

Screening and characterization of β -glucosidase production by *Saccharomyces cerevisiae*

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ABSTRACT

Beta-Glucosidases (BGS) are the group of hydrolase enzymes, involved in the degradation processes and many biological processes. Due to demand, intensive screening of BGS is required to explore the natural microbial source of BGS. The current study deals with isolation and identification of BGS producing *S. cerevisiae* from Thai fruits & beverages and assessment of impact of pH, temperature, and salt concentration on BGS production. About 34 samples were collected. Yeast cells were isolated by plate method and characterized. About ten different strains were isolated and identified. The strain has been confirmed as *S. cerevisiae* through ribosomal sequencing. The optimization of BGS production was achieved by Box-Behnken design and Response Surface Methodology and confirmed that pH 4.0, temperature at 40 °C, and 0.5% of NaCl are optimum conditions. The kinetic analysis suggested that 24 h of incubation achieve the maximum yield. The reported *S. cerevisiae* strain could be the safer source for BGS. Further studies on enzyme recovery and purification will unbolt the way to attain high-quality microbial enzyme.

INTRODUCTION

Beta-Glucosidases (β -D-glucoside glucohydrolases; EC3.2.1.21) (BGS) are the group of heterogeneous glycoside hydrolase enzymes that can break the β -glucosidic linkages of both disaccharide and oligosaccharide or either of them and other conjugates of glucose. Glucosidases are involved in the degradation process of cellulosic biomass, glycolipids, cyanogenesis, and other secondary metabolites. BGS are classified based on several criteria, majorly based on substrate specificity, and nucleotide sequence identity (Henrissat and Davies, 1997). Plant BGS involved in hydrolysis of β -glucoside. BGS has a great potential to be used in various biotechnological processes like liberation of aromas, flavours, and isoflavone aglycones to oligosaccharides and alkylglycosides (Pyo *et al.*, 2005; Yin *et al.*, 2005; Otieno *et al.*, 2006). In addition, microbial BGS has been reported to have the ability to transform plant glucoside isoflavones into aglycones, which is connected to cancer prevention, menopausal symptoms (Pyo *et al.*, 2005),

inflammation and cardiovascular disease (Hu *et al.*, 2009). BGS are present in many organelles and almost in all the living systems from bacteria to mammals with a variety of functionality. BGS are involved in the cellulolytic process of bacteria and fungi. The activity of enzyme is depends on length of the glucose chain and also participate in defense mechanism of plants and insects (Kubicek *et al.*, 1993). BGS in yeasts, such as *Debaryomyces hansenii*, are responsible for the release of the flavoring compounds like nerol, geraniol, linalool, benzyl and phenylethyl alcohols (Rosi *et al.*, 1994). Some of the yeast species were found in grapes with glycosidase activity such as *Hanseniaspora*, *Pichia*, *Candida*, *Saccharomycodes*, *Metschnikowia*, and *Brettanomyces* (Spagna *et al.*, 2002). Delcroix *et al.* (1994) reported about presence of β -glucosidase activity in *Saccharomyces cerevisiae* and some recent studies suggested that fruits, such as apricot, fig, grapes, lychee, mangosteen, and papaya, are the best source of *S. cerevisiae* with BGS (Guimaraes *et al.*, 2006; Maragatham and Panneerselvam, 2011; Khanda and Zainab, 2014). Moreover, BGS have various industrial applications as reviewed by Bhatia *et al.* (2002). Even though much detailed information on different BGS with distinct substrate specificity is available, several research units are intensively working about BGS to figure out the molecular aspects of their substrate specificity and assembly. The source of BGS also influences on their functionality.

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Several factors affect the BGS production, which can be optimized for the optimum production. One of the statistical and experimental designing approaches is a response surface methodology (RSM) (Xu *et al.*, 2010).

The current investigation was conceived and carried out the screening, species identification, and cultural characterization of *S. cerevisiae* with BGS activity from Thai fruits and fruit-derived beverages along with reference strains of *S. cerevisiae*. The impact of three variables like pH, temperature, and concentration of sodium chloride was determined during BGS production by *S. cerevisiae* both in separate and combined manner (RSM).

MATERIALS AND METHODS

Samples and isolation

About 34 Thai fruits and fruit-derived beverages such as coconut, custard apple, durian, grapes, grape juices, sugarcane, lychee, lychee juices, mango, mangosteen, rambutan, and strawberry were collected from the local market, Chiang Mai, Thailand. Five *S. cerevisiae* strains were obtained from Thailand Institute of Scientific and Technological Research (TISTR), which were coded as TISTR 5003, TISTR 5024, TISTR 5051, TISTR 5059, and TISTR 5197. The yeast colonies were isolated as described earlier (Guimaraes *et al.*, 2006).

Biochemical and molecular identification

The isolated yeast strains and the TISTR strains were inoculated in YPD and incubated at 30 °C for 12 h. After incubation, yeast suspensions were prepared and adjusted to 0.2 OD at 600 nm.

All the strains were assessed for D-glucose and galactose fermentation and growth in the presence of D-Ribose, D-Xylose and L-Rhamnose (Vaughan-Martini and Martini, 1993). The results of the biochemical test provide the information about the presence of *S. cerevisiae* in isolates. Then the suspected strain was confirmed through 5.8S rRNA based molecular identification. The nucleotide sequencing of an isolated strain was outsourced from KU-Vector Custom DNA Synthesis Service, Kasetsart University, Bangkok, Thailand.

The sequencing was carried out using the Big Dye Terminator Cycle Sequencing Kit and sequencing machine model ABI 377 (PE Applied Biosystem). The sequence analysis was performed using the BLASTN, National Centre for Biotechnology Information (NCBI) (<http://blast.ncbi.nlm.nih.gov/blast.cgi>). The phylogenetic relationship between the deduced amino acid sequences was constructed by the neighbor-joining method.

Determination of β -glucosidase activity

The β -glucosidase activity was determined by measuring the rate of hydrolysis of *p*-nitrophenyl- β -D-glucopyranoside (*p*NPG, Sigma Chemical Co., Mo., USA) (Hernandez *et al.*, 2003). The β -glucosidase activity was expressed as relative activity (%).

Optimization of growth condition for maximum β -glucosidase production

Strains were inoculated in YPD broth with different pH range (3.5, 4.0, 4.5, 5.0, 5.5, and 6.0) and incubated at 30 °C. The pH of the medium was adjusted using 50 mM citrate phosphate buffer (Arroyo-Lopez *et al.*, 2009). The another set of tubes were prepared with constant pH, determined from previous assay, and incubated at different temperature ranging from 30 to 60 °C (30, 40, 45, 50, 55, and 60 °C). The next set of tubes was prepared with constant pH and was incubated at predicted optimum temperature, but the concentration of NaCl in the medium was varied from 0.3 – 0.8% (w/v) (0.3, 0.4, 0.5, 0.6, 0.7, and 0.8). All the tubes were incubated for 48 h and then supernatants were collected by centrifugation at 4 °C for 10 min at 5,000 \times g for the assessment of β -glucosidase activity.

Optimization of growth condition by Response Surface Methodology (RSM)

RSM was employed to optimize the multiple variants that influence the BGS activity. The Stat-Ease software (Design-Expert 6.0.2, Delaware, USA Echip, 2000-trial version) was used for experimental design and statistical analysis. A three-factor with three-level of Box–Behnken design was selected to evaluate the effect of combination of three independent variables such as pH, temperature (°C), and concentration of NaCl (%; w/v), coded as X1, X2 and X3, respectively. The values were selected from the individual assessment of the influence of selected variables. The minimum and maximum values for pH, temperature, NaCl concentration were set as 3.5 and 4.5, 30 and 50 °C, 0.4 and 0.6%, respectively. The response values for reactive activity (%) of β -glucosidase were -1 (Lower), 0 (Middle), and +1 (Higher). The complete design consisted of 17 experimental sets including five replicates of the center point (WORAHARN *et al.* 2015). The reactive activity (%) of β -glucosidase produced from *S. cerevisiae* was statistically determined by actual response value. The Analysis of Variance (ANOVA) was used for data analysis and $p < 0.05$ was considered as significant. The experimental data were established by second-order polynomial regressed equations. Optimum parameters were defined by the Design-Expert software version 6.0.2.

Kinetics study

The optimum conditions for the maximum β -glucosidase activity were determined from the RSM. β -glucosidase activity was kinetically assessed to find out the optimum incubation time of selected strain to produce/ recover maximum enzyme activity. Strains were inoculated and incubated at the optimum condition for 48 h and samples were collected at different intervals (0, 1, 3, 6, 9, 12, 24, 36, and 48). The samples were subjected to activity determination as detailed above.

Statistical analysis

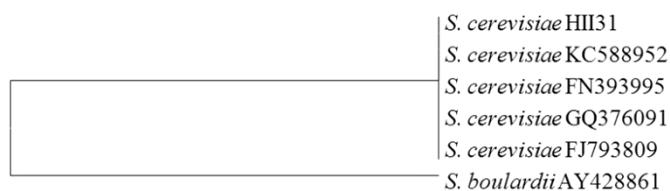
Analysis of variance (ANOVA) with a confident interval of 95% ($p < 0.05$) was reported, and all the experiments were

performed in triplicates. The statistical program SPSS (version 17.0) was used for the analysis of significant differences in enzyme activity at a different temperature, pH, sodium chloride concentration, and kinetic analysis.

RESULTS AND DISCUSSION

Isolation and confirmation of yeast strain

Samples were collected, and yeast cells were isolated. Isolated yeast cultures were biochemically tested such as ability to ferment d-glucose and galactose, and ability to grow in the presence of d-ribose, d -xylose, and l-rhamnose, for predicting the species of the strain along with selected reference strains. The results of the biochemical tests and predicted species of the strains were tabulated (Table 1). There are four different strains were isolated and were predicted as *Pichia spartinae*, *S. kluyveri*, *P. strasbergensis*, and *S. cerevisiae* based on the biochemical profile. Strains *P. spartinae* and *S. kluyveri* were detected in three and five fruits samples respectively. *P. strasbergensis* and *S. cerevisiae* were isolated from only strawberry and sugarcane, respectively (Table 1). This data suggested that *S. kluyveri* is the most commonly resisting yeast strain of tested fruits at the time of sampling and analysis. Since, the objective of the investigation was to isolate *S. cerevisiae*, that particular strain (designated as HII31) was subjected to molecular-based confirmation by partial sequencing of 5.8S rRNA coding gene and phylogenetic analysis by the neighbor-joining method, and the results confirmed that strain as *S. cerevisiae*. The strain HII31 has 100% similarity with *S. cerevisiae* strain W46. The strain was submitted to the NCBI GenBank database with the accession number of KC588952. The phylogenic tree of the selected strain HII31 is presented in Figure 1.



0.1

Fig. 1: Phylogenetic pattern of isolated *S. cerevisiae* HII31

Optimization of enzyme production

The production and release of any particular microbial enzyme are affected by several physical factors; most influential factors are pH, temperature and salt concentration of the medium. Thus, the impact and influences of pH, temperature, and NaCl concentration during BGS production has been studied in an independent manner and combined manner by BBD and RSM. Based on the previous reports (Spagna *et al.*, 2002; Hernandez *et al.*, 2003; Dhake and Patil, 2005) range of pH, temperature and NaCl concentration were selected and tested by separate experiments. RSM is accepted and proved technique for the

optimizing of multiple variables to achieve a maximum yield of desired products (Hajj *et al.*, 2012).

Optimization of multiple variables for the maximum yield of BGS has been carried out by RSM and Box-Behnken design (BBD) of the experiment. Three levels (level codes -1, 0, 1) of three independent variables such as pH (X1), temperature (X2), and NaCl (X3), were selected in the current study. Influence of pH (3.5, 4.0, and 4.5), temperature (30, 40, and 50 °C), and NaCl concentration (0.4, 0.5, and 0.6%) on BGS production has been evaluated. Based on the RSM and BBD, seventeen experimental sets were designated and carried out. The predicted and actual values, represented as a relative activity, were represented in Table 2. The results suggested an increase in pH, temperature, and NaCl concentration beyond the level of 4, 40 °C and 0.5% affected the BGS activity, respectively. The optimum pH, Temperature, and NaCl content of the medium for BGS activity by isolated *S. cerevisiae* was detected as 4, 40 °C and 0.5% respectively (Figure 2A, B, C). The optimum incubation period for the enzyme activity was 24 h (Figure 2D). Reports revealed that some cations have the ability to induce (Na^+ and Ca^{2+}) and inhibit (Cu^{2+} and Mg^{2+}) the BGS production (Chang *et al.*, 2012). Moreover, presence of Na^+ in the surrounding environment of yeast facilitates the effective BGS transport through membrane (Dhake and Patil, 2005). Thus the concentration of NaCl was also selected as one of the influencing factor of BGS production. The impact of selected three factors, (pH, temperature, and NaCl) on BGS activity has been evaluated by RSM. About seventeen experiments were executed to validate the predicted values of relative activity of BGS that was obtained by multiple regression analysis. The results suggested that both predicted and actual conditions for the maximum production of BGS, in terms of relative activity, were similar (Table 2). Some of the variations were observed in relative activity (%) of BGS in individual experiments with same conditions, which are not statistically significant ($p > 0.05$). The response surface data like regression coefficient, R^2 and probability values for the relative activity of BGS were obtained from Export Design Program V.6.0.2 and tabulated (Table 3).

Relative activity (%) = $98.000 + 1.025 \times (X1) - 0.130 \times (X2) + 0.570 \times (X3) - 6.595 \times (X1)^2 - 6.170 \times (X2)^2 - 2.300 \times (X3)^2$ – Equation 1

Where X1 = pH, X2 = Temperature (°C), and X3 = % NaCl

The equation 1 shows the predicted quadratic polynomial model of BGS production. The coefficient of determination (R^2) was used for the checking of the predicted model under a numerical method. The maximum R^2 (97.63%) and maximum adjusted R^2 (96.21%) reveals that the model equation was adequate for predicting the maximum relative activity or maximum production of BGS by HII31. In general, the fitted model is described by lack-of-fit values for the variation in the experimental data (Trinh and Kang, 2010). For a successful experimental result, the lack-of-fit value should be statistically non-significant ($p > 0.05$).

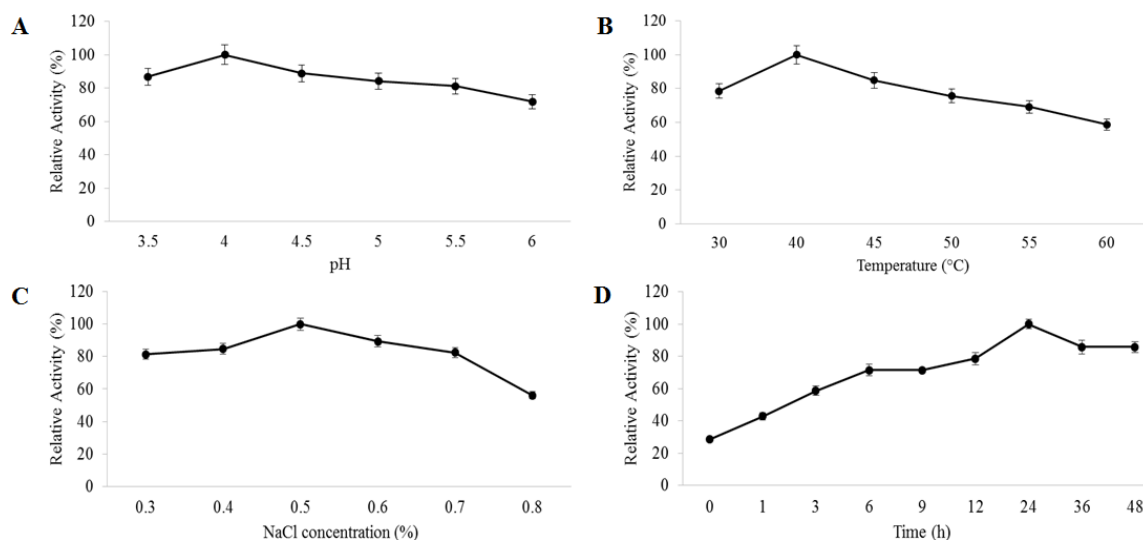


Fig. 2: The influence of pH (A), Temperature (B), NaCl concentration (C) of the medium, and incubation time (D) on BGS activity by *S. cerevisiae* HII31.

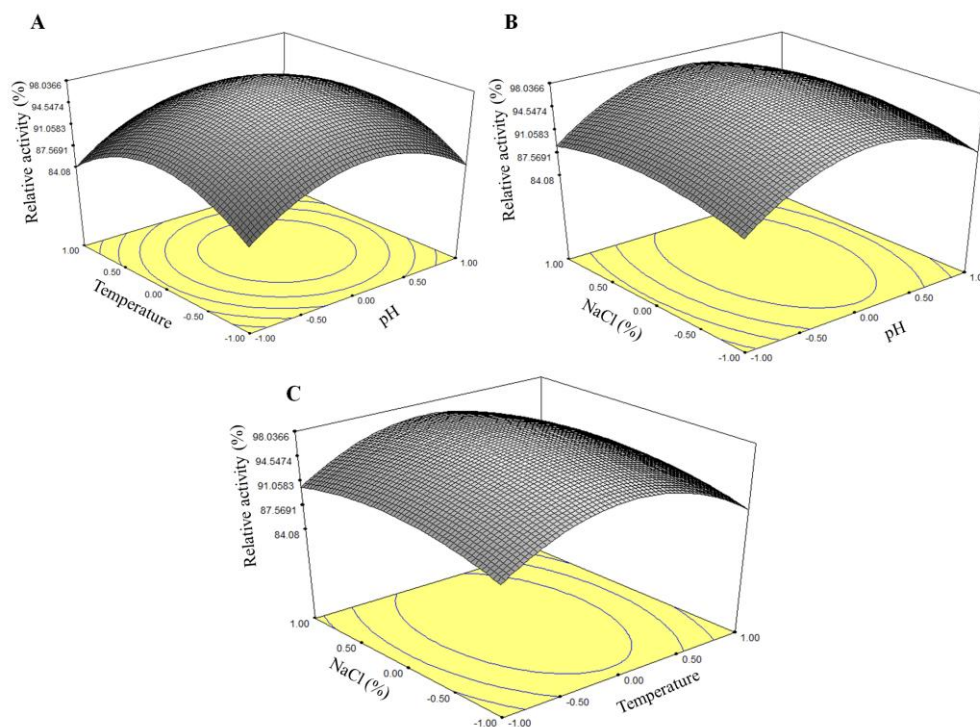


Fig. 3: Representation of response surface plots showing the influence of combined factors like pH and temperature (A), pH and NaCl concentration (B), temperature and NaCl concentration (C) on BGS activity by HII31.

The quadratic polynomial model for the % relative activity of BGS and the variance analysis has been represented in Table 3. The linear parameters (X_1) and quadratic parameters (X_1^2 , X_2^2) were found as significant at the level of $p < 0.05$ and $p < 0.01$, respectively, and in all possibilities lack-of-fit was predicted as not significant ($p > 0.05$) (Table 4). These results are indicated that the relationship between response values and the independent variables were sufficient to represent the real optimal conditions for BGS activity by HII31. The response surface plots represented the optimum conditions such as pH,

temperature, and NaCl concentration for BGS activity by HII31 (Figure 3). The influence of pH and temperature on BGS activity with constant NaCl concentration (0.5 %) was predicted as 3.75 - 4.25 and 35- 45 °C, respectively (Figure 3A). The influence of pH and NaCl concentration on BGS activity with the temperature at 40 °C was predicted as 3.75 - 4.25 and 0.45 - 0.60 %, respectively (Figure 3B). The influence of temperature and NaCl concentration on BGS activity with constant pH of 4.0 was predicted as 35 - 45°C and 0.45 - 0.60 %, respectively (Fig. 3C). Whereas, BBD revealed that pH 4.0, the temperature at 40 °C, and

0.5 % of NaCl was the precise conditions for BGS activity (Table 2). The influence of incubation time for the recovery of maximum enzyme activity by HII31 has been accessed along with reference strains. A study by Hernandez *et al.* (2003) on wine strain of *S. cerevisiae* for BGS activity suggested that optimal pH was 4 and the temperature ranging from 40-50 °C. BGS produced by *S. cerevisiae* strain (AL 41), isolated from Sicilian must and wines, has been reported for the enzyme stability up to 35 days and it had optimum pH of 3.5-4.0 and temperature at 20 °C

(Spagna *et al.*, 2002). Whereas, in the present study, negative regulation of BGS production, in terms of relative activity, was noticed after 24 h of incubation, this might be resultant of utilization of BGS enzyme by yeast cells.

This phenomenon has not been observed in reference strains used in this study (Table 5). Thus further detailing is required in the form of scientific investigations to address the impact of incubation time on BGS production/activity by HII31.

Table 1: Source, biochemical test profile and growth of selected strains.

Sources (No. of strains)	Biochemical characterization					Predicted as
	Fermentation of		Growth in the presence of			
	D -glucose	Galactose	D-Ribose	D-Xylose	L-Rhamnose	
Mangosteen (1)	+	-	-	-	-	<i>Pichia spartinae</i>
Coconut (3)	+	+	-	-	-	<i>Saccharomyces kluyveri</i>
Mango (5)	+	+	-	-	-	<i>S. kluyveri</i>
Custard apple (2)	+	+	-	-	-	<i>S. kluyveri</i>
Grapes and grape juices (5)	+	+	-	-	-	<i>S. kluyveri</i>
Lychee and lychee juices (5)	+	-	-	-	-	<i>P. spartinae</i>
Rambutan (5)	+	-	-	-	-	<i>P. spartinae</i>
Strawberry (2)	+	v	s	+	-	<i>Pichia strasbergensis</i>
Durian (1)	+	+	-	-	-	<i>S. kluyveri</i>
Sugarcane (5)	+	v	-	-	-	<i>S. cerevisiae</i>
TISTR 5003	+	v	-	-	-	<i>S. cerevisiae</i>
TISTR 5024	+	v	-	-	-	<i>S. cerevisiae</i>
TISTR 5051	+	v	-	-	-	<i>S. cerevisiae</i>
TISTR 5059	+	v	-	-	-	<i>S. cerevisiae</i>
TISTR 5197	+	v	-	-	-	<i>S. cerevisiae</i>

+: positive, -: negative, s: slow utilization, v: variable.

Table 2: The Box–Behnken design and experimental values of relative activity (%) of β -glucosidase activity by *S. cerevisiae*.

Run order	Independent variables			Relative activity (%)	
	pH (X1)	Temperature (°C) (X2)	NaCl (%) (X3)	Actual values	Predicted values
1	4.5	30	0.5	86.39	86.39
2	4.0	50	0.4	88.83	88.83
3	4.5	40	0.4	89.56	89.56
4	3.5	30	0.5	84.34	84.34
5	4.0	40	0.5	98.00 ^a	98.00
6	4.0	30	0.4	89.09	89.09
7	3.5	40	0.4	87.51	87.51
8	3.5	50	0.5	84.08	84.08
9	4.0	50	0.6	89.97	89.97
10	3.5	40	0.6	88.65	88.65
11	4.5	50	0.5	86.13	86.13
12	4.0	40	0.5	97.00 ^b	98.00
13	4.0	40	0.5	99.00 ^c	98.00
14	4.0	30	0.6	90.23	90.23
15	4.0	40	0.5	96.00 ^d	98.00
16	4.0	40	0.5	100.00 ^e	98.00
17	4.5	40	0.6	90.70	90.70

^{a-e} values are not significantly differ among the multiple experimental trails.

Table 3: Regression coefficients, R², and probability values of response surface data for reactive activity (%) of β -glucosidase.

Term	Coefficient estimate	Standard error coefficient	Statistic value (T)	P (Prob > F)
Constant	98.00	0.45	217.78	<0.0001
pH	1.02	0.35	2.91	0.0159
Temperature (°C)	-0.13	0.35	-0.37	0.7208
NaCl (%)	0.57	0.35	1.63	0.1380
pH × pH	-6.59	0.49	-13.45	<0.0001
Temperature (°C) × Temperature (°C)	-6.17	0.49	-12.59	<0.0001
NaCl (%) × NaCl (%)	-2.30	0.49	-4.69	0.0008

Standard deviation = 1.00, R² = 97.63%, Adjust R² = 96.21.

Table 4: Variance analysis of relative activity (%) of β -glucosidase activity.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	6	411.95	411.95	68.66	68.66	<0.0001
Linear	3	11.14	11.14	3.71	3.71	0.0291
Square	3	365.69	365.69	121.90	121.90	<0.0001
Interaction	-					
Residual Error	10	10.00	10.00	1.00		
Lack-of-Fit	6	0.00	0.00	0.00	0.00	1.0000
Pure Error	4	10.00	10.00			
Total	16	421.95				

DF: Degree of freedom.

Table 5: β -glucosidase activity of *S. cerevisiae* strains.

Time (h)	Relative activity (%)					
	HII31	TISTR 5003	TISTR 5024	TISTR 5051	TISTR 5059	TISTR 5197
0	28.57 ± 1.43	14.29 ± 1.43	42.86 ± 2.14	44.29 ± 2.21	32.86 ± 1.64	14.29 ± 0.71
1	42.86 ± 2.14	15.71 ± 1.57	45.71 ± 2.29	28.57 ± 1.43	35.71 ± 1.79	21.43 ± 1.07
3	58.57 ± 2.93	17.14 ± 1.71	47.14 ± 2.36	42.86 ± 2.14	38.57 ± 1.93	28.57 ± 1.43
6	71.43 ± 3.57	18.57 ± 1.86	50.00 ± 2.50	35.71 ± 1.79	42.86 ± 2.14	40.00 ± 2.00
9	71.43 ± 3.57	21.43 ± 2.14	52.86 ± 2.64	60.00 ± 3.00	50.00 ± 2.50	40.00 ± 2.00
12	78.57 ± 3.93	20.00 ± 2.00	65.71 ± 3.29	71.43 ± 3.57	42.86 ± 2.14	50.00 ± 2.50
24	100.00 ± 5.00 [#]	54.29 ± 2.71	70.00 ± 3.50	64.29 ± 3.21	54.29 ± 2.71	60.00 ± 3.00
36	85.71 ± 4.29	78.57 ± 3.93*	92.86 ± 4.64 [#]	78.57 ± 3.93*	60.00 ± 3.00	71.43 ± 3.57
48	85.71 ± 4.29	77.14 ± 3.86	92.86 ± 3.94	71.43 ± 3.57	64.29 ± 3.21*	72.86 ± 3.64*

* Significant value ($p < 0.05$) with other strains; [#] Non-significant value ($p > 0.05$)

CONCLUSION

The current study established and revealed the isolation, identification of BGS producing *S. cerevisiae* strain (HII31) from Thai fruit-derived beverages and culture conditions for the maximum production of BGS. Further meticulous research on enzyme recovery and purification will unbolt the way to attain high quality safe microbial enzyme with industrial and pharmaceutical demands. The selected high potential strain with β -glucosidase activity will be used as starter culture for plant-derived food biotransformation in near future.

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DECLARATION

Screening and isolation part of the study was performed at Health Innovation Institute, Chiang Mai, Thailand and the identified strain is belongs to Health Innovation Institute. The RSM studies were carried out at Faculty of Pharmacy, Chiang Mai University.

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