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ORIGINAL ARTICLE

Production of β-glucosidase from okara fermentation using *Kluyveromyces marxianus*

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Abstract The effective utilization of okara (soybean residue) has become a considerable challenge in recent years. In this paper, the potential advantages of β-glucosidase production from okara fermented by *Kluyveromyces marxianus* were evaluated and the properties of the β-glucosidase were also characterized. The results showed that okara can significantly induce the production of β-glucosidase from *K. marxianus*. The β-glucosidase activity was up to 4.5 U/mg under optimized fermentation conditions. The optimal parameters were as follows: fermentation temperature 35 °C, cultivation time 98 h, inoculum concentration 10%, and 30 g/L of okara. After two steps of purification using ammonium sulfate precipitation and Sephadex G-75 column chromatography, the activity of β-glucosidase was 71.4 U/mg. The native enzyme was an approximately 66 kDa dimer consisting of two different subunits (22 and 44 kDa). The kinetic parameters of the *K. marxianus* β-glucosidase, using *p*NPG as substrate, were V_{max} 8.34 μmol min^{-1} mg^{-1} and K_m 7.42 mM. The β-glucosidase showed high thermostability and acid–alkali tolerance as well as low inhibition by DMSO (10–50%). In conclusion, this study supports the notion that okara fermentation by *K. marxianus* could be a useful process to produce *β-glucosidase*.

Keywords Okara · β-Glucosidase · *Kluyveromyces marxianus* · Fermentation · Purification · Characteristic

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Introduction

Okara (soybean residue) is a byproduct of tofu or soymilk production. A considerable amount of okara is produced by the food industry, particularly in Asian countries. The soybean curd-manufacturing sectors of Korea, Japan, and China generate approximately 310,000, 800,000, and 2,800,000 tons of annual okara, respectively (Li et al. 2011). Large quantities of okara discarded as a waste seriously pollute the environment. However, okara still contains most of the carbohydrates, some of the protein and a small portion of the oil of soybean (van der Riet et al. 1989). Several strategies have been proposed to avoid the waste of nutrients in okara. Quitain et al. (2006) extracted oil from okara by physical methods. Yeast fermentation is also a low-cost method to increase the added-value of

okara such as obtaining erythritol from okara by *Y. lipolytica* (Liu et al. 2017). Yeast *Kluyveromyces marxianus* strains isolated from a wide variety of dairy products, such as kefir grains, soft cheeses, and aged cheeses (Padilla et al. 2012; Fadda et al. 2017) are generally considered as safe as *S. cerevisiae* and *Kluyveromyces lactis* (Lopes et al. 2014). *Kluyveromyces marxianus* from different sources showed interesting traits, including the livability in multitudinous substrates and at high temperatures (Fonseca et al. 2008).

β-Glucosidases (EC 3.2.1.21) are enzymes that catalyze the hydrolysis of *β*-1,4-glycosidic linkages and partially hydrolyze *β*-1,1, *β*-1,2, *β*-1,3 and *β*-1,6 glycosidic linkages (Wallecha and Mishra 2003). Most of these enzymes hydrolytically bind to a terminal and nonreducing *β*-D-glycosidic linkages to release *β*-D-glucose and the corresponding aglycone, mediating transglycosylation reactions. *β*-glucosidases are widely used in the food industry and other fields. For example, *β*-glucosidase hydrolyzes aromatic precursors to debitter and enhance the aroma of wine (Belancic et al. 2003) and generates high-purity isoflavone aglycones. Microbes are commonly used to produce *β*-glucosidase, such as *Aspergillus niger* (Seidle and Huber 2005) and *Aspergillus oryzae* (Watanabe et al. 2016). Plants also can produce *β*-glucosidase, such as tea leaves (Sener 2015). However, the diverse applications of these enzymes warrant the continued searching for new *β*-glucosidase producers that could expand the availability of enzymes with certain specificities and be used under feasible operating conditions. *Kluyveromyces marxianus* can secrete extracellular enzymes including pectinases, polygalactoronases, inulinase, proteases (Foukis et al. 2012), and *β*-galactosidase (Padilla et al. 2012). Rajoka et al. (2004) found that *K. marxianus* can be an efficient producer of a thermostable *β*-glucosidase.

In our previous study, the nutritional values and processing characteristics of *K. marxianus* fermented okara was significantly improved (Hu et al. 2019), which may be related to the induction of *β*-glucosidase (Su et al. 2018), but it was not clear. Many reports have shown that fibrous substances can induce the production of *β*-glucosidase (Rajoka et al. 2004; Seidle and Huber 2005; Baffi et al. 2011), so okara may be a well considerable inducer for *β*-glucosidase. Therefore, we used okara as a matrix using the yeast *K. marxianus* fermentation to produce *β*-glucosidase in this study. The results of this study can provide a new way to utilize the abundant soybean byproduct and might have a significant contribution to the industrial production of *β*-glucosidase.

Materials and methods

Materials and reagents

Soybeans (Heihe 43, Heilongjiang, China) were kindly supplied by the Shandong Shengfeng Seeds Co., Ltd. (Shandong, China). One hundred grams of raw soybeans preserved at room temperature (25 °C) were soaked in a 5-fold amount of water for 12 h. Then the soaked soybeans were grounded into fine particles with a 9-fold amount of water (80 °C), at a low speed for 3 min using a soymilk machine (JYL-Y20, Joyang Co., Ltd., Shandong, China), and filtered through a 60-mesh sieve to obtain the okara. The okara was stored at − 20 °C until use.

Kluyveromyces marxianus from kefir grains was isolated and identified previously by colony morphology and sequence analysis of the 26S rDNA D1/D2 region (Su et al. 2018) and deposited at the China General Microbiological Culture Collection Center (CGMCC) under accession number 13907 (Beijing, China).

The bicinchoninic acid (BCA) and glucose oxidase/peroxidase (GOD-PAP) kits were purchased from the Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The sodium dodecyl sulfate (SDS) gel electrophoresis kit (12% pre-casted gel), cellobiose, and galactose were obtained from the Beijing Dingguo Changsheng Biotechnology Co., Ltd. (Beijing, China). Carboxymethyl cellulose, microcrystalline cellulose, and salicin were obtained from the Shanghai Yuanye Biotechnology Co., Ltd. (Shanghai, China). Beef extract, peptone, and yeast extract were purchased from Beijing Aoboxing Bio-tech Co., Ltd. (Beijing, China). All other chemicals used in this study were of analytical grade and were purchased from Beijing Chemical Works (Beijing, China).

Identification of *β*-glucosidase production

At first, the production of the *β*-glucosidase level on okara by *K. marxianus* fermentation was determined using the esculin medium. (Renchinkhandand et al. 2017). One hundred μL of *K. marxianus* suspension (10^7 cells/mL) was evenly coated to a plate (9.0 cm × 9.0 cm) with modified esculin medium (1 g/L esculin (6,7-dihydroxycoumarin 6-glucoside), 1 g/L ferric ammonium citrate, 1 g/L KH_2PO_4, 0.5 g/L $MgSO_4$, 2 g/L agar, and 2.5 g/L peptone, 10 g/L yeast extract or 10 g/L okara, pH 5.0). A medium containing no yeast extract and okara was used as a control. During the incubation period at 30 °C for 0–120 h, the amount of *β*-glucosidase production was observed by observing the speed and depth color of the dark brown precipitate (Li et al. 2017).

Crude enzyme preparation

The *K. marxianus* was activated in basal medium (8 g beef powder, 10 g peptone, 4 g yeast extract, 20 g glucose, 2 g potassium hydrogen phosphate, 2 g diammonium hydrogen citrate, 5 g sodium acetate, 0.2 g magnesium sulfate, 0.04 g manganese sulfate, and 10 mL Tween 80 in 1000 mL water, pH 5.7 ± 0.2) and the cultures were incubated for 15 h at 30 °C and 120 rpm on an incubator shaker. After cultivation, the cells were centrifuged (5 050 g for 20 min at 4 °C), washed twice with distilled water to remove the medium, and then adjusted to a concentration of 10^7 cells/mL for inoculation.

Thirty grams of okara soaked in distilled water at a ratio of 1:5 (w/v) were placed in 500 mL Erlenmeyer flask and sterilized at 121 °C for 15 min. After cooling, *K. marxianus* inoculum solution was added at a ratio of 6% (v/w) in the okara medium. After fermentation, a clear supernatant containing β-glucosidase was obtained by centrifugation (5 050 g for 20 min at 4 °C) and used as the crude enzyme for further experiments.

β-Glucosidase activity assay

The β-Glucosidase activity was assayed according to a modified method (Belancic et al. 2003) using *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG) as a substrate. A reaction mixture comprising 0.2 mL of 20 mM *p*NPG in 10 mM sodium acetate buffer (pH 5.0) and 0.2 mL of the enzyme fraction was incubated at 60 °C for 30 min. The reaction was terminated by the addition of 0.4 mL of 1 M Na_2CO_3, and the liberation of *p*-nitrophenol (*p*NP) was evaluated by measuring the absorbance at 405 nm. (Infinite M200, Tecan (Shanghai) Trading Co., Ltd., Shanghai, China). One unit (U) of β-glucosidase activity was defined as the amount of enzyme in sodium acetate buffer (10 mM, pH 5.0) that released 1 μmol *p*NP per minute at 60 °C. The protein concentration was estimated using the BCA kit (bovine albumin at a concentration of 1 mg/mL was used as calibrant).

Effect of carbon sources on β-glucosidase production

Different carbon sources for inducing the production of β-glucosidase were evaluated by previously described methods (Baffi et al. 2011; Harrer et al. 1983) with modification. The carbon sources added to the medium were monosaccharide (glucose and galactose), disaccharide (sucrose, cellobiose, and maltose), and polysaccharide (carboxymethyl cellulose) at a concentration of 5 g/L and 10 g/L (Table 1); they were separately added to the yeast media (YP medium, 10 g/L yeast extract, 20 g/L peptone, pH 5.0) and sterilized at 121 °C for 20 min. With the *K. marxianus* suspension inoculated, the medium was cultured at 30 °C for 48 h under aerobic conditions. The same concentrations of okara were added in the YP medium in the same manner. At the same time, a solution consisting of 30 g/L of pure okara was used to estimate β-glucosidase production. Following the crude β-glucosidase activity, the effect of different carbon sources on the β-glucosidase production was assessed.

Table 1 The effects of various carbon sources on the induction of β-glucosidase from *K.marxianus*

Carbon sources	Concentration (g/L)	
	5.0	10.0
Monosaccharide		
Glucose*	111.79 ± 2.83%^{f}	148.30 ± 3.17%^{f}
Galactose*	112.42 ± 3.25%^{f}	152.43 ± 2.52%^{e}
Disaccharide		
Sucrose*	123.43 ± 2.66%^{d}	182.90 ± 1.36%^{c}
Maltose*	124.45 ± 2.37%^{c}	146.90 ± 3.76%^{g}
Cellobiose*	148.03 ± 4.02%^{a}	260.02 ± 3.83%^{a}
Polysaccharides		
Microcrystalline cellulose	121.44 ± 2.85%^{e}	156.29 ± 2.36%^{d}
Carboxymethyl cellulose*	56.64 ± 2.84%^{g}	86.30 ± 1.65%^{h}
Okara*	136.53 ± 2.33%^{b}	230.15 ± 2.26%^{b}
Okara**	466.03 ± 4.29%	

The relative activity was defined as 100% cultured in the YP medium (10 g/L yeast extract, 20 g/L peptone, pH 5.0)

*YP medium with different carbon sources

**Solution of pure okara at 30 g/L, natural pH

Different letters in the same row indicate significant differences at $p < 0.05$

Fermentation optimization and purification of β-glucosidase

Based on the single factor tests (Supplementary Fig. 1), three factors of cultivation temperature, cultivation time, and inoculum concentration were considered for response surface methodology (RSM) to optimize the β-glucosidase production in okara using Box–Behnken design (Bello et al. 2012). With the β-glucosidase activity being response value, three factors and three levels of response surface design were chosen for the optimization of the *K. marxianus* fermentation conditions (Table 2). The best conditions could be predicted according to the following quadratic models:

$$Y = \beta_0 + \sum_{j=1}^{k} \beta_j X_j + \sum_{j=1}^{k} \beta_{ij} X_{ij}^2 + \sum_{j>1}^{k} \beta_{ij} X_i X_j$$

Table 2 Box–Behnken experimental design and the results of β-glucosidase activity from *K.marxianus* in pure okara solution

Run number	A	B	C	β-glucosidase activity (U/mg)
1	− 1 (30)	− 1 (72)	0 (10)	2.69
2	1 (40)	− 1	0	2.84
3	− 1	1 (120)	0	2.84
4	1	1	0	2.97
5	− 1	0 (96)	− 1 (8)	2.14
6	1	0	− 1	2.41
7	− 1	0	1 (12)	2.09
8	1	0	1	2.2
9	0 (35)	− 1	− 1	2.69
10	0	1	− 1	3.07
11	0	− 1	1	2.69
12	0	1	1	2.66
13	0	0	0	4.5
14	0	0	0	4.54
15	0	0	0	4.56
16	0	0	0	4.38
17	0	0	0	4.58

A, cultivation temperature (°C); B, cultivation time (h); C, inoculum concentration (%)

where Y is the predicted response, X_iX_j represents coded independent variables, β_0 is the offset term, and β_i, β_{ii} and β_{ij} are the interaction coefficients of the linear, quadratic and second-order terms, respectively.

To improve the purification of β-glucosidase, the crude β-glucosidase was precipitated firstly with ammonium sulfate at a concentration range of 20–80% saturation at 4 °C overnight and the precipitated enzyme was collected after centrifugation at 5 050 g at 4 °C for 30 min. Then, the collections were dissolved in 20 mM acetate buffer (pH 5.0) and dialyzed in 0.1 M distilled water for one day in a cold room, with continuous stirring. The enzyme dialysate was applied to a Sephadex G-75 column (Jinan Bona Biological Technology, Co, Ltd., Shandong, China) (40 × 3.5 cm) and eluted with distilled water at a flow rate of 0.5 mL/min. The protein fractions were detected at 280 nm, collected, and analyzed for β-glucosidase activity.

Molecular weight determination

The active fraction was analyzed by 7% nondenaturing polyacrylamide gel electrophoresis (Native PAGE) and 12% SDS-PAGE (Lee et al. 2013). After electrophoresis, the two gels were stained with Coomassie blue. To determine the band of the β-glucosidase, these gels were also immersed in a solution containing 0.1 mM sodium acetate buffer solution (pH 5.0), 0.1% esculin, and 0.3% 0.1 mM $FeCl_3$ at 60 °C for 30 min, and then soaked in a 10% glucose solution to stop the reaction. The position of a black band was used to determine the molecular mass of the β-glucosidase (Kwon et al. 1994).

Substrate specificity analysis

The relative activities of β-glucosidase against 10 kinds of substrates including aryl-glycosides (20 mM; *p*NPG, *o*-nitrophenyl-D-β-galactopyranoside (*o*NPG)), esculin, and salicin), disaccharides (20 mM; cellobiose, maltose, lactose, and sucrose), and polysaccharides (1%; carboxymethyl cellulose and starch) were tested (Mallek-Fakhfakh and Belghith 2016). The activities were determined by the glucose oxidase/peroxidase (GOD-PAP) method using a commercial kit except for *p*NPG and *o*NPG (Table S1).

Kinetic parameters

The kinetic parameters (K_m and V_{max}) for the β-glucosidase were obtained at 1.7–10 mM *p*NPG from the reaction rates (mmol mg^{-1} min^{-1}). The K_m and V_{max} values were determined according to the Lineweaver–Burk plot.

Optimal temperature of β-glucosidase

The optimal temperature for the β-glucosidase activity was determined to range from 20 to 80 °C. To assess the thermal stability of the β-glucosidase, the purified enzyme was incubated at different temperatures (40–65 °C) for various amounts of time (1–5 h) and the residual activities

were estimated according to the β-glucosidase activity assay.

Optimal pH of β-glucosidase

The optimal pH for the β-glucosidase activity was estimated ranging from pH 3.0 to 8.0 (citrate buffer, pH 3.0–3.5; sodium acetate buffer, pH 4.0–7.0; phosphate buffer, pH 7.5–8.0; 0.1 M). The pH stability was analyzed by preincubating 100 µL of β-glucosidase in 900 µL of the different buffers at 4 °C for 12 h or 24 h. The activities were estimated according to the β-glucosidase activity assay.

Effects of metal ions on β-glucosidase

The purified β-glucosidase was preincubated at 4 °C for 24 h at room temperature in the presence of different metal ions (K^+, Ca^{2+}, Na^+, Mg^{2+}, Mn^{2+}, Ba^{2+}, Fe^{3+}, Zn^{2+}, Ag^+, and Cu^{2+}) at 10 mM concentration, and β-glucosidase activity was evaluated according to the β-glucosidase activity assay.

Effects of organic solvents on β-glucosidase

The purified enzyme was used to investigate the effects of organic solvents, such as alcohols (methanol, ethanol, 1-propanol, 1-butanol, and 1-pentanol) at 1 M concentration and DMSO (10–50%) according to the β-glucosidase activity assay.

Statistical analysis

The least significant difference (LSD) was carried out using multiple regression analysis (ANOVA) with an SPSS 19.0 for Windows (SPSS Inc, Chicago, IL, USA), and $p < 0.05$ was considered to be statistically significant. The optimization of the culture conditions was analyzed with Design-Expert 8.0 (STAT-EASE Inc., MN, USA). All data are expressed as the means ± standard deviations (SD) and were calculated in triplicate.

Results and discussion

Okara induced the production of β-glucosidase

The production of β-glucosidase can be demonstrated by the color depth of a dark brown region (Renchinkhandand et al. 2017) using esculin medium. As shown in Fig. 1, β-glucosidase secretion showed different patterns on the esculin medium with or without okara. In 24 h, a dark brown region appeared on the esculin plates with okara, which means that β-glucosidase was secreted rapidly, and the color of dark brown region get more intense in 48–96 h. In contrast, the plate supplemented with yeast extract didn't appear similar color until 72 h incubation, and the control medium in the first line didn't appear a dark brown region until 96 h. The color difference on these three lines fully demonstrated that okara could significantly promote the secretion of β-glucosidase from *K. marxianus*.

β-Glucosidase is a type of cellulase, which can be induced by the addition of carbohydrates using microorganism-driven production (Rajoka et al. 2004). To further investigate the ability of okara to induce β-glucosidase from *K. marxianus*, the effects of various carbon sources on β-glucosidase induction were investigated in more detail. The results showed that except for carboxymethyl cellulose, most of the carbohydrates (monosaccharides, disaccharides, polysaccharides, and okara) used in this study exerted a positive effect on the induction of β-glucosidase expression (Table 1). Among them, the cellobiose showed a very strong β-glucosidase induction. In the presence of cellobiose at 5 g/L and 10 g/L, the β-glucosidase activity was 1.48-fold and 2.60-fold higher than that of control, respectively. Cellobiose has shown the strongest induction effect (Baffi et al. 2011), possibly because β-glucosidase can convert cellobiose and cellotriose into glucose upon hydrolyzing cellulose, which can participate in cell metabolism, or because cellobiose is important in the regulation of β-glucosidase-encoding genes (Seidle and Huber 2005). The effects of different carbon sources for the production of β-glucosidase by *K. marxianus* fermentation had the following order: cellobiose > okara > sucrose > microcrystalline cellulose > galactose > glucose > maltose. Rajoka et al. (2004) had also reported that cellobiose and sucrose greatly improved β-glucosidase production.

It is worth noting that the amount of β-glucosidase produced in pure okara solution was much higher than those reached in other carbon sources. This was a very favorable consequence. It has recently been reported that *K. marxianus* cultured in wheat bran, which contains 46.4% of total dietary fiber, can significantly increase the β-glucosidase secretion (Zhang et al. 2019), indicating the potential application of the cellulose-rich material in the production of β-glucosidase. The okara contains more than 50% dietary fiber (Li et al. 2011; Hu et al. 2019), and may contribute to the production of large amounts of β-glucosidase; thus, the fermentation conditions of *K. marxianus* in pure okara solution need to be optimized to obtain more β-glucosidase.

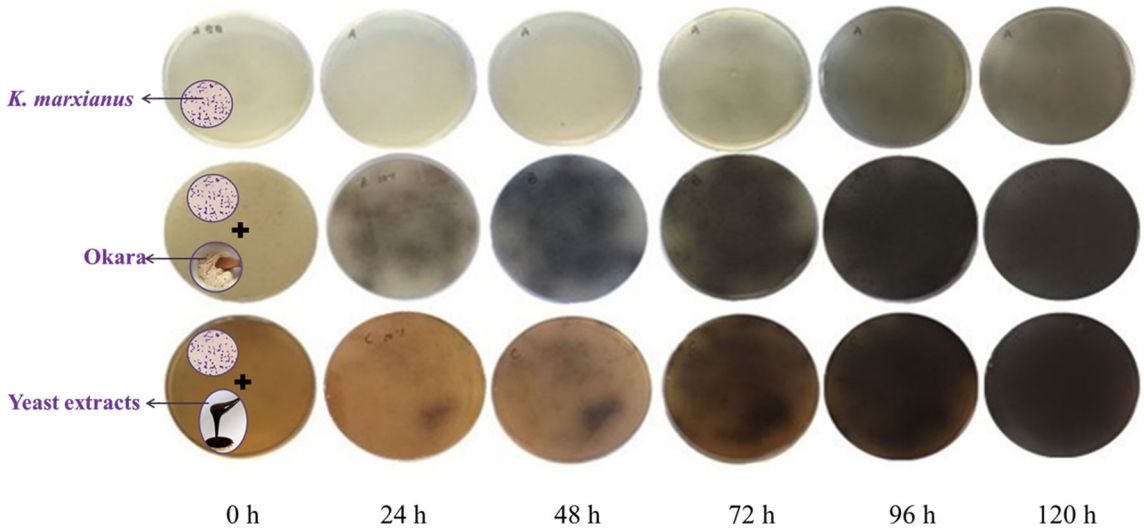


Fig. 1 Okara induces the production of extracellular β-glucosidase. Esculin iron agar plates with unsupplemented (Up), supplemented with okara (Middle) and with yeast extract (Down), were observed at 30 °C during 0–120 h in the presence of *K. marxianus*

Optimization and purification of β-glucosidase from fermentation okara

According to the multiple regression analysis of RSM, a second-order polynomial equation describing the correlation of the β-glucosidase production and the relevant independent variables (X_1, X_2 and X_3) is described as below:

$$Y = 4.51 + 0.083X_1 + 0.079X_2 - 0.084X_3 - 5.000E^{-003}X_1X_2 - 0.040X_1X_3 - 0.10X_2X_3 - 1.12X_1^2 - 0.55X_2^2 - 1.18X_3^2$$

where Y represents the β-glucosidase activity and X_1, X_2 and X_3 represent the experimental values of cultivation temperature, cultivation time and inoculum concentration, respectively.

As presented in Table 3, model terms with values of $P > F$ ($P < 0.0001$) are considered to be highly significant The lack of fit F-value of 0.8603 suggests that the lack of fit is not significant. The correction coefficient R^2 (0.9979) indicated that the model could explain 99.79% of the variation of response value. Thus, the result of ANOVA analysis implied that the model of RSM can be taken. The maximal activity of β-glucosidase was measured as 4.5 U/mg and reached at Design-Expert 8.0 predicted conditions of cultivation: fermentation temperature 35 °C, cultivation time 98 h, inoculum concentration 10%, and 30 g/L of okara. Rajoka et al. (2004) also reported that the temperature of 35 °C was most favorable for the secretion of β-glucosidase from *K. marxianus.*

For the first step of purification, an ammonium sulfate solution of different saturation was used to precipitate the enzyme protein, and 60% saturation ammonium sulfate was selected (Supplementary Fig. 2), the β-glucosidase was purified by 8.44 times. Then, the Sephadex G-75 chromatography was used as the second purification step, and the β-glucosidase activity was only detected in F1 fraction (Fig. 2a). After two steps of purification, the activity of β-glucosidase was 71.4 U/mg corresponding to 15.87-fold purification and 14.28% of yield.

To further confirm the purity of the β-glucosidase in F1 fraction, Native PAGE, esculin chromogenic assay, and SDS-PAGE with Coomassie blue staining were used (Fig. 2b). The purified enzyme showed a single protein band on the Native PAGE with an apparent molecular mass of approximately 66 kD with a black band indicating β-glucosidase activity when esculin chromogenic assay was developed. Three bands appeared on SDS-PAGE at 22, 44 and 66 kDa, respectively, and no band showed β-glucosidase activity. The best explanation was that the enzymatic activity of 22 and 44 kDa under denaturing conditions (SDS, reducing agent) were prohibited and the β-glucosidase was an apparent molecular mass of approximately 66 kDa containing two subunits (approximately 22 and 44 kDa). As for the SDS-PAGE 66 kDa band, no enzyme activity was appeared, which may be related to the changes in the spatial structure of the enzyme molecule caused by SDS reduction, which needs further research and explanation. The molecular mass of β-glucosidases from various microbial sources (Yeom et al. 2012; Kuo et al. 2018; Watanabe et al. 2016; Baffi et al. 2011; Yoshida et al. 2010) usually range from 30 to 500 kDa as monomers or multimers (dimers to octamers). β-Glucosidases from *Dekkera bruxellensis* yeast (Kuo et al. 2018) and *Agaricus bisporus* fungi (Ašić et al. 2015) exhibited a dimeric structure. β-Glucosidases from *K. marxianus* reported by

Table 3 ANOVA for the quadratic model of the *β*-glucosidase activity from *K. marxianus* in pure okara solution

Variable	Sum	Degree of freedom	Mean square	F value	*P*
Model	13.96	9.0	1.55	362.60	< 0.0001
A	0.054	1	0.054	12.72	0.0091
B	0.050	1	0.050	11.59	0.0114
C	0.056	1	0.056	13.11	0.0085
AB	1.000E−004	1	1.000E−004	0.023	0.8828
AC	6.400E−003	1	6.400E−003	1.50	0.2659
BC	0.042	1	0.042	9.82	0.0165
A^2	5.30	1	5.30	1239.21	< 0.0001
B^2	1.30	1	1.30	302.80	< 0.0001
C^2	5.86	1	5.86	1369.45	< 0.0001
Residual	0.030	7	4.279E−003		
Lack of fit	4.675E−003	3	1.558E−003	0.25	0.8603
Pure error	0.025	4	6.320E−003		
Cor total	13.99	16			
R^2 = 0.9979					

A, cultivation temperature (°C); B, cultivation time (h); C, inoculum concentration (%)

Yoshida et al. (2010) is a tetramer. These results have shown that the *β*-glucosidase obtained in this study has a different structure, which may be due to the induction of okara.

As is known to all, the cost and the loss of activity of enzymes has always been a problem that must be solved in isolation and purification. Many chromatographies such as anion exchange (Mallek-Fakhfakh and Belghith 2016), hydrophobic interaction (Hernandez-Guzman et al. 2016), and size-exclusion chromatography (Ašić et al. 2015; Mallek-Fakhfakh and Belghith 2016) have been applied to the isolation of *β*-glucosidases. Most of the purifications include two or more chromatography steps. However, the purification process developed in this study was evenly simple and effective, which could be attributable to the crude enzyme solution containing few protein impurities and the effectiveness of the ammonium sulfate purification step, which increased the efficacy of the subsequent dialysis and gel filtration. The use of simple purification technologies significantly contributes to cost savings in enzyme preparations.

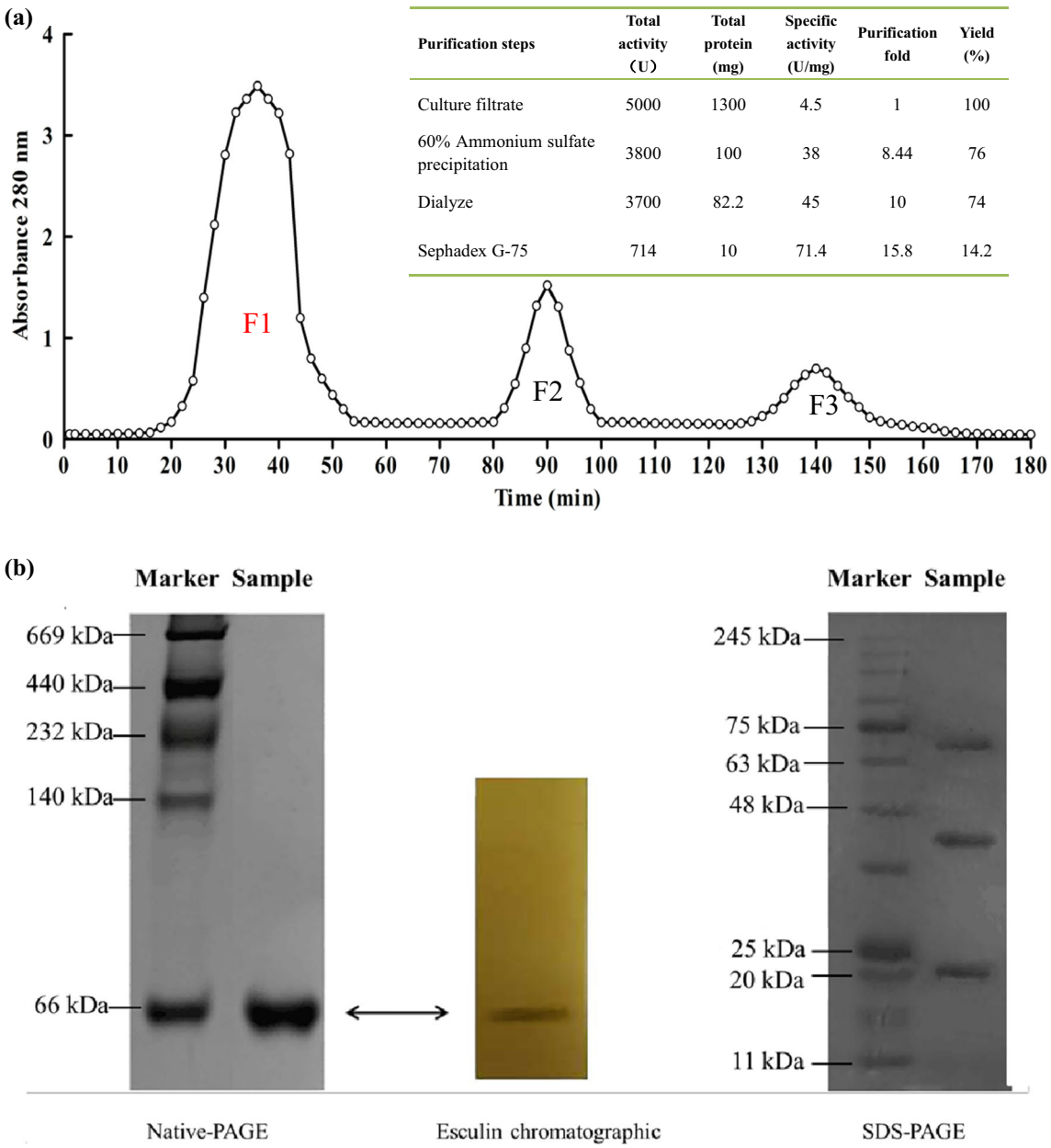
Substrate specificity and kinetic parameters of *β*-glucosidase

Most microbial *β*-glucosidases are classified into glycoside hydrolase family 3 (GH3) based on their amino acid sequence and substrate specificity, which is characterized by broad substrate specificity. (Guo et al. 2015). The substrate specificity of the *β*-glucosidase from *K. marxianus* was summarized in Table S1. In the aryl glycoside substrate, *β*-glucosidase had the highest activity with *p*NPG, followed by esculin and salicin, but it was inactive with *o*NPG. In the disaccharide substrate, *β*-glucosidase showed the highest hydrolysis activity with cellobiose, followed by maltose. No *β*-glucosidase activity was detected in substrates of lactose, sucrose, and polysaccharides. Because the substituent group and its position on the phenyl ring greatly impact the electron-withdrawing ability (Seidle and Huber 2005), this factor may have contributed to the observation that esculin was a better substrate than salicin. We also observed that the enzyme hydrolyzed glucosidic bonds of cellobiose (*β*-1,4) and maltose (α-1,4) but not *β*-1,4 glucosidic bonds in carboxymethyl cellulose. These results indicated that *β*-glucosidase from *K. marxianus* belongs to GH3 (Krogh et al. 2010).

The kinetic parameters for *p*NPG were calculated using Lineweaver–Burk plots and the linear regression (Supplementary Fig. 3). The V_{max} and K_m values determined for *p*NPG were 8.34 μmol min^{-1}·mg^{-1} and 7.42 mM, respectively. The activity of the purified enzyme was 71.4 U/mg, being higher than that of the extracellular *β*-glucosidase activity (6.98 U/g) from *K. marxianus* reported by Zhang et al. (2019).

Effects of temperature, pH, metal ions, and organic solvents on *β*-glucosidase

The effects of temperature on the stability of the *β*-glucosidase were shown in Fig. 3a, b. The *β*-glucosidase produced by okara using *K. marxianus* showed maximal catalytic activity at 60 °C that was higher than those previously reported for enzymes from fungi (Belancic et al. 2003; Watanabe et al. 2016; Belancic et al. 2003; Ašić



Purification steps	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification fold	Yield (%)
Culture filtrate	5000	1300	4.5	1	100
60% Ammonium sulfate precipitation	3800	100	38	8.44	76
Dialyze	3700	82.2	45	10	74
Sephadex G-75	714	10	71.4	15.8	14.2

Fig. 2 Purification of β-glucosidase activities with gel filtration columns. **a** Sephadex G-75; **b** Apparent molecular mass identification. Native PAGE (Left), Esculin chromatograph (Middle) and SDS-PAGE (Right)

et al. 2015) and bacteria (Bello et al. 2012). And it remained stable and retained almost full activity after 5 h incubation at 55 °C. The use of this enzyme in reactions at elevated temperatures would increase the solubility of the reactants and products, which would be conducive to the enzymatic hydrolysis of greater amounts of biomass.

The purified β-glucosidase showed maximal catalytic activity at pH 5.0 (Fig. 3c, d). The stability assay results showed that the enzyme was active over a wide range of pH values (from pH 3.0 to 8.0), as more than 38% of maximal enzyme activity was retained after 24 h. Notably, the enzyme retained almost 60% of its initial activity after 12 h at 4 °C and pH 3.0 and retained 42% of its initial activity at pH values of 7.0 to 7.5, providing further evidence of its acid–alkali tolerance under lingocellulose saccharification conditions (Ma et al. 2015).

As shown in Fig. 3e, the β-glucosidase activity was increased by approximately 20% in the presence of Mg^{2+} and K^{+} while Ba^{2+}, Fe^{3+}, Cu^{2+}, Zn^{2+}, and Ag^{+} significantly inhibited the enzyme.

In terms of tolerance to organic solvents, the β-glucosidase activity remained nearly constant at 10–50% DMSO. In contrast, almost all assayed alcohols inhibited the β-glucosidase, including methanol and 1-butanol.

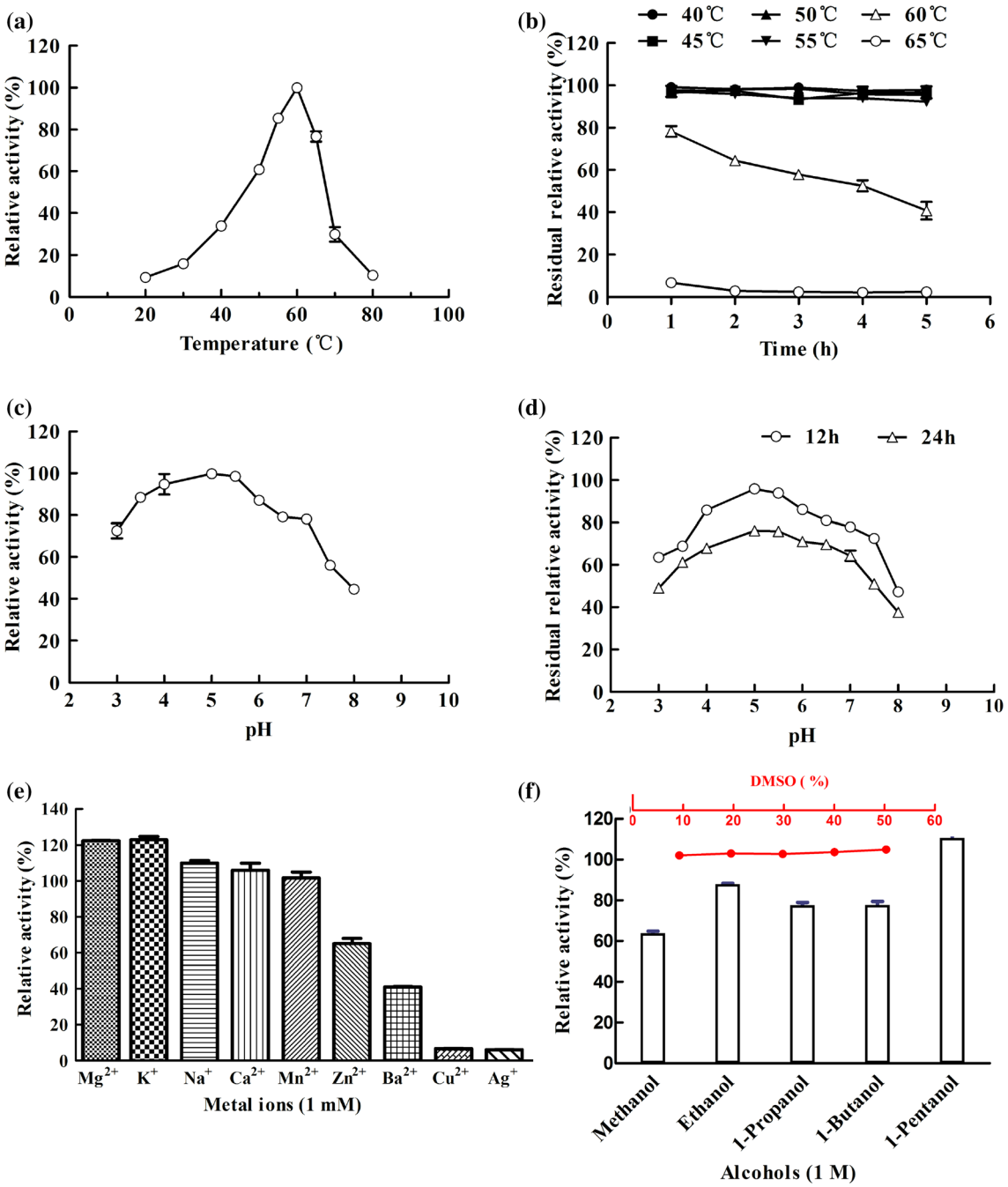


Fig. 3 Characteristics of the *β*-glucosidase from *K. marxianus*. **a** Effect of temperature on *β*-glucosidase activity, with a relative activity of 100% at 60 °C; **b** Thermal stability of the *β*-glucosidase after incubation for different amounts of time; the control was incubated at 4 °C; **c** Effect of pH on *β*-glucosidase activity, with a relative activity of 100% at pH 5.0; **d** pH stability of *β*-Glucosidase after incubation in citrate (pH 3.0–4.0), sodium acetate (pH 5.0–6.0), and phosphate (pH 6.0–8.0) for different amounts of time. The relative activity was defined as 100% at pH 5.0; **e** Effect of metal ions on *β*-glucosidase activity. The relative activity was defined as 100% without any additive (Control); **f** Effects of DMSO and alcohols on *β*-glucosidase. The relative activity was defined as 100% without any additive (Control). The values are shown as the mean ± SD of three independent experiments

Interestingly, 1-pentanol slightly activated the enzyme, possibly through its transglycosylation, leading to the combination of *p*NPG and 1-pentanol accelerating the reaction(Watt et al. 1998). DMSO is generally used to promote the dissolution of low water-soluble substrates in industrial processing. However, most of the previously reported *β*-glucosidases were inhibited by DMSO (Mallek-Fakhfakh and Belghith 2016). Thus, the *K. marxianus β*-glucosidase obtained in this study has wide broad industrial applications.

Conclusion

This paper focused on the advantage of okara in the production of the β-glucosidase by *K. marxianus* fermentation. The results fully demonstrated that the fermentation of *K. marxianus* with a small amount of okara or pure okara can produce a large amount of β-glucosidase. The β-glucosidase from *K. marxianus* after only two purification steps can reach a high purity with a resistance of high temperature, pH, and wide range of DMSO concentrations. In conclusion, the research provides conditions for the improvement of the β-glucosidase production using okara as substrate and a practical technical solution for the added-value processing of okara.

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