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Contributed Paper

Medium Optimization for β-Galactosidase Production by a Thermotolerant Yeast

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ABSTRACT

Kluyveromyces sp. CK8 was previously isolated from rotten fruit in northern Thailand and found to be capable of growing at 45°C. This research aimed to identify the yeast isolate and to optimize its production of β-galactosidase in synthetic medium using lactose as the sole carbon source. Molecular identification via phylogenetic analysis, based on comparative sequence analysis of the 26S rRNA gene at the D1/D2 domain, revealed that isolate CK8 was *Kluyveromyces marxianus*. The yeast isolate produced β-galactosidase 5.33 U/ml culture when cultivated at 37°C for 18 h in Yeast-Malt extract (YM) medium containing 1% (w/v) lactose as the sole carbon source. Optimization of medium composition for enzyme production was conducted using 2-Level Factorial experimental design to determine the most relevant variables of the culture medium composition. Among five parameters, yeast extract, $(NH_4)_2SO_4$ and KH_2PO_4 were significant effectors at $p<0.05$. A central composite design (CCD) and response surface plot predicted the highest β-galactosidase activity of 8.94 U/ml culture in the medium containing (g/l) of the following: yeast extract (0.23), $(NH_4)_2SO_4$ (9.64), KH_2PO_4 (7.0), lactose (20) and $MgSO_4.7H_2O$ (0.1). A validation time course culture study conducted for 18 h in the optimal medium found that the actual maximum value of enzyme activity was 8.82 U/ml culture which is 98.66% of the predicted value.

Keywords: β-galactosidase, thermotolerant yeast, medium optimization, 2-level factorial experimental design, central composite design

1. INTRODUCTION

β-galactosidase or lactase (β-D-galactosidegalactohydrolase, EC 3.2.1.23) is an enzyme that catalyzes the hydrolysis of lactose to D-glucose and D-galactose. It is an important enzyme which is widely used in food processing industries especially in the dairy industry for lactose-free milk production for lactose intolerant consumers. In addition, this enzyme has been widely investigated for galactooligosaccharide (GOS) synthesis in the functional food industry [1]. The confirmed health claims of GOS have significantly increased the public's demand for food containing GOS, particularly in

Japan and Europe [2]. Many organisms naturally synthesize β-galactosidase, including microbes, plants and animals. Traditionally, the most widely used β-galactosidases in food industries were obtained from microbial sources such as *Saccharomyces cerevisiae*, *Kluyveromyces* spp. and *Aspergillus* spp. [3]. Additionally, products obtained from these microorganisms are generally recognized as safe (GRAS) for human consumption [4, 5].

Recently, it is accepted that thermostability has been recognized as an important characteristic of industrial enzymes due to the capability of the longer period of uses [6]. Moreover, the thermostability of β-galactosidase was suggested to be an important factor that positively influences GOS synthesis [7]. The recombinant thermostable β-galactosidases from *Bacillus stearothermophilus* and *Thermotoga maritime* were characterized and suggested to be efficiently used in GOS synthesis [6, 7]. In previous research, we found that *Kluyveromyces* sp. isolate CK8 obtained from rotten fruit in northern Thailand was capable of ethanol fermentation at 45°C. This yeast strain also showed the ability to utilize lactose as the sole carbon source for growth and ethanol production. Therefore, we anticipated that yeast isolate CK8 would be able to produce thermostable β-galactosidase. However, this isolate produced intracellular β-galactosidase in low quantities and an optimal medium for achieving higher productivity was required. The objectives of the current research were to identify the species of *Kluyveromyces* sp. isolate CK8, and to optimize its culture medium for β-galactosidase production.

2. MATERIALS AND METHODS

2.1 Microorganisms and Seed Preparation

Kluyveromyces sp. CK8 was maintained on yeast-malt extract (YM) agar containing w/v of 0.3% yeast extract, 0.3% malt extract, 0.5% peptone, 1.0% glucose and 2.0% agar, and periodically transferred into the fresh medium. The reference strains of *Kluyveromyces marxianus* BCC 7025 and *K. marxianus* BCC 7049 were obtained from the BIOTEC Culture Collection (BCC), National Center for Genetic Engineering and Biotechnology, Thailand. The seed culture used for the enzyme production experiment was prepared by inoculating the yeast strain into 50 ml of sterile YM broth in a 125 ml Erlenmeyer flask and incubating the culture at 37°C for 18 h using an orbital shaker at 180 rpm.

2.2 Identification and Phylogenetic Analysis

The morphological and biochemical characteristics of the selected yeast strain were studied by the standard methods described by Yarrow [8]. The genomic DNA was extracted according to the method described by Sambrook et al. [9]. The D1/D2 domain of 26S rDNA was amplified by polymerase chain reaction (PCR) as described by Libkind et al. [10]. Then, PCR product size was confirmed by 1.0% (w/v) agarose gel electrophoresis and followed by purification using the GF-1 AmbiClean Kit (Vivantis, USA). The D1/D2 26S rDNA fragment was sent to the 1st BASE Company, Singapore, for sequencing. The sequence obtained was aligned using BLAST analysis. Then, the multiple alignments were performed by using BioEdit 7.0 and phylogenetic tree was constructed by MEGA 4.0 program [11].

2.3 Enzyme Preparation and Assay

Yeast cells were harvested and resuspended in sodium phosphate buffer pH 7.0. The assay for β-galactosidase activity was determined by the modified method described by Miller [12]. Yeast culture was centrifuged at 10,000×*g* at 4°C for 10 min. Cell pellets were resuspended in 0.1 M sodium phosphate buffer (pH 7.0) and washed twice with the same buffer. Yeast cell permeabilization was carried out using 50% (v/v) ethanol and shaken for 15 min at 4°C as described by Panesar et al. [13]. The permeabilized yeast cells were centrifuged and washed twice with sterile distilled water and suspended in the same buffer. A volume of 0.1 ml of the cell suspension was combined with 0.9 ml 0.1 M sodium phosphate buffer pH 7.0 and 0.2 ml of 4 mM *o*-nitrophenyl-β-D-galactopyranoside (ONPG) was added. The reaction mixture was incubated at 37°C for 10 min and the reaction was stopped by adding 1 ml of 0.5 M sodium carbonate solution (pH 10.0). The amount of *o*-nitrophenol released in the supernatant was measured by the absorbance at 420 nm using a spectrophotometer. One unit of enzyme activity was defined as the amount of enzyme that liberated 1 μmole of *o*-nitrophenol per minute under standard assay conditions.

2.4 Fractional Factorial Design

A fractional factorial design 2^{5-1} was used for screening the most important factors in medium composition that affected enzyme production. The liquid media composed of yeast extract, lactose, $(NH_4)_2SO_4$, KH_2PO_4 and $MgSO_4.7H_2O$ were prepared at the high and low levels according to 2-Level fractional factorial design (Table 1). The data obtained from the experiment was investigated by regression analysis using Design Expert program version 7.0. The experiments were conducted at 37°C, 180 rpm for 18 h.

Table 1. The high and low level of each variable applied in 2-Level fractional factorial design.

Independent variable	Low level (-)	High level (+)
Yeast extract (g/L)	0.3	3.0
Lactose (g/L)	5.0	20.0
$(NH_4)_2SO_4$ (g/L)	1.2	8.8
KH_2PO_4 (g/L)	1.0	5.0
$MgSO_4.7H_2O$ (g/L)	0.1	1.0

2.5 Central Composite Design (CCD)

The most significant medium compositions obtained from fractional factorial design were evaluated for the significant effects and the optimum quantity was determined statistically using a central composite design and a response surface methodology. In this study, the statistical design for three variables was created for five different levels of yeast extract, $(NH_4)_2SO_4$ and KH_2PO_4 towards 14 treatments with 3 center point treatments (Table 2 and 5).

Table 2. Different levels designed by the seventeen factorial designs of three independent variables.

Independent variable	Symbol	Level				
		-1.68	-1	0	+1	+1.68
Yeast extract	A	0	0.06	0.15	0.24	0.3
$(NH_4)_2SO_4$	B	5	7.03	10	12.97	15
KH_2PO_4	C	3	4.42	6.50	8.58	10

In the enzyme production experiment, the time course of enzyme production by *K. marxianus* CK8 was studied by preparing 50 ml of optimal medium in 125 ml Erlenmeyer flasks to which 1% (v/v) seed culture was added. Time course was carried out at 37°C, 180 rpm for 24 h and sampling in triplicate at 3 h intervals.

3. RESULTS AND DISCUSSION

In the past, classification of yeasts was primarily based on morphology and physiology, in particular the capacity to assimilate carbon sources. The colony morphology of *Kluyveromyces* sp. isolate CK8 on YM agar medium was cream-white with a smooth surface after incubation for 48 h at 37°C (Figure 1a). Microscopically, the cells are spheroidal, ovoidal or elongate. Asexual reproduction by multilateral budding and pseudohyphae formation were observed, but true hyphae were not formed (Figure 1b and 1c). The physiological characterization of isolate CK8 was similar to the two type strains obtained from BCC, *K. marxianus* BCC 7025 and *K. marxianus* BCC 7049, isolate CK8 was similarly able to assimilate D-glucose, D-galactose, sucrose, maltose, lactose, raffinose, trehalose, inulin and D-fructose as found with those two type strains. Additionally, all three strains showed a similar pattern in fermentation of D-glucose, D-galactose, sucrose, raffinose, inulin and D-fructose.

Finally, the isolate CK8 could be presumptively identified as *Kluyveromyces marxianus* based on its carbon compound utilization and maximum growth temperature of 45°C. Lachance (1998) reported that *K. marxianus* is the only inulin-assimilating *Kluyveromyces* species, but did not assimilate or ferment α-glucoside and grow well at 37°C [14].

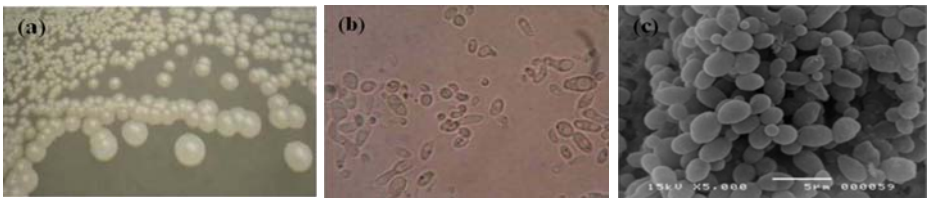

Figure 1. Morphology of *Kluyveromyces* sp. CK8 on YM agar plate (a), under light microscope 1000× (b) and under scanning electron microscope (S.E.M., bar = 5 μm) (c).

3.1 Molecular Taxonomic Identification and Phylogenetic Analysis

Molecular taxonomic analysis was investigated by using the results of sequencing analysis of the D1/D2 domain of 26S rDNA in isolate CK8. This genomic region was amplified by PCR and the DNA fragment with approximately 600 bp. The phylogenetic relationships were obtained by comparing the 26S rDNA D1/D2 sequences of the isolate CK8 strain with other *Kluyveromyces* species. *Saccharomyces cerevisiae* strain CBS 1171 was used as an out-group. The nucleotide sequences of the DNA fragment of the 26S rRNA D1/D2 gene was aligned using BLAST analysis, which clearly demonstrated that isolate CK8 showed a 99% similarity to *Kluyveromyces marxianus*. The phylogenetic tree was demonstrated in Figure 2.

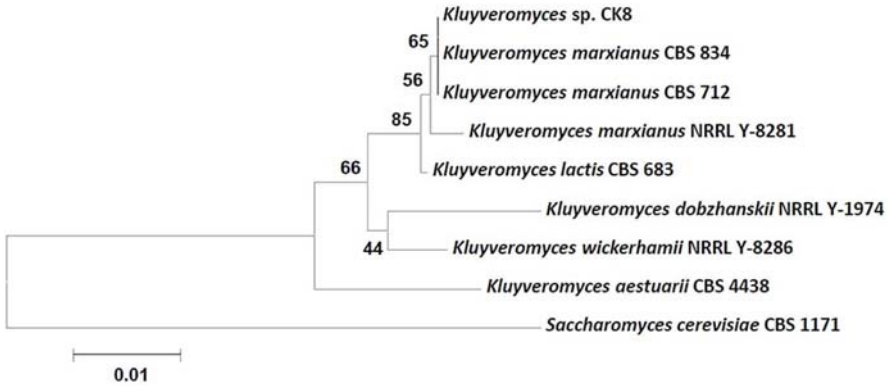

Figure 2. The phylogenetic tree of *Kluyveromyces* sp. CK8 among other yeast species in the genus *Kluyveromyces*.

3.2 Fractional Factorial Design

The medium composition containing yeast extract, lactose, $(NH_4)_2SO_4$, KH_2PO_4 and $MgSO_4.7H_2O$ was analyzed to determine the most important factors affecting β-galactosidase production. According to 2^{5-1} fractional factorial design matrix, total 16 different media were designated for β-galactosidase production with 3 center point treatments (Table 3). The enzyme activity was assayed and the analysis was performed by linear regression regarding the first order model. It was revealed that $(NH_4)_2SO_4$ and KH_2PO_4 were positive factors while yeast extract was a negative factor significantly influencing the enzyme production at $p<0.2$ (Table 4). Therefore, these three medium compositions were required to determine their optimum level by the central composite design (CCD). Lactose was classified as a non-significant factor influenced in the positive way ($p<0.5602$) on the enzyme production in contrast to MgSO4 which was indicated as a non-significant factor ($p<0.8105$) but influenced negatively. Therefore, they were fixed at their high (20 g/l lactose) and low level (0.1 g/l MgSO4), respectively, in further CCD experiment. The positive effect of lactose for production of the enzyme by *K. marxianus* CK8 revealed that the high concentration of lactose was not required as it was classified as a non-significant factor for enzyme production.

Table 3. The treatments of each variable used in 2-Level fractional factorial design for investigation of factors effecting on β-galactosidase production.

Trials	Yeast extract (g/l)	Lactose (g/l)	$(NH_4)_2SO_4$ (g/l)	KH_2PO_4 (g/l)	$MgSO_4.7H_2O$ (g/l)	β-gal activity (U/ml)
1	0.30	5.00	1.20	1.00	1.00	1.22
2	3.00	5.00	1.20	1.00	0.10	1.23
3	0.30	20.00	1.20	1.00	0.10	1.87
4	3.00	20.00	1.20	1.00	1.00	1.10
5	0.30	5.00	8.80	1.00	0.10	2.26
6	3.00	5.00	8.80	1.00	1.00	1.32
7	0.30	20.00	8.80	1.00	1.00	3.32
8	3.00	20.00	8.80	1.00	0.10	1.18
9	0.30	5.00	1.20	5.00	0.10	2.61
10	3.00	5.00	1.20	5.00	1.00	1.69
11	0.30	20.00	1.20	5.00	1.00	2.64
12	3.00	20.00	1.20	5.00	0.10	1.80
13	0.30	5.00	8.80	5.00	1.00	2.63
14	3.00	5.00	8.80	5.00	0.10	1.85
15	0.30	20.00	8.80	5.00	0.10	2.77
16	3.00	20.00	8.80	5.00	1.00	1.20
17	1.65	12.50	5.00	3.00	0.55	1.75
18	1.65	12.50	5.00	3.00	0.55	1.88
19	1.65	12.50	5.00	3.00	0.55	1.79

Table 4. The linear regression analysis of 2-Level fractional factorial design for screening of parameters influencing enzyme production by *Kluyveromyces* sp. isolate CK8.

Variable	Coefficient	*p*-value
Yeast extract	- 0.50	0.0005
Lactose	0.064	0.5602
$(NH_4)_2SO_4$	0.15	0.1814
KH_2PO_4	0.23	0.0532
$MgSO_4.7H_2O$	- 0.026	0.8105
R^2= 0.7078		

3.3 Central Composite Design (CCD)

To find the optimal quantities of the significant compositions identified by the fractional factorial design including yeast extract, $(NH_4)_2SO_4$ and KH_2PO_4, total 15 different media were created according to design matrix of central composite design for enzyme production (Table 5). The enzyme activity was determined and analyzed by multiple linear regressions. The activity values were fitted to quadratic model and the polynomial equation was

generated as equation 1.

β-galactosidase activity (U/ml) = -5.05436 +33.16760 (A)+1.03237 (B)+1.23944 (C)-0.40074 (A)(B)-0.18621 (A)(C)-0.021594 (B)(C)-59.14882 (A^2)-0.036370 (B^2)-0.061779 (C^2) (1)

Where A, B and C are yeast extract, $(NH_4)_2SO_4$ and KH_2PO_4, respectively.

Table 5. Central Composite designed experiments.

Trials	Code setting levels			Actual levels			Actual activity (U/ml)	Predicted activity (U/ml)
	A	B	C	Yeast extract (g/l)	$(NH_4)_2SO_4$ (g/l)	KH_2PO_4 (g/l)		
1	-1	-1	-1	0.06	7.03	4.42	5.25	5.58
2	1	-1	-1	0.24	7.03	4.42	7.18	7.68
3	-1	1	-1	0.06	12.97	4.42	6.26	6.68
4	1	1	-1	0.24	12.97	4.42	8.31	8.36
5	-1	-1	1	0.06	7.03	8.58	6.34	6.72
6	1	-1	1	0.24	7.03	8.58	8.68	8.68
7	-1	1	1	0.06	12.97	8.58	7.36	7.29
8	1	1	1	0.24	12.97	8.58	8.73	8.82
9	-1.68	0	0	0	10	6.50	6.09	5.68
10	1.68	0	0	0.30	10	6.50	8.92	8.74
11	0	-1.68	0	0.15	5	6.50	7.62	7.10
12	0	1.68	0	0.15	15	6.50	8.23	8.15
13	0	0	-1.68	0.15	10	3	7.68	7.11
14	0	0	1.68	0.15	10	10	8.49	8.45
15	0	0	0	0.15	10	6.50	8.37	8.54
16	0	0	0	0.15	10	6.50	8.59	8.54
17	0	0	0	0.15	10	6.50	8.55	8.54

The results were presented as contour and quadratic response surface plot as shown in Figure 3 with ANOVA (Table 6). The model was significant and reliable with a p-value of 0.0041 and R^2 of 0.9211. It was found that yeast extract (p=0.002), $(NH_4)_2SO_4$ (p=0.0446) and KH_2PO_4 (p=0.0162) maintained their significant impacts on β-galactosidase production at the optimum levels (A, B and C) by enhancing the enzyme activity. However, the higher concentration of those components significantly diminished the enzyme activity at $p<0.1$ (A^2, $p<0.0121$; B^2, p=0.0554; C^2, p=0.0977)

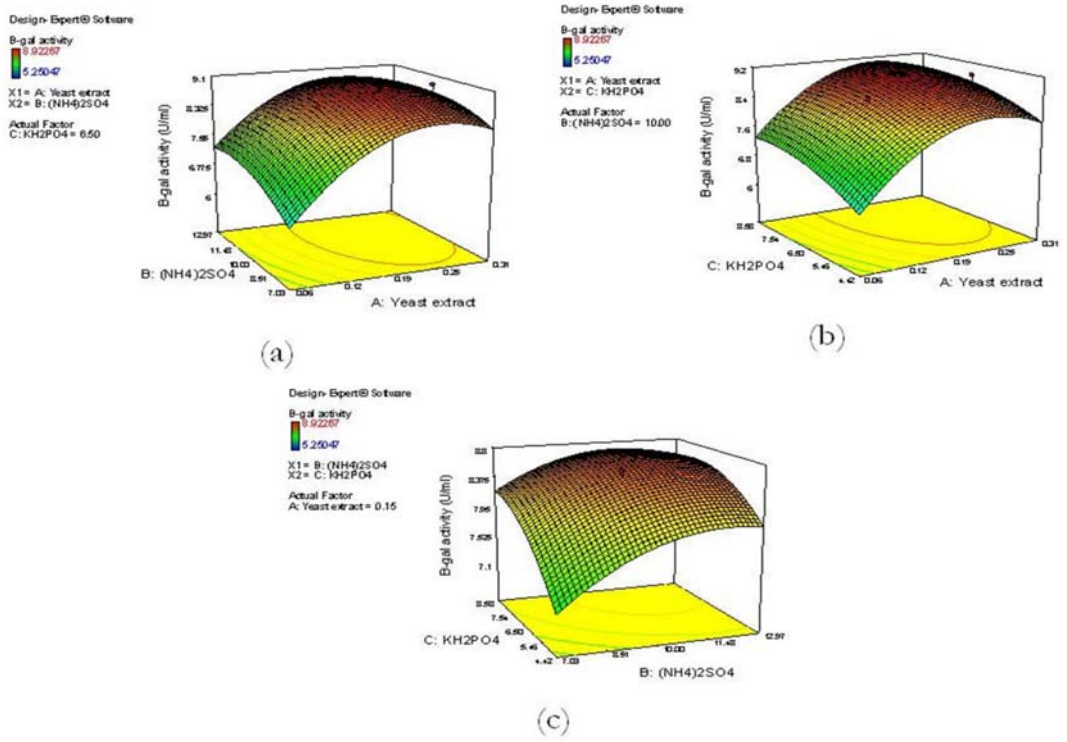

Figure 3. Responses surface plot of b-galactosidase production by *K. marxianus* CK8 cultivated at 37°C, 180 rpm for 18 h.

Table 6. The result of central composite design.

Term	Coefficient	*p*-value
Intercept	8.54	0.0041
A-Yeast extract	0.91	0.0002
B-$(NH_4)_2SO_4$	0.31	0.0446
C-KH_2PO_4	0.40	0.0162
AB	-0.11	0.5431
AC	-0.035	0.8412
BC	-0.13	0.4481
A^2	-0.47	0.0121
B^2	-0.32	0.0554
C^2	-0.27	0.0977

The optimum medium composition predicted by this equation was 0.23 g/l yeast extract, 9.64 g/l $(NH_4)_2SO_4$, 7 g/l KH_2PO_4, 20 g/l lactose and 0.1 g/l $MgSO_4.7H_2O$ with the predicted β-galactosidase activity value of 8.94 U/ml culture. The β-galactosidase activity was higher compare to 5.33 U/ml culture obtained from the cultivation in Yeast-Malt extract (YM) medium containing 1% (w/v) lactose as the sole carbon source at 37°C for 18 h. Consideration on compositions of the optimal medium obtained from this experiment, the newly obtained medium was markedly cheaper

compare to YM medium. This would be advantageous for enzyme production in the large scale.

3.4 Time Course Study of Enzyme Production

The time course study of β-galactosidase production by *K. marxianus* CK8 was carried out to validate a maximum enzyme production during 18 h cultivation. The actual value of enzyme activity was 8.82 U/ml culture at 18 h cultivation which calculated to be 98.66% of the predicted value. Manera et al. (2008) optimized culture medium for production of β-galactosidase from *K. marxianus* CCT 7082 and found that the optimum concentrations (g/l) of constituents were a lactose of 28.2, yeast extract 17.0, and $(NH_4)_2SO_4$ 8.8 at pH 6.0. Under these conditions the enzyme activity from *K. marxianus* CCT 7082 was 10.6 U/ml [15] which was higher than the enzyme activity obtained from *K. marxianus* CK8. This might be due to not only the difference of yeast strain, but the different enzyme preparation method might be also influenced as our experiment prepared the enzyme in form of permeabilized cell while Manera et al. (2008) used the enzyme extracted by glass bead [14]. However, the concentration of yeast extract in the optimal medium obtained from our experiment was only 0.23 g/l while 17 g/l was included in the optimal medium reported by Manera et al. [15].

4. Conclusion

A thermostolerant yeast strain, *Kluyveromyces* sp. CK8 was identified to be *Kluyveromyces marxianus*, based on morphological, physiological characteristics and the sequence analysis of the D1/D2 domain on 26S rRNA gene. In addition, the optimal medium composition for β-galactosidase production by *K. marxianus* CK8 was determined to be yeast extract 0.23 g/l, $(NH_4)_2SO_4$ 9.64 g/l, KH_2PO_4 7 g/l, lactose 20 g/l and $MgSO_4.7H_2O$ 0.1 g/l. The maximum enzyme production was 8.82 U/ml culture at 18 h cultivation.

Acknowledgements

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