Permeabilization of *Saccharomyces fragilis* IZ 275 cells with ethanol to obtain a biocatalyst with lactose hydrolysis capacity

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ABSTRACT. The permeabilization was used to transform microorganisms in cell biocatalysts with high enzymatic activity. The *Saccharomyces fragilis* IZ 275 yeast cells were permeabilized with ethanol, as permeabilizing agent. To optimize the permeabilization conditions were used the design of Box-Behnken 15 trials (3 central points). The independent variables and their levels were ethanol (29, 32 and 35%), temperature (15, 20 and 25°C) and time (15, 20 and 25 min). The answer (Y) function has beta-galactosidase activity (U mg^{-1}). The optimum conditions for obtaining a high enzymatic activity were observed in 35% ethanol concentration, temperature 15°C and 20 min. treatment time. The maximum activity of the enzyme beta-galactosidase obtained was 10.59 U mg^{-1}. The permeabilization of the *S. fragilis* IZ 275 cells was efficient.

Keywords: permeabilizing agent, beta-galactosidase, microbial biotechnology, optimal conditions.

Permeabilização de células de *Saccharomyces fragilis* IZ 275 com etanol para obtenção de biocatalisador com capacidade de hidrólise de lactose

RESUMO. A permeabilização foi usada para transformar células de microrganismos em biocatalisadores com alta atividade enzimática. As células de levedura de *Saccharomyces fragilis* IZ 275 foram permeabilizadas com etanol, como agente permeabilizante. Para otimizar as condições de permeabilização foi utilizado o delineamento de Box-Behnken com 15 ensaios (3 repetições no ponto central). As variáveis independentes e seus níveis foram etanol (29, 32 e 35%), temperatura (15, 20 e 25°C) e tempo (15, 20 e 25 min.). A função resposta (Y) foi atividade de beta-galactosidase (U mg^{-1}). As condições ótimas para a obtenção de uma alta atividade enzimática foram observadas em 35% de concentração de etanol, temperatura de 15°C e tempo de tratamento de 20 minutos. A máxima atividade da enzima beta-galactosidase obtida foi de 10.59 U mg^{-1}. A permeabilização das células de *S. fragilis* IZ 275 foi eficiente.

Palavras-chave: agente permeabilizante, beta-galactosidase, biotecnologia microbiana, condições ótimas.

Introduction

Saccharomyces fragilis is described as a homothallic, hemiascomycetous yeast and production of several enzymes among them beta-galactosidase (Llorente et al., 2000; Dagbagli & Goksungur, 2008). The major common feature of *S. fragilis* is the capacity to assimilate lactose and to use this sugar as a carbon source. The long history of safe association with food products helped *S. fragilis* achieve GRAS (Generally Regarded As Safe) and QPS (Qualified Presumption of Safety) in the United States and European Union, respectively. This designation means that there are few restrictions on application and largely enhances their potential in the biotechnology sector (Fukuhara, 2006; Schaffrath & Breunig, 2000). *S. fragilis*, has been more widely adopted by industry, mainly because it possesses traits that are desirable for biotechnology applications. These include the capacity to assimilate sugars, namely lactose and inulin; an extremely rapid growth rate, with typical generation times of about 70 min; thermotolerance, with the ability to growth up to 52°C; and a high secretory capacity (Fonseca, Heinzle, Wittmann, & Gombert, 2008).

Beta-galactosidase is one among other enzymes with industrial potential used in the hydrolysis of lactose in milk and cheese whey, generating food with low levels of lactose, whose result is a better solubility and digestibility of milk and dairy products, making them ideal for consumers intolerant to this sugar (Husain, 2010). To detect enzyme activity, two different substrates are commonly used. X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) is employed for *in*

vivo detection of enzyme activity on plates since it readily enters the cells. X-Gal is, however, unsuitable for quantitative measurements. For that purpose ONPG (o-nitrophenyl-β-D-galacto pyranoside) has to be used which in turn has the disadvantage of being unable to enter into intact cells. Therefore, the cells have to be permeabilized before determination of enzyme activity (Kippert, 1995).

Beta-galactosidase from yeast cells is an intracellular enzyme and it is necessary the use of techniques to obtain it. Physicochemical methods and enzyme can be used, but are not suitable, because the drastic applied treatments can lead to degradation of the compound of interest (Coelho, Salgado, & Ribeiro, 2008; Panesar, Panesar, Singh, & Bera, 2007).

Cell permeabilization techniques are often useful for a lot of applications relating to enzyme technology. For example, permeabilization procedures are usually rapid and do not destroy cellular enzymes. Thus, the total amount of an enzyme associated with a cell can be assayed after permeabilization. A number of permeabilization methods for yeast have been developed, such as use of detergents, organic solvents, and desiccation (Becker, Caldwell, & Zachgo, 1996). Cell permeabilization is influenced by several operating conditions that need to be optimized. However, the traditional optimization method in which the level of one parameter is varied at a time over a certain range, while keeping the other variables constant, is generally time consuming, requiring a large number of tests (Sen & Swaminathan, 1997), and does not reflect the interaction effects among the variables and, consequently, does not depict the net effect of the various factors on the enzyme activity (Dagbagli & Goksungur, 2008). These drawbacks can be overcome by using statistical experimental factorial designs, and the experimental data of responses are usually fitted to second order polynomial functions by the response surface methodology (RSM).

This study aimed to assess cell permeabilization *Saccharomyces fragilis* IZ 275 cells in different conditions time, temperature and concentration of ethanol using Box-Behnken design and response surface methodology.

Material and methods

Microorganism

The strain SF IZ 275 from the Dairy Science and Technology, Graduation and Research Center, *Universidade Norte do Paraná* (UNOPAR) was obtained from Foundation Andre Tosello - Tropical Culture Collection. Stock cultures were maintained at 4ºC in PDA (Potato Dextrose Agar) agar test tubes.

Inoculum preparation

The SF IZ 275 strain were initially grown in PDA agar test tube and incubated at 35ºC for 24 h. Three loop colonies were then transferred into separate 250 mL Erlenmeyer flasks containing 50 mL of malt extract (15 g L^{-1}). The flasks were incubated at 35ºC and shaken at 150 rpm for 24 h. The optical density (OD) was then determined at a wavelength of 570 nm using a spectrophotometer and OD was adjusted to 0.6.

Obtaining SF IZ 275 cells and permeabilization

The strain SF IZ 275 was used as inoculum for beta-galactosidase production using cheese whey as media. Cheese whey obtained from a local dairy cooperative was deproteinized by adding lactic acid to pH 4.6 and heating at 90°C for 30 min. Then it was filtered through whatman nº 1 filter paper to remove coagulated protein and adjusted to pH 5.0 with NaOH solution (50%). It was then pasteurized under 65°C for 30 min. The pasteurized cheese whey was inoculated with 10% inoculum (at Optical Density, $OD_{670nm} = 0.6$) of SF IZ 275 and incubated at 35°C, 150 rpm for 24 h. The cells were harvested from 5 mL of fermentation media by centrifugation at 5000 rpm for 20 min and washed with 0.1 M phosphate buffer (pH 6.8) solution. The biomass concentration was monitored spectrophoto metrically with a Femto 700 Plus spectrophotometer (Sao Paulo, Brazil). Absorbance of prepared biomass solution was measured at 570 nm and the biomass concentration was estimated from a biomass dry weight vs. absorbance calibration curve obtained previously.

Cell permeabilization was performed in 25 mL Erlenmeyer flasks each containing 5 mL of the reacting suspension consisting of 5 mg (dry wt) of SF IZ 275 cells in 0.1 M potassium phosphate buffer (pH 6.8) and ethanol according to the experimental design (Table 1). Flasks were incubated on an orbital shaker at 150 rpm at a temperature and for a time depending on the above design. The supernatant was removed by centrifugation at 5000 rpm for 5 min and the cells were washed twice with the same buffer. The final pellet was resuspended in 1 mL of 0.1 M potassium phosphate buffer (pH 6.8) and the enzyme activity of permeabilized cells was determined as described later.

Beta-galactosidase activity

The determination of beta-galactosidase activity was performed according to Inchaurrondo, Yautorno and Voget (1994). The beta-galactosidase activity was assayed using the chromogenic substrate ONPG (o-nitrophenyl-β-galactopyranoside).

50 μL sample of permeabilized cell suspension (0.05 mg) was mixed with 2 mL of 1.25 mM ONPG in 0.1 M potassium phosphate buffer (pH 6.6) and incubated for 5 min at 37°C. The reaction was discontinued by adding 0.5 mL of 1 M sodium carbonate. Liberated ONPG was measured spectrophotometrically at 420 nm. One unit of beta-galactosidase activity (U) is defined as the amount of enzyme which hydrolysis of 1 μmol of substrate (ONPG) per minute under the conditions of the assay. All activity tests were performed in triplicate and expressed as mean values.

Experimental design

The permeabilization of SF IZ 275 cells for high activity of beta-galactosidase was performed using a factorial design and analysis by the response surface method. Controlled conditions for ethanol concentration, temperature and incubation time were tested according to 3-factor 3-level (-1; 0; +1) Box-Behnken Design (Montgomery, 2005) with three replicates at the central point summarizing 15 experimental runs (Table 1).

The mathematical model for the response function (y = beta-galactosidase activity) was expressed as:

$$A = \beta_0 + \sum_{i=1}^{n} \beta_i X_i + \sum_{i=1}^{n} \beta_{ii} X_i^2 + \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} \beta_{ij} X_i X_j \qquad \text{Equation (1)}$$

where β_0 is the estimated coefficients in response surface and Xi Xj are real variables on the dependent variable. Statistical analysis of the mathematical model was performed using ANOVA ($p < 0.05$) and regression analysis using Statistica 6.0. Response surface was generated to evaluate the response.

Storage stability

The ability of permeabilized cells treated with ethanol to retain beta-galactosidase activity during storage was studied. A suspension of 1 g of cells (wet wt) in 20 mL potassium buffer 0.1 M (pH 7.0) was stored at 4°C. At daily intervals for 9 days a predetermined volume of suspension was separated by centrifugation and beta-galactosidase activity was determined in the separated cells.

Results and discussion

The optimization of the cell permeabilization conditions was carried out to find the optimal values of independent variables (ethanol concentration, temperature and treatment time), which would give maximum beta-galactosidase activity. Based on the Box-Benhken Design (BBD), the experimental levels of beta-galactosidase activity under each set of condition were determined and compared with the corresponding predicted levels suggested (Table 1). The maximum experimental value for beta-galactosidase activity was 2.12 U mg^{-1}, while the value of predicted response was 2.06 U mg^{-1}. Approximately 97% of validity was achieved, indicating the model exerted an adequate prediction on the enzyme activity. The close correlation between the experimental and predicted data indicates the appropriateness of the experimental design. The maximum beta-galactosidase activity (2.12 U mg^{-1}) was achieved in the following condition, ethanol 35% (v v^{-1}), 15°C and 20 min (Table 1).

Table 1. Effect of ethanol concentration, time and temperature in the beta-galactosidase activity of permeabilized *Saccharomyces fragilis* IZ 275 cells.

Standard Run*	Variables (real value)			Response	
	X1	X2	X3	Beta-galactosidase Activity (U mg^{-1})	
	Ethanol Concentration (%)	Temperature (°C)	Time (min.)	Experimental	Predictive
1	-1 (29)	-1 (15)	0 (20)	1.36	1.40
2	1 (35)	-1 (15)	0 (20)	2.12	2.06
3	-1 (29)	1 (25)	0 (20)	1.43	1.49
4	1 (35)	1 (25)	0 (20)	1.97	1.93
5	-1 (29)	0 (20)	-1 (15)	1.40	1.43
6	1 (35)	0 (20)	-1 (15)	1.95	2.07
7	-1 (29)	0 (20)	1 (25)	1.66	1.54
8	1 (35)	0 (20)	1 (25)	2.03	2.01
9	0 (32)	-1 (15)	-1 (15)	1.95	1.88
10	0(32)	1 (25)	-1 (15)	1.84	1.76
11	0 (32)	-1 (15)	1 (25)	1.72	1.80
12	0 (32)	1 (25)	1 (25)	1.82	1.89
13	0 (32)	0 (20)	0 (20)	2.05	1.94
14	0 (32)	0 (20)	0 (20)	1.90	1.94
15	0 (32)	0 (20)	0 (20)	1.86	1.94

*Run was randomly.

Based on the results for obtaining high activity of beta-galactosidase from permeabilized cells of SF IZ 275, the effect of linear terms of ethanol concentration was significant (p < 0.05), indicating the establishment of the concentration for the highest design limit (35%). The linear and quadratic terms of the temperature and time were not significant (p > 0.05), indicating that established temperature and time for the smaller range limit, 15 - 25°C and 15 - 20 min, respectively proved to be sufficient for the process.

Statistical analysis

The proposed second degree polynomial was fitted to the data using multiple linear regressions to determine the optimum cell permeabilization conditions that resulted in the maximum beta-galactosidase activity. The effects of ethanol, temperature and time were quantitatively evaluated using response surface curves. By applying multiple regression analysis on the experimental data, the following second degree polynomial was found to represent the relationship between the independent variables tested (Equation 2).

The predicted levels of beta-galactosidase activity using Equation (2) are given in Table 1 along with experimental data. The significance of the fit of the second-order polynomial for the beta-galactosidase activity was assessed by carrying out analysis of variance (ANOVA) with results shown in Tables 2.

The coefficient of determination (R^2) of the model was 0.89648 (Table 2), which indicated that the model adequately represented the real relationship between the variables under consideration. An R^2 value of 0.89648 means that 89.6% of the variability was explained by the model, which is acceptable for biological system and only 10.4% was as a result of chance. The coefficient of variation (C.V.) obtained was 12.95%. The Coefficient of Variation (C.V.) indicates the degree of precision with which the treatments were carried out. A low value of C.V. suggests a high reliability of the experiment (Mason, Gunst, & Hess, 1989).

Results obtained after carrying out ANOVA is presented in Table 2. Values of 'Prob. > F' less than 0.05 indicate the model terms are significant. Values greater than 0.10 indicate the model terms are not significant. The 'Lack of Fit' F–value of 2.02396 implies that there is insignificant lack of fit. The 'Lack of Fit' (Prob > F) value of 0.347587 implies that there is only 34.75 % chance that the 'Lack of Fit' F–value could occur due to noise.

Optimization of permeabilization of *Saccharomyces fragillis* IZ275 cells

In order to optimize variables that influence beta-galactosidase activity from permeabilized SF IZ 275 cells, response surface plots were generated from the regression model. The three-dimensional response surfaces for beta-galactosidase activity: ethanol concentration, temperature and time were plotted (Figure 1).

Figure 1a shows the effects of temperature and ethanol concentration on beta-galactosidase activity. Permeabilization with low concentration of ethanol and low temperature of process showed the lowest enzyme activity. The beta-galactosidase activity was higher in the temperature range of 15 – 25°C and concentration of ethanol up to ca. 35%. Panesar et al. (2007), showed that the optimum process conditions for cell permeabilization of *Kluyveromyces marxianus* NCIM 3465 were 50% (v v^{-1}) ethanol concentration, 25°C temperature and treatment time of 15 min.

$$Y = -112.741 + 6.26\,X_1 + 0.972X_2 + 0.515X_3 - 0.019X_1X_2 - 0.015X_1X_3 + 0.010X_2X_3 - 0.080X_1^2 - 0.015X_2^2 - 0.006X_3^2 \qquad \text{Equation (2)}$$

Table 2. Analysis of variance (ANOVA) for beta-galactosidase activity of permeabilized *Saccharomyces fragillis* IZ 275 by quadratic model.

Sources	Sum ofsquares	df	Meansquares	F value	p–value [Prob > F]
X_1 – Ethanol concentration (%) (L)	15.20761	1	15.20761	57.90435	0.016835★
X_2 – Temperature (°C) (L)	0.02101	1	0.02101	0.08001	0.803876
X_3 – Time (min) (L)	0.02880	1	0.02880	0.10966	0.772010
X_1X_2 (L)	0.31360	1	0.31360	1.19406	0.388577
X_1X_3 (L)	0.19803	1	0.19803	0.75400	0.476757
X_2X_3 (L)	0.27562	1	0.27562	1.04947	0.413359
X_1^2 (Q)	1.92296	1	1.92296	7.32186	0.113744
X_2^2 (Q)	0.51004	1	0.51004	1.94203	0.298112
X_3^2 (Q)	0.08216	1	0.08216	0.31282	0.632231
Lackof Fit	1.59467	3	0.53156	2.02396	0.347587
PureError	0.52527	2	0.26263		
Total SS	20.47769	14			

*Significant p < 0.05. R-squared – 0.896; Adjusted R–squared – 0.710; Standard deviation – 1.209; C.V. % – 12.95.

Figure 1b depicts the response surface plot as a function of time versus ethanol concentration. Change of time does not significantly affect the curvature of the surface. From a graphical representation there is a dependence of beta-galactosidase activity on the concentration of permeabilizing agent (ethanol 32 - 35%). A maximum permeabilization of 2.816 mmol L^{-1}ONP min^{-1} g^{-1} was obtained by treating cells with 75% (v v^{-1}) of ethanol at 20°C for 15 min. (De Faria et al., 2013). Figure 1c shows high permeabilization effectiveness within the range of time and temperature studied, while below and above these ranges a significant decrease of activity can be noticed. This confirms that the range these variables were chosen properly and sufficient for the process.

In this study it was also clear that the effect of ethanol concentration on beta-galactosidase activity was more important than the temperature and time. In a preliminary study by our group showed that within the tested time and temperature range at different concentrations of ethanol 50, 75 and 100% there was a high activity of beta-galactosidase with no significant difference.

Trawczynska and Wójcik (2015) defined the optimum operating conditions for yeast cell permeabilization at 53% of ethanol concentration, temperature of 14.8°C and treatment time of 40 min. The use of the whole yeast cells as biocatalysts is a very promising alternative and has gained a lot of interest in recent years (Yu et al., 2007).

Whole cells of SF IZ 275 exhibited no beta-galactosidase activity. The mechanism of enzyme release has not been fully studied. However, cell wall lysis is not believed to be the mode of enzyme solubilization. Perhaps the solvent extracts a lipid component from the yeast cell membrane, allowing leakage of intracellular or periplasmic protein. A similar procedure utilizing lower concentrations of solvent (< 20%) has been reported for the measurement of intracellular enzyme *in situ* (Wendorff & Amundson, 1971). In this case, no intracellular enzyme leaks out, rather small molecular weight substrate molecules diffuse into the cell.

The validity of the results predicted by the regression model, was confirmed by carrying out repeated experiments under optimal permeabilization conditions (i.e. ethanol concentration; 35% - v v^{-1}, temperature; 15°C and time; 20 min.).
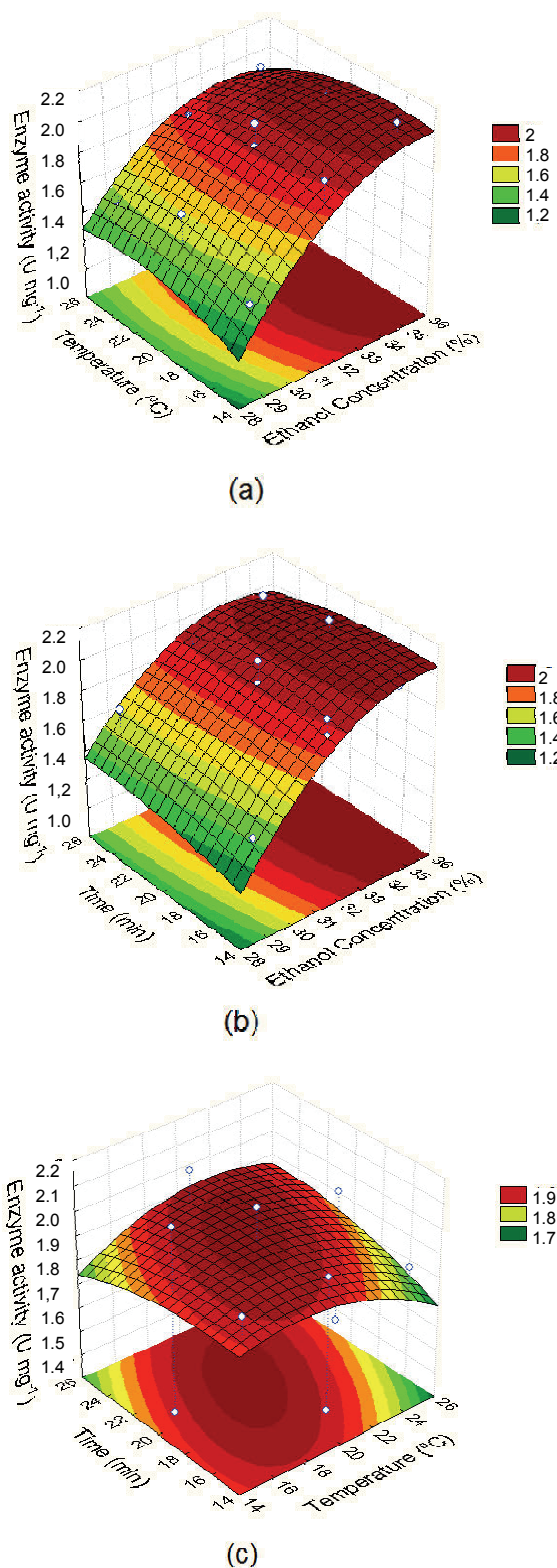

(a)

(b)

(c)

Figure 1. Response surface plot representing the effect of a) temperature and ethanol concentration, b) time and ethanol concentration, c) time and temperature on beta-galactosidase activity (U mg^{-1}) of permeabilized *Saccharomyces fragilis* IZ 275 cells.
Source: Authors.

The results obtained from three replications demonstrated that the average of the maximum beta-galactosidase activity (2.12 U mg^{-1}) obtained was close to the predicted value (2.06 U mg^{-1}). The excellent correlation between the predicted and measured values from these experiments indicates validity of response model.

The R^2 statistic indicates the percentage of the variability of the optimization parameters, which is explained by the model. The response surface plots were used to determine the optimum level of the significant variables for maximal beta-galactosidase activity of permeabilized yeast cells.

Application of Box-Behnken design in the enzyme activity by SF IZ 275, it was presented as progress in predicting conditions for cell permeabilization. The response surface proved to be a powerful tool for bioprocess optimization converting to a mathematical model which predicts the location of the optimum range. The maximum enzyme activity of 2.12 U mg^{-1} was reached at 35% ethanol concentration (v v^{-1}), 15°C and 20 min. The permeation process can be used for other studies requiring permeabilized cells to obtain other metabolites of interest.

Storage stability

Yeast cells after permeabilization at the optimal conditions have been tested with respect to maintaining enzymatic activity during storage. The cells showed 78% loss of enzymatic activity when they were stored in phosphate buffer pH 6.8 at 5 °C, for a period of 9 days (Figure 2).
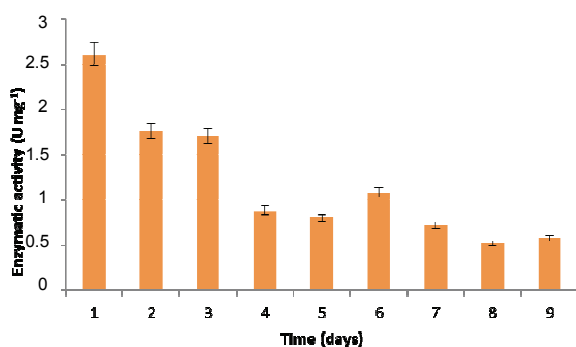
Figure 2. Storage stability of the beta-galactosidase activity.
Source: Authors.

Based on the results it can be seen that there was a significant decrease in enzyme activity between day 1 (2.61 U mg^{-1}) and days 2 and 3 (1.76 and 1.70 U mg^{-1}, respectively) of storage. After day 3 there was a significant decrease remaining until day 9 (0.57 U mg^{-1}).

Conclusion

Ethanol alcohol can effectively improve the permeability of SF IZ 275 cells. Statistical optimization of permeabilization of cell membrane by ethanol has been successfully carried out using RSM based on the 15 factorial Box-Behnken design. The proposed mathematical model with estimated parameters describes well the permeabilization process. The optimum operating conditions for the permeabilization process to achieve maximum enzyme activity were ethanol concentration of 35%, 15°C temperature and process duration of 20 min. Under these conditions of process variables the predicted value of maximum enzyme activity was found to be 10.59 U mg^{-1}. The fact that ethanol alcohol permeabilized SF IZ 275 cells retained enzyme activity for a certain period suggesting that these permeabilized cells could be used as a source of biocatalyst for different applications. Furthermore, the use of permeabilized cells can help to overcome the problems and costs associated with enzyme extraction and purification from yeast cells and in the development of a low-cost technology for biotechnological application.

Acknowledgements

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