the scission of an oligosaccharide to short-chain sugar products. Moreover, the presence of starch-degrading enzymes in uncooked broken rice flour, such as glucoamylase, may synergistically hydrolysis oligosaccharide to short-chain sugar products from the action of RSDE to glucose as reported by Lomthong *et al*. [2]. The environmental impact includes reduced energy consumption during hydrolysis as well as the expense of additional glucoamylase enzymes required in the traditional procedure [14].

**Table 5:** Effects of metal ions and reagents on the activity of purified RSDE produced from *Laceyella sacchari* P43.

| Metal ion and reagents | Relative activity (%) |
|---|---|
| Control | 100 |
| Metal ions (1 mM) | |
| $Zn^{2+}$ | 64.2±0.63 |
| $Co^{2+}$ | 122.6±2.87 |
| $Mg^{2+}$ | 102.6±3.90 |
| $Mn^{2+}$ | 150.4±3.90 |
| $K^{+}$ | 103.9±2.60 |
| $Na^{+}$ | 106.7±1.62 |
| $Ca^{2+}$ | 121.3±3.35 |
| $Fe^{3+}$ | 85.5±1.81 |
| $Hg^{2+}$ | 42.4±3.00 |
| $Cu^{2+}$ | 50.3±2.60 |
| Detergents (1%, (v/v)) | |
| Triton ×-100 | 97.4±2.10 |
| Tween 20 | 79.5±1.80 |
| Tween 80 | 86.5±2.65 |
| SDS | 41.4±2.76 |
| Inhibitors (5 mM) | |
| N-BS | 30.6±2.35 |
| EDTA | 49.5±1.57 |

RSDE: Raw starch degrading enzyme, SDS: Sodium dodecyl sulfate, EDTA: Ethylenediaminetetraacetate, N-BS: N-Bromo succinimide.

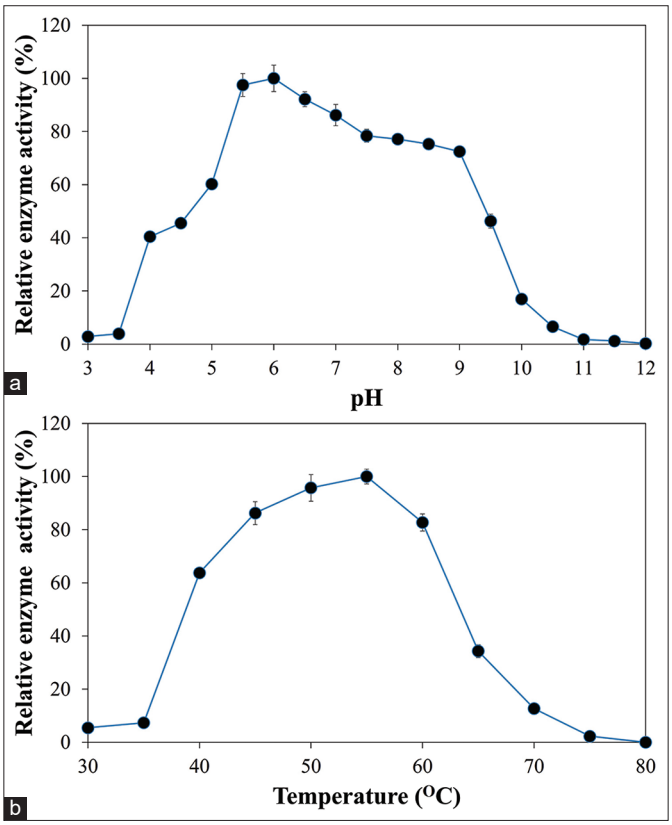


**Figure 5:** Effects of pHs and temperatures on raw starch degrading enzyme activity against cassava starch (2.0%). (a) pH and (b) temperature.

Scanning electron micrographs of native and digested broken rice powder are shown in Figure 8. Before digestion, the native broken rice flour was shaped and smoothed on the surface. However, the digested powder displayed a rough surface and tiny holes around the structure,

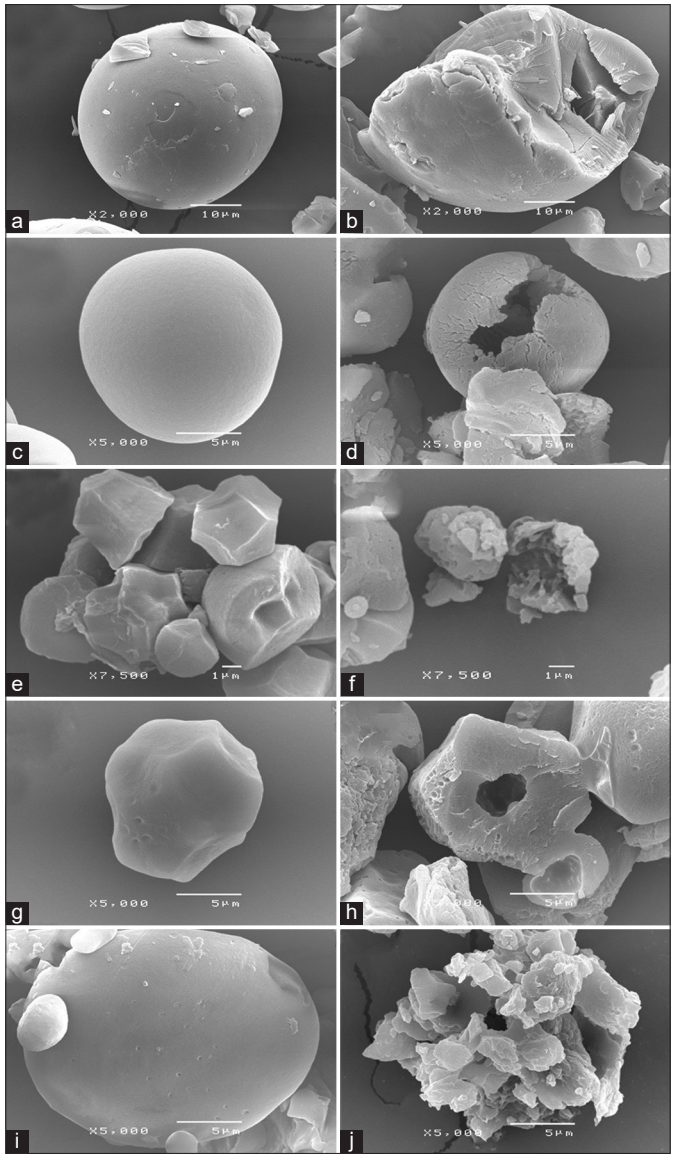


**Figure 6:** Electron micrographs of native and digested various kinds of the substrate by raw starch degrading enzyme produced from *Laceyella sacchari* P43 after incubation at 55°C pH 9.0 for 1.0 h. (a and b) Potato starch, (c and d) Cassava starch, (e and f) Rice flour, (g and h) Corn starch, (i and j) Wheat flour.

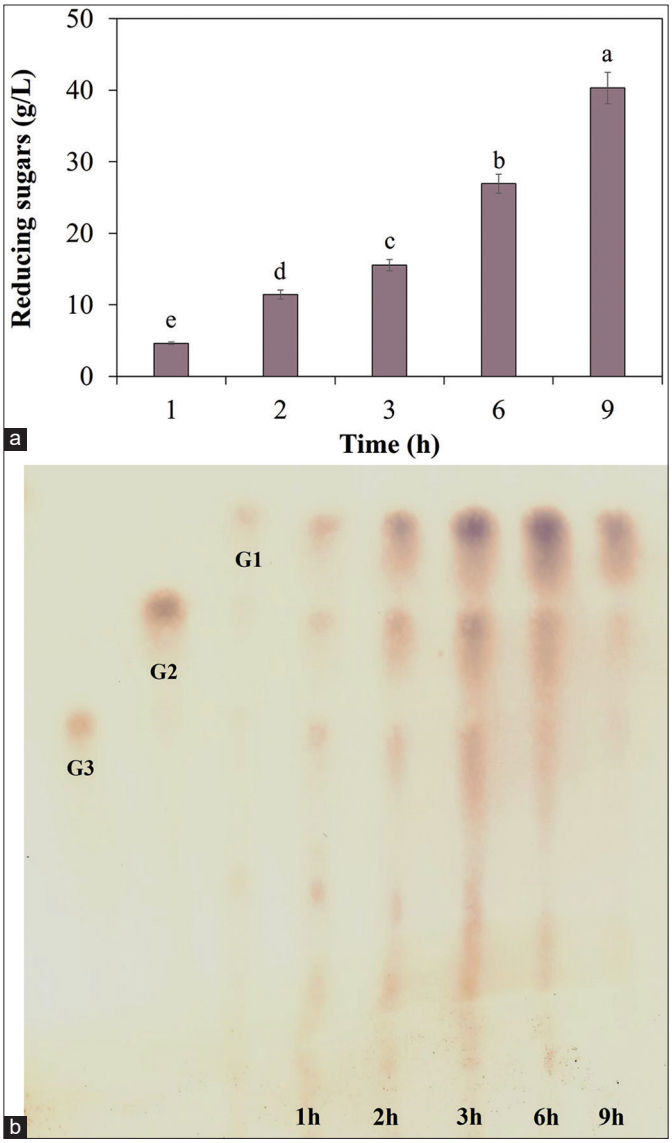


**Figure 7:** Quantitative and qualitative results of sugar syrup from the hydrolysis of broken rice powder (100 g/L) by a raw starch degrading enzyme produced from *Laceyella sacchari* P43 at 55°C pH 6.0 for 9 h. (a) Time course reducing sugars liberated from the hydrolysis reaction, where error bars = ± SD; different lowercase letters above the bar indicate significant ($P < 0.05$) difference among means. (b) TLC chromatogram of hydrolysis products. G1, glucose; G2, maltose; G3, maltotriose.

confirming the capacity of the RSDE derived from the P43 strain to hydrolyze broken rice flour powder for sugar syrup production.

## 4. CONCLUSION

The thermophilic bacterium *L. sacchari* P43 was a potential strain for RSDE production using the low-cost agricultural crop and by-products from the brewing industry as substrates, including broken rice powder and dried brewer's yeast as carbon and nitrogen sources at 8.0 and 4.7 g/L, respectively. The optimized medium resulted in a 5.03-fold improvement in RSDE production and an 8.5-fold reduction in cost compared to the non-optimized medium. The fed-batch fermentation was carried out in a 3.0 L airlift fermenter to improve the enzyme production up to 5.88 times over un-optimized medium and conditions. The RSDE could hydrolyze various kinds of starch and flour and showed the ability to produce sugar syrup from low-grade broken rice substrate without adding glucoamylase at 55°C, which could lower energy consumption and operating expenses. This study provided an alternative thermophilic bacterium strain for RSDE production without the cooling system, which showed the potential for further industrial application.

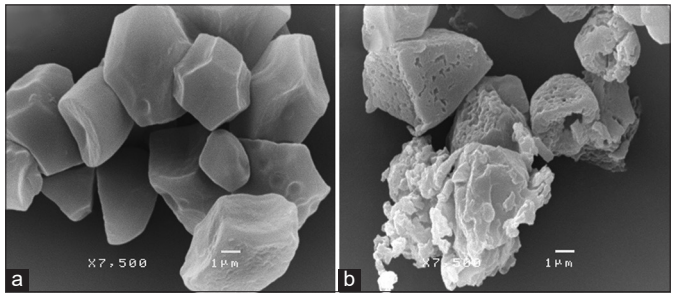


**Figure 8:** Electron micrographs of native (a) and digested (b) broken rice powder by crude raw starch degrading enzyme produced by *Laceyella sacchari* P43 after incubation at 55°C pH 6.0 for 9 h.

**Table 6:** Relative activity of purified RSDE produced from *Laceyella sacchari* P43 on various substrates (2% (w/v)) after incubation at 55°C pH 6.0 for 1.0 h.

| Substrates | % Relative activity |
|---|---|
| Potato starch | 100.0±0.00[a] |
| Cassava starch | 66.7±2.36[c] |
| Rice flour | 88.2±0.76[b] |
| Corn starch | 61.4±2.10[d] |
| Wheat flour | 29.4±0.38[e] |

Values are averages of three determinations. Different letters within the same column are statistically different at $P<0.05$. RSDE: Raw starch degrading enzyme.

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## 6. AUTHOR CONTRIBUTIONS

All authors have read and approved the final manuscript. WK: Investigation, data acquisition, data analysis/interpretation. AP: Data acquisition. CS: Data analysis/interpretation, supervision. VK: Technical or material support, supervision. TL: Concept and design, data acquisition, data analysis/interpretation, drafting the manuscript, and final approval.

## 7. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

## 8. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

## 9. DATA AVAILABILITY

All the data generated and analyzed are included in this research article.

## 10. PUBLISHER'S NOTE

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