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CLONING OF SUCROSE ISOMERASE ENCODING GENE FROM *KLEBSIELLA SINGAPORENSIS* ISB-36 AND ITS EXPRESSION IN *PICHIA PASTORIS*

Cao Xuan Bach, Dang Thi Kim Anh, Nguyen Thanh Thuy, Truong Tu Anh, Nguyen Thi Dieu Linh, Vu Nguyen Thanh✉

Food Industries Research Institute, 301 Nguyen Trai, Thanh Xuan, Hanoi, Vietnam

✉To whom correspondence should be addressed. E-mail: thanhvn@firi.vn

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SUMMARY

Given potential health benefits including low glycemic index, tooth friendly, suitable to infants, elderly and diabetic patients, isomaltulose was considered as a promising alternative sweetener to sucrose. Due to the presence of liposaccharide endotoxin in *Serratia plymuthica* CBS 574.44, a Gram-negative bacterium, and minute amount of formaldehyde carried over, purification of isomaltulose requires rigorous controls in industry. To reduce the cost associated with product purification, here we propose the use of recombinant enzyme in isomaltulose production. The mature gene coding for sucrose isomerase synthase (*K.SI36.PalI*) from *Klebsiella singarporensis* ISB 36, which isolated from woodborer in Vietnam, was expressed in *Pichia pastoris* X33. The nucleotide sequence of *K.SI36.PalI* gene was similar to AY040843.1 of *Klebsiella* sp. LX3 except one nucleotide C^{1025} in AY040843.1 replaced by T^{1025} in *K.SI36.PalI*. This leads to single amino acid difference in deduced protein sequence (from 342Ser to 342Phe). Furthermore, the addition of two amino acids (Glu and Phe) was observed at N-terminus. The calculated molecular weight of sucrose isomerase from *K.SI36.PalI* was 67.46 kDa and the pI was 6.55. There was one potential glycosylation site at 466Asn. The maximum sucrose isomerase activity in the culture broth reached 36,6 $U.mL^{-1}$in 1 L shake-flask. The purified recombinant enzyme was most active at 40°C and pH 7.0. At the optimum condition, within 6 hours, the enzyme converted 94% of sucrose in a 40% sucrose solution into isomaltulose. This was the first study on the expression of sucrose isomerase synthase gene in *P. pastoris*, and the results showed the efficient conversion of sucrose isomerase recombinant.

Keywords: *food sweetener, isomaltulose, Klebsiella, Pichia pastorsis, recombinant enzyme, sucrose isomerase.*

INTRODUCTION

Although playing the most important role in the food industry as a natural sweetener, sucrose, or table sugar, may cause negative effects, such as obesity, dental caries, heart diseases and is not suitable for people with diabetes (Lina *et al.,* 2002). Isomaltulose, a structural isomer of sucrose has been found as a promising alternative. Unlike other sucrose substitutes, isomaltulose can be assimilated by the human body and served as the source of energy (Tiefenbacher, 2017). It is tooth-friendly, promoting the growth of intestine bifidobacteria, causing low glycemic response and suitable for diabetic patients, elderly and infants(Lina*et al.,* 2002). In the food industry, isomaltulose is the precursor for the production of isomalt through hydrogenation (BeMiller, 2019).

In nature, isomaltulose occurs in small amounts in sugar cane syrup and honey (O'Donnell, Kearsley, 2012). It can be produced from sucrose by enzymatic rearrangement. The enzyme responsible for the process is termed as sucrose isomerase (EC 5.4.99.110) which catalyzes the conversion of sucrose (α-D-glucopyranosyl-(1-2)-β-D-fructofuranoside) into isomaltulose (6-O-alpha-D-glucopyranosyl-D-fructofuranose) and various amounts of D-glucose, D-fructose, and trehalulose (1-O-alpha-D-glucopyranosyl-beta-D-fructose) (Véronèse, Perlot, 1998; Véronèse, Perlot, 1999; Xu *et al.*, 2013). Many bacterial species sucrose isomerase producing has been reported, including *Serratia plymuthica*, *Erwinia rhapontici*, *Enterobacter* sp., *Klebsiella singaporensis*, *Raultella planticola*, *Enterobacter* sp., *Pantoea dispersa*, and *Pseudomonas mesoacidophila* (Goulter *et al.*, 2012;

Mu *et al.*, 2014a). Several of sucrose isomerase coding genes have been cloned, expressed and characterized (Cha *et al.*, 2009; Park *et al.*, 2010; Mu *et al.*, 2014b). In general, sucrose isomerases are functioning in near-neutral pH and are thermolabile. For example, *PalI* from *Klebsiella* sp. LX3 expressed in *Escherichia coli* DH5α demonstrated the highest activity at pH 6.0 and 35°C. The half-life of the enzyme was merely 1.8 min at 50°C (Zhang *et al.*, 2002). Surface display may alter the properties of sucrose isomerase. Sucrose isomerase from *Enterobacter* sp. FMB-1 expressed in *E. coli* was the most active at pH 6.0, while the surface-displayed enzyme in *Saccharomyces cerevisiae* showed the highest activity at pH 7.0. The latter was also slightly more stable to the effect of temperature than the one expressed in *E. coli* (Lee *et al.*, 2011a; Wei *et al.*, 2013).

Industrially, isomaltulose is produced from food-grade sucrose by using the formaldehyde-inactivated cells of *Protaminobacter rubrum* (=*Serratia plymuthica*) CBS 574.44 as biocatalyst and followed by purification and crystallization (Krastanov, Yoshida, 2003; Kawaguti, Sato, 2010; Goulter *et al.*, 2012; Mu *et al.*, 2014b). Although *S. plymuthica* is a nonpathogenic species, due to the presence of liposaccharide endotoxin in the cell wall and formaldehyde used for bacterial inactivation, the purification step should be carried out with rigorous monitoring (Orsi, Sato, 2016). In this study, we propose a new approach, whereas isomaltulose be produced from sucrose using recombinant enzyme expressed extracellularly by *Pichia pastoris*. The conversion mixture is expected to be free from endotoxin and formaldehyde, and thus reduces the cost for recovery.

MATERIALS AND METHODS

Microorganism

Strain *Klebsiella singaporensis* ISB-36 demonstrating sucrose isomerase activity was previously isolated from the excrement of woodborer *Colosternapollinosa sulphurea* Heller on *Anisoptera costata* Korth collected in Bàu Bàng forest station at Bến Cát, Bình Dương, Vietnam (Nguyễn Thanh Thủy *et al.*, 2013). The strain was identified as *K. singaporensis* as it shared 100% nucleotide homology with the *rpoB* sequence JX425353 of LMG 23571, the type strain of *K. singaporensis* (JX425353.1).

Cloning and expression

A gene coding for mature sucrose isomerase without sequence for signal peptide (*K.SI36.PalI*) was isolated by PCR amplification reaction from *K. singaporensis* ISB-36 using primer pair SI12-EcoRI (AGC TGA ATT CGC ACC ATC CTT GAA TCA G) and SI13-XbaI-R (TTG TTC TAG ATT ACC GCA GCT TAT ACA C) designed based on the GenBank sequence AAK82938 encoding sucrose isomerase of *K. singaporensis* LX3 (*K.LX3.PalI*). The location of cleavage sites for signal peptide sequence was predicted using SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/). For PCR, HF-Phusion DNA polymerase (Thermofisher, United States) was used. The amplified product was digested with *Eco*RI and *Xba*I (Fermentas, United States), purified using GeneJET Gel Extraction kit (Fermentas, United States), and ligated to pPICZαA (Invitrogen, United States) previously digested with the same enzymes using T4 ligase (Fermentas, United States). The ligation product was transformed into *E. coli* DH5α (Invitrogen, United States) by heat shock and spread on LB low-salt agar (0.5% yeast extract, 1% tryptone, 0.5% NaCl, 1.5% agar, pH 7.0) supplemented with 50 mg/L zeocin (Invitrogen) and incubated at 37°C. After 24 h, colonies were picked, grown in liquid LB-zeocin and the plasmids were extracted, digested with *Eco*RI and *Xba*I to examine the presence of the heterologous inserts.

The pPICZαA containing *K.SI36.PalI* gene from *K. singaporensis* ISB-36 was opened with *Mss*I (Fermentas, United States) and transformed into *P. pastoris* X33 (Invitrogen, United States) using a Micropulser Electroporator (BioRad, United States) according to the manufacturer's instructions. The transformants were selected using YPD-zeocin agar (1% yeast extract, 2% bacto peptone, 2% glucose, 1.5% agar, 100 mg.L^{-1} zeocin). Purified transformants were grown in expression medium BMM with methanol as the sole carbon source (100 mM potassium phosphate, pH 6.0, 1.34% yeast nitrogen base (YNB, Difco), 0.4 ppm biotin, 0.5% methanol).

Sucrose isomerase activity assay

Sucrose isomerase activity was routinely determined based on the formation of reducing sugar (isomaltulose) from the non-reducing precursor (sucrose) using DNS assay (Ghose, 1987). Since D-glucose, D-fructose, trehalulose can be the by-product of the enzymatic rearrangement, the conversion products were assessed by HPLC. Enzymatic

reactions were carried out in 20% of sucrose solution in 20 mM sodium phosphate buffer, pH 7.0. The reaction was performed in 1.5 mL microtubes at desired temperatures. After 30 min, sucrose isomerase was inactivated by heating at 100°C for 10 min and then the amount of reducing sugar was determined by DNS method. One unit of sucrose isomerase is determined as the amount of enzyme required for conversion of sucrose into 1 μmol of reducing sugar per min at a given testing condition. The assay was carried out in triplicate and the mean value was given.

For HPLC analyses, reverse-phase column Cosmosil ODS 5C18MSII (Nacalai Tesque, United States) was used. Fractions were eluted with water at the rate of 0.6 mL/min at 25°C and detected by a RID 6A Refractive Index Detector (Shimadzu, Japan). Sugar standards, including D-glucose, D-fructose, sucrose (Sigma, United States), and isomaltulose (Wako pure Chemical Industries, Japan) were used.

Enzyme production and purification

The yeast *P. pastoris* X33 harboring *K.SI36.PalI* gene was grown in 50 mL of BMG (100 mM potassium phosphate, pH 6.0, 1.34% YNB, 0.4 ppm biotin, 1% glycerol) at 28°C with shaking at 150 rpm for 24 h. The cells were harvested by centrifugation and reinoculated in 200 mL expression medium BMM and incubated at the same condition. For remaining inducing state for the recombinant enzyme synthesis, 0.5% methanol was added every 24 h. After 96 h, the fermentation broth was centrifuged at 6000 × g for 30 min at 4°C and cells were discarded. The obtained supernatant was concentrated using Viva flow 200, 30 kDa MWCO (Sartorius, Germany). The enzyme was further purified using Superdex 200 (GE Healthcare, United States) gel filtration. Sample of 5 mL was loaded into XK 26/40 column (GE Healthcare, United States) and eluted with 20 mM sodium phosphate buffer, pH 7.0 at the rate of 1 $mL.min^{-1}$ with column temperature maintained at about 10°C. Fractions of 5 mL were collected and tested for sucrose isomerase activity.

Effect of pH and temperature

Purified sucrose isomerase was used to assess the effect of pH and temperature on the activity and the stability of the enzyme. Testing the enzyme activity at different temperatures (20-80°C) was carried out in 20 mM sodium phosphate buffer, pH 7.0. For testing the effect of pH, following buffer systems (50 mM) were used: pH 4, 5, 6 - citrate-phosphate; pH 7, 8 - sodium phosphate; pH 9, 10 - glycine sodium hydroxide. To determine thermostability, enzyme was incubated at 40, 50, 60°C, pH 7.0 and the remaining activity was measured at different time intervals. Similarly, for assessing the pH stability, the enzyme was incubated at different pHs, but the temperature was kept at 40°C.

Enzyme kinetic

Conversion kinetics were studied using sucrose substrate concentrations of 20 and 40%. An initial sucrose isomerase activity of 10 $U.mL^{-1}$was used and the reaction was carried out at pH 7.0, 40°C in a volume of 5 mL. Samples collected at different time intervals were heat-treated (100°C, 10 min) then analyzed by DNS and HPLC.

RESULTS

Cloning and sequence analysis of *K.SI36.PalI* gene

The PCR amplification of *K.SI36.PalI* gene from genomic DNA of *K. singaporensis* ISB36 using primers SI12-EcoRI and SI13-XbaI-R yielded in a fragment of about 1700 bp (Figure 1(a)). After ligating with *pPICZαA* and transforming into *E. coli* DH5α, the insert was sequenced using the primers α-factor and 3'-AOX1. The closest GenBank sequence was AAK82938 of *K. singaporensis* LX3 with 99,94% similarity (differ 1 nucleotide over 1713 nucleotides in mature gene). There was a nucleotide substitution leading to the alteration from Ser at the amino acid position 342 of *K. singaporensis* LX3 to Phe. In our construct, the native 28 amino acid signal peptide sequence was replaced by the α-factor sequence of the plasmid. The mature sequence of the *K.SI36.PalI* gene was expected to differ from *K. singaporensis* LX3 also by two amino acid (Glu and Phe) introduced with the primer (*Eco*RI recognition site) at the N- terminus. The predicted protein would have 572 amino acids and with estimated molecular weight of 67.46 kDa, pI of 6.55. The enzyme was expected to have a low level of glycosylation as there was only one potential glycosylation site at 466Asn identified.

After transformation to *P. pastoris* X33, 24 yeast clones were selected and tested for sucrose isomerase activity. All selected clones expressed sucrose isomerase activity after 72 h of cultivation. The mean activity was 13.4 $U.mL^{-1}$ and the maximum activity obtained was 32 $U.mL^{-1}$ (Figure 2).

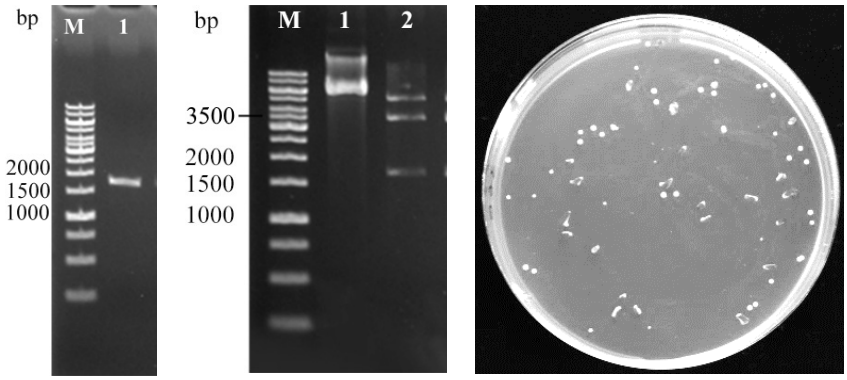

Figure 1. Cloning of *K.SI36.PaII* gene from *K. singaporensis* ISB36. (a): PCR product amplified with primers SI12-EcoRI and SI13-XbaI-R. (b): pPICZαA containing *K.SI36.PaII* gene (1) before and (2) after digestion with *Eco*RI and *Xba*I; (c): *P. pastoris* clones growing on YPD-zeocin agar.

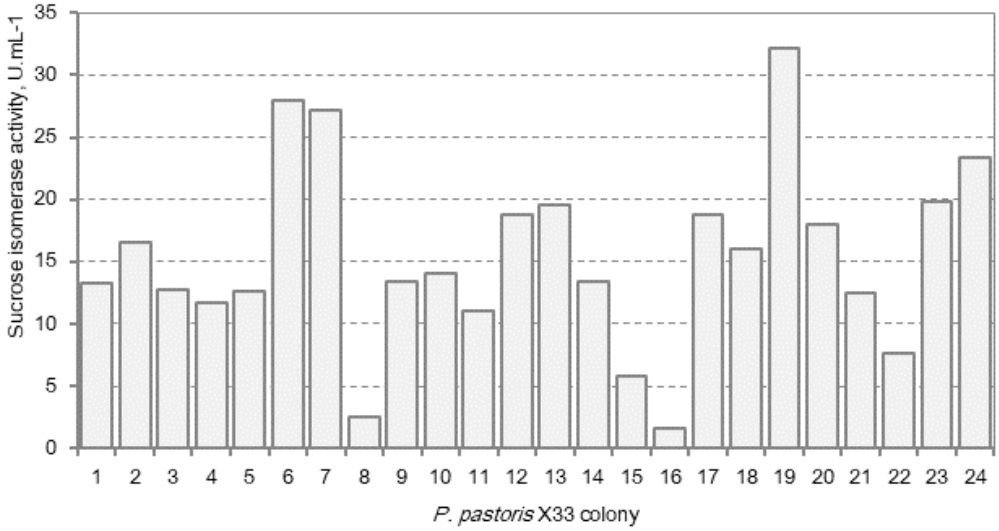

Figure 2. Screening of *P. pastoris* X33 clones containing *K.SI36.PaII* gene for sucrose isomerase activity.

Fermentation and purification of the enzyme

Based on initial screening, clone X33.SI36#7 was selected from top high sucrose isomerase activity (#19, #6, #7 and #24) for enzyme expression and characterization. The yeast was cultivated in a 2 L fermentor with pH maintained at 5.0 and dissolved oxygen above 10%. After 96 h, wet biomass reached 90 $g.L^{-1}$ and extracellular protein concentration was 10 $\mu g.mL^{-1}$. Sucrose isomerase activity of the culture supernatant was 36.6 $U.mL^{-1}$. After 10-fold concentration, the activity of 300.8 $U.mL^{-1}$was achieved. After gel-filtration purification, the sucrose isomerase activity was 46.5 $U.mL^{-1}$. SDS-PAGE indicated the presence of one protein band just below the 66.2 kDa marker protein, slightly smaller than the predicted size (67.46 kDa). The absence of the smear zone above the band indicated a low level of glycosylation (Figure 3).

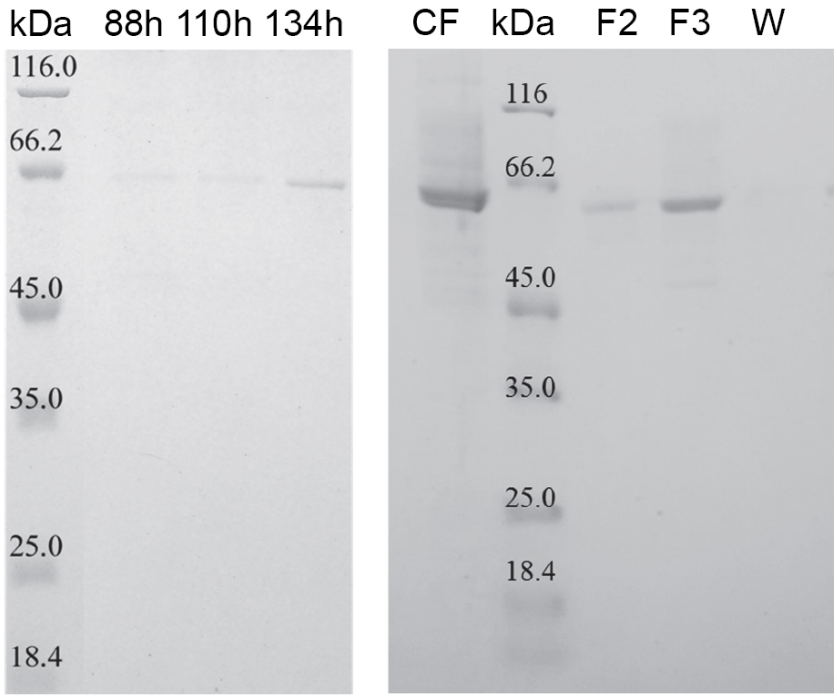

Figure 3. SDS-PAGE analysis of sucrose isomerase. (a): Culture supernatant after 88, 110, and 134 h of fermentation; (b): CF - supernatant after concentration; F2, F3, W - fractions 2, 3 and waste of gel-filtration chromatography; kDa – standard protein markers and sizes in kDa.

Effect of temperature

Sucrose isomerase of *K. singaporensis* ISB36 expressed in *P. pastoris* X33 showed maximum activity at 40°C. At higher temperatures, the enzyme rapidly lost the function. At 50°C and 60°C the activity was only 20% and 7% of that at 40°C, respectively. The enzyme was thermolabile with the half-life less than 10 min at 50°C, and less than 1 h at 40°C. The data obtained by this study agree well with the previous reports. Enzyme sucrose isomerase synthase from *K. singaporensis* LX3 (*K.LX3.PalI*) was most active at 35-40°C and at 50°C and 60°C, the activity was only 47% and 6% of the optimum (Zhang *et al.,* 2002). Similarly, sucrose isomerase from *Enterobacter* sp. FMB-1 has an optimum temperature of 45°C and lost activity at 60°C (Lee *et al.*, 2011b).

Effect of pH

The enzyme had no activity outside the pH 5.0 to pH 10.0 range (Figure 5) and it functioned best at pH 7.0. At pH 6.0 or pH 8.0, the activity of the enzyme reduced by 35%. At low temperature, the enzyme was rather stable at different pHs after 72 h of incubation at 4°C in the buffers with pH 5.0 and pH 7.0, the residual activity of the enzyme detected was 75% and 80%, respectively. At the temperature optimum (40°C), the enzyme lost activity rapidly when incubated in the buffer with pH 5.0 and with a lesser extent at pH 6.0 and pH 7.0.

Reaction kinetic

The conversion of sucrose into isomaltulose by recombinant sucrose isomerase was rapid and efficient. After 2 h of incubation with 10 $U.mL^{-1}$ sucrose isomerase, 60% of sucrose in the 40% sucrose solution was converted into isomaltulose and after 6 h, the conversion was nearly complete (94%) (Figure 6). HPLC analysis indicated the presence of a minor amount of D-glucose, D-fructose, and trehalulose (4-5% in total, data not shown).

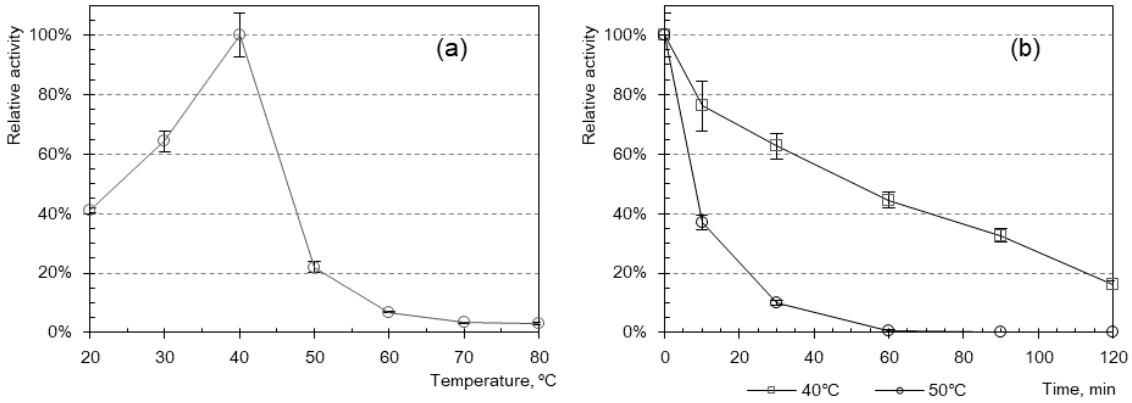

Figure 4. The effect of temperature on sucrose isomerase activity. (a): activity at different temperatures; (b): thermostability of the enzyme.

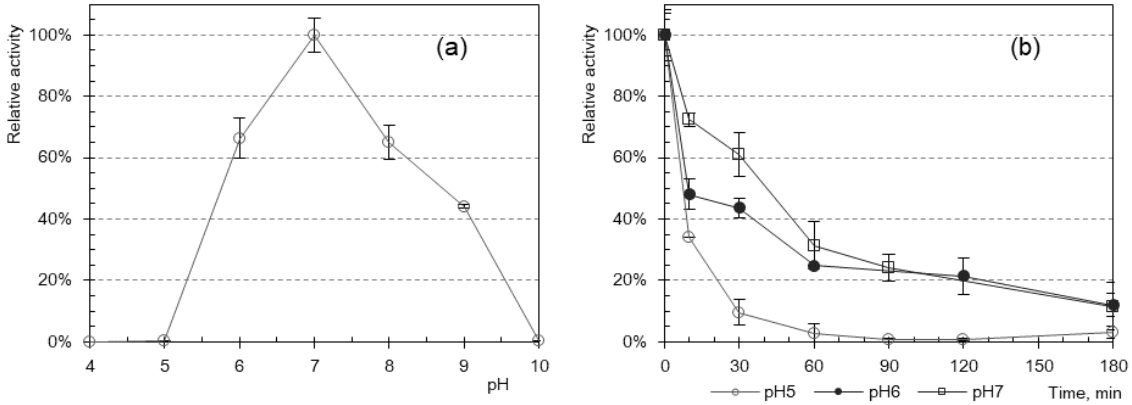

Figure 5. The effect of pH on sucrose isomerase activity. (a): activity at different pHs; (b): the stability of enzyme at different pHs.

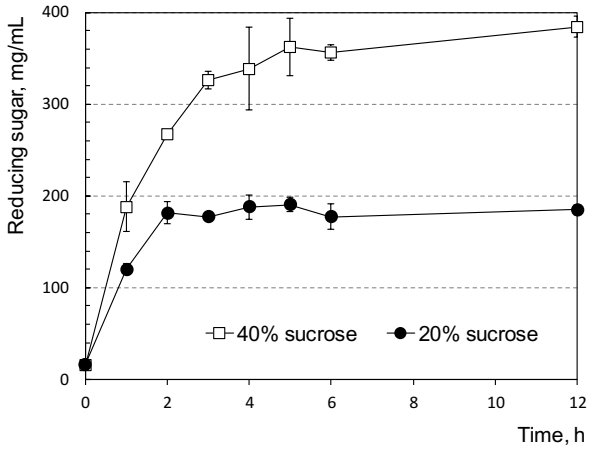

Figure 6. Kinetic of isomerization at different initial sucrose concentrations.

CONCLUSION

With one amino acid substitution, sucrose isomerase obtained from *K. singaporensis* ISB36 was nearly identical to the previously studied of *K. singaporensis* LX3 (Zhang *et al*., 2002; Zhang *et al*., 2003). The enzyme expressed in *P. pastoris* demonstrated similar properties with the enzyme expressed in *E. coli* in terms of pH, temperature optima and stability. The recombinant enzyme could efficiently convert sucrose into isomaltulose with a minor amount (less than 5%) of by-products. This is the first report on the expression of sucrose isomerase using *P. pastoris* system. The use of recombinant sucrose isomerase could reduce the risk associated with endotoxin and formaldehyde in isomaltulose production.

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TÁCH DÒNG GEN MÃ HÓA SUCROSE ISOMERASE TỪ *KLEBSIELLA SINGAPORENSIS* ISB-36 VÀ BIỂU HIỆN TRÊN *PICHIA PASTORIS*

Cao Xuân Bách, Đặng Thị Kim Anh, Nguyễn Thanh Thủy, Trương Tú Anh, Nguyễn Thị Diệu Linh, Vũ Nguyên Thành

Viện Công nghiệp Thực phẩm, 301 Nguyễn Trãi, Thanh Xuân, Hà Nội, Việt Nam

TÓM TẮT

Với những lợi ích sức khỏe như chỉ số đường huyết thấp, không gây sâu răng, phù hợp với trẻ sơ sinh, người già và bệnh nhân tiểu đường, isomaltulose được coi là một chất ngọt thay thế đầy hứa hẹn cho sucrose. Do sự hiện diện của endotoxin liposaccharide trong *Serratia plymuthica* CBS 574.44, một vi khuẩn Gram âm, và formaldehyde tồn dư, tinh chế isomaltulose đòi hỏi được kiểm soát chặt chẽ trong công nghiệp. Để giảm chi phí tinh chế sản phẩm, chúng tôi đề xuất sử dụng sucrose isomerase tái tổ hợp trong sản xuất isomaltulose. Gen trưởng thành mã hóa enzyme sucrose isomerase (*K.SI36.PalI*) từ *Klebsiella singarporensis* ISB 36 phân lập từ ấu trùng xén tóc ở Việt Nam được biểu hiện trong *Pichia pastoris* X33. Gen *K.SI36.PalI* tương đồng với trình tự AY040843.1 của *Klebsiella* sp. LX3 ngoại trừ một nucleotide tại vị trí C^{1025} trong AY040843.1, được thay bằng T^{1025} trong trình tự của *K.SI36.PalI*. Điều này dẫn đến sự khác biệt một amino acid thành phần (342Ser thành 342Phe). Thêm vào đó, hai amino acid Glu và Phe được quan sát thấy ở đầu N. Khối lượng phân tử tính toán của sucrose isomerase từ *K.SI36.PalI* là 67,46 kDa, pI là 6,55. Một vị trí glycosyl hóa tiềm năng tại 466Asn. Hoạt tính sucrose isomerase trong canh trường đạt 36,6 $U.mL^{-1}$ trong bình tam giác 1 L. Enzyme tinh chế hoạt động mạnh nhất ở pH 7,0 và 40°C. Ở điều kiện tối ưu, enzyme chuyển hóa 94% sucrose trong dung dịch sucrose 40% thành isomaltulose trong vòng 6 giờ. Đây là nghiên cứu đầu tiên về biểu hiện của sucrose isomerase ở *P. pastoris* và kết quả đã cho thấy sự chuyển hóa hiệu quả của enzyme tái tổ hợp.

Từ khoá: *chất ngọt thực phẩm, isomaltulose, Klebsiella, Pichia pastorsis, enzyme tái tổ hợp, sucrose isomerase.*