EFFECT OF CALCIUM IONS ON ETHANOL PRODUCTION FROM MOLASSES BY *SACCHAROMYCES CEREVISIAE*

By

SUNEE CHOTINEERANAT[1], RUNGTIVA WANSUKSRI[1], KUAKOON PIYACHOMKWAN[1], PATHAMA CHATAKANONDA[2], PIPAT VEERATHAWORN[3] and KLANARONG SRIROTH[1,4]

[1]*Cassava and Starch Technology Research Unit, National Center for Genetic Engineering and Biotechnology, Pathumthani, Thailand*

[2]*Kasetsart Agricultural and Agro-Industrial Product Improvement Institute, Kasetsart University, Bangkok, Thailand*

[3]*Mitr Phol Sugarcane Research Centre Co., Ltd*

[3]*Department of Biotechnology, Kasetsart University, Bangkok, Thailand*

aapkrs@ku.ac.th

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Abstract

ONE OF THE most widely used feedstocks for potable or biofuel ethanol fermentation is blackstrap molasses, a by-product of cane sugar production. Inconsistent quality of molasses frequently found in industrial production, however, makes ethanol production much less efficient and cost-ineffective. Besides fermentable sugars (sucrose, glucose and fructose) present in molasses, calcium is also found in the range of 10–20% of the total carbonated ash (15% by weight). In the sugar process, calcium is usually applied as lime, CaO, during defecation, which sometimes results in over-liming if juice quality is poor. In this study, the role of calcium residues (0–0.72% w/v of Ca^{2+} in the form of calcium chloride) on fermentation efficiency of yeast was evaluated using model solutions of sucrose, glucose and fructose (20% w/v). The results suggest a detrimental effect of calcium ions on yeast performance which was concentration dependent. A slight decrease in fermentation rates and ethanol yields was evident when calcium was present at 0.18% w/v Ca^{2+} in all sugar solutions. This effect was more pronounced when calcium ion concentration increased. At 0.72% w/v of Ca^{2+}, the rates of fermentation and ethanol yields of all sugars were considerably decreased (the ethanol yields decreased by 14–25% relative to the control sample, i.e. no calcium ion added). At a very high concentration of Ca^{2+} (2.16% w/v), yeast fermentation of sucrose was almost absolutely inhibited. This might be, in part, due to the inhibition effect of invertase enzyme for conversion of sucrose to invert sugars, a limiting step in ethanol fermentation of sucrose by yeast. The pretreatment of molasses by acid and heat prior to fermentation was then introduced to remove calcium which improved the fermentation efficiency.

Introduction

The most widely used feedstock for potable or biofuel ethanol fermentation by yeast, *Saccharomyces cerevisiae*, is sugarcane in forms of syrup and blackstrap molasses, a by-product from cane sugar production. Molasses is rich in many nutrients that are essential for microbial fermentation. It consists mainly of sugars including sucrose, glucose and fructose (approximately 30–40, 4–9 and 5–12 g/100g molasses, respectively) (Chen and Chou, 1993).

Sucrose, the most available fermentable sugar in molasses, is first inverted by yeast-secreted invertase sugars which are subsequently converted, under anaerobic conditions, by yeast to ethanol and carbon dioxide.

Besides these fermentable sugars, there are other components, viz. protein, minerals (potassium, calcium, sodium, magnesium, copper, iron, manganese, zinc, chloride and sulfur) and vitamins (biotin, folic acid, riboflavin, thiamine and niacin) some of which are essential for yeast growth and metabolism.

Among various minerals present in molasses, calcium is found as the second after potassium, in the range of 0.3–0.9 and 1.5–6.0% by weight of molasses with 75% dry substance for calcium and potassium, respectively (Higginbotham and McCarthy, 1998). In the sugar process, calcium is typically applied as lime, CaO, during defecation, which sometimes results in over-liming if juice quality is poor. Calcium can be discharged into the final molasses which presumably affects the ethanol production efficacy in various process stages, including yeast fermentation and ethanol distillation.

In this study, the role of calcium residues (0–0.72% w/v of Ca^{2+} in the form of calcium chloride) on fermentation efficiency of yeast was evaluated using model solutions of sucrose, glucose and fructose (20% w/v).

Materials and methods

Materials

Molasses was obtained from MitrPhol sugar factory, Thailand. An invertase enzyme (β-fructofuranosidase) was supplied by Novozymes Co. Ltd. (Basgsvaerd, Denmark). Yeasts, *Saccharomyces cerevisiae*, were a commercial dry active product (BioFerm-XR, North American Bioproducts Corporation - NABC, USA).

Effect of calcium on yeast fermentation of sucrose at high calcium concentrations (0–2.16% w/v of Ca^{2+})

The sucrose solution (20% w/v) containing different calcium concentrations (0–2.16% w/v of Ca^{2+} in the form of $CaCl_2.2H_2O$) was prepared with the addition of some nutrients (10g/L of yeast extract; 1 g/L of KH_2PO_4; 1g/L of $(NH_4)_2SO_4$ and 0.5 g/L of $MgSO_4.7H_2O$) at pH 4.5. Yeasts, *Saccharomyces cerevisiae* (BioFerm-XR) were added (0.6 g/L) and the fermentation was achieved at 32°C for 48 h in a 5L-fermentor with controlled temperature and pH. The samples were collected at different time intervals and analysed for sugar and ethanol contents by High Performance Liquid Chromatography methods using a Sugar-Pak TMI column (6.5 x 300 mm; Waters Corporation, MS, USA) at 80°C with a Refractive Index detector (Water 410 Differential Refractometer, Waters Corporation, MS, USA) and the eluent of 0.05 g/L Calcium Titriplex® dihydrate at the flow rate of 0.5 mL/min, according to ICUMSA Method GS7-23 (ICUMSA, 1994). The fermentation efficiencies were reported as a percentage of experimental to theoretical yields of ethanol.

Effect of calcium on yeast fermentation of sucrose, glucose and fructose at low calcium concentrations (0–0.72% w/v of Ca^{2+})

The sugar solutions, i.e. sucrose, glucose and fructose (20% w/v) containing different calcium concentrations (0, 0.18, 0.36 and 0.72% w/v of Ca^{2+} using 0, 0.5, 1.0 and 2.0% w/v of $CaCl_2.2H_2O$, respectively) were prepared with the addition of some nutrients (10 g/L of yeast extract; 1 g/L of KH_2PO_4; 1 g/L of $(NH_4)_2SO_4$ and 0.5 g/L of $MgSO_4.7H_2O$) at pH 4.5. Yeasts, *Saccharomyces cerevisiae* (BioFerm-XR) were added (0.6 g/L) and the fermentation was achieved at 32°C for 48 h in a 1 L-flask without controlled temperature and pH.

The samples were collected at different time intervals and analysed for sugar and ethanol contents by High Performance Liquid Chromatography methods using a Sugar-Pak TMI column (6.5 × 300 mm; Waters Corporation, MS, USA) at 80°C with a Refractive Index detector (Water 410 Differential Refractometer, Waters Corporation, MS, USA) and the eluent of 0.05 g/L Calcium Titriplex® dihydrate at the flow rate of 0.5 mL/min, according to ICUMSA Method GS7-23 (ICUMSA, 1994). The fermentation efficiencies were reported as a percentage of experimental to theoretical yields of ethanol.

Effect of calcium concentration on hydrolytic activity of invertase enzymes

The sucrose solutions (10% w/v) containing different calcium concentrations, i.e. 0, 0.18, 0.36, 0.72, 1.44, 2.16 and 2.88% w/v (in a form of $CaCl_2.2H_2O$) were thoroughly mixed with invertase enzymes (the enzyme concentration was 5.84 mg/mL) at pH 4.5.

The mixtures were incubated at 32 and 55°C for 1 h. Subsequently, the enzyme activities were terminated by boiling for 15 min and the aliquots were used to quantify the amount of reducing sugars by Somogyi-Nelson method (Somogyi, 1952).

Fermentation of molasses with acid pretreatment

The qualities of molasses were analysed including total soluble solids by a refractometer, sulfated ash (GS1/3/4/7/8-11 method) and conductivity ash (GS1/3/4/7/8-13 method) according to ICUMSA method (ICUMSA, 1994) and pH by a pH meter.

The amount of fermentable sugars was quantified using a High Performance Liquid Chromatography (ICUMSA Method GS7-23; ICUMSA, 1994) equipped with a Sugar-Pak TMI column (6.5 × 300 mL, Water Corporation, MS, USA) at 80°C and operated with 0.05 g/L calcium titriplex® dehydrate (Ca-EDTA) at the flow rate of 0.5 mL/min. The calcium content was estimated by Inductively Coupled Plasma technique (AOAC, 2000).

Molasses was pretreated with sulfuric acid prior to yeast fermentation. Sulfuric acid (1N H_2SO_4) was added to molasses until the pH of sample reached 3.0. The mixture was then heated to 95°C for 0.5 h and then left overnight at room temperature.

The precipitates were then removed by filtration. The pretreated molasses was subsequently fermented by adjusting the total soluble solid content of molasses to 25°Brix at pH 4.5. Some essential nutrients including 10 g/L of yeast extract; 1 g/L of KH_2PO_4; 1 g/L of $(NH_4)_2SO_4$ and 0.5 g/L of $MgSO_4.7H_2O$ were added to ensure a proper growth of yeast during ethanol fermentation.

The fermentation was achieved by *Saccharomyces cerevisiae*, without and with 0.36% calcium (or 1% w/v of calcium chloride, $CaCl_2.2H_2O$), at 32°C for 48 h.

The samples were collected at different time intervals and analysed for sugar and ethanol contents by chromatographic methods as described previously.

Results and discussion

Sucrose, a major sugar component found in cane molasses, is a fermentable sugar that can be converted to ethanol by yeast fermentation. During fermentation, yeasts convert sucrose to invert sugars by invertase enzymes secreted by yeasts themselves.

In this study, ethanol was produced from sucrose solutions containing different calcium concentrations (0–2.16 % Ca^{2+}). Figure 1 demonstrates the effect of calcium ions on the ethanol production of sucrose by yeasts.

Calcium induced a reduction in ethanol production. The adverse effect was more pronounced when the calcium concentrations increased; the ethanol yield could be reduced up to 86% (the ethanol yields and fermentation efficiencies were decreased from 0.508 to 0.070 g/g sugar as invert and from 99.38 to 13.71% for samples with 0 and 2.16% Ca^{2+}, respectively (Table 1).

A reduction of ethanol production during sucrose fermentation induced by calcium was likely caused by an inhibition effect of invertase by calcium.

In fact, the conversion of sucrose to invert sugars is reported as the limiting step for ethanol production by yeast fermentation of sucrose.

Takeshige and Ouchi (1995) reported the effect of some metal ions on ethanol productivity by yeasts, which were strain-dependent.

The yeast strain with low ethanol productivity exhibited a lower invertase activity when it utilised molasses containing metal ions, in particular copper, potassium and calcium, indicating an inhibition effect on invertase activity.

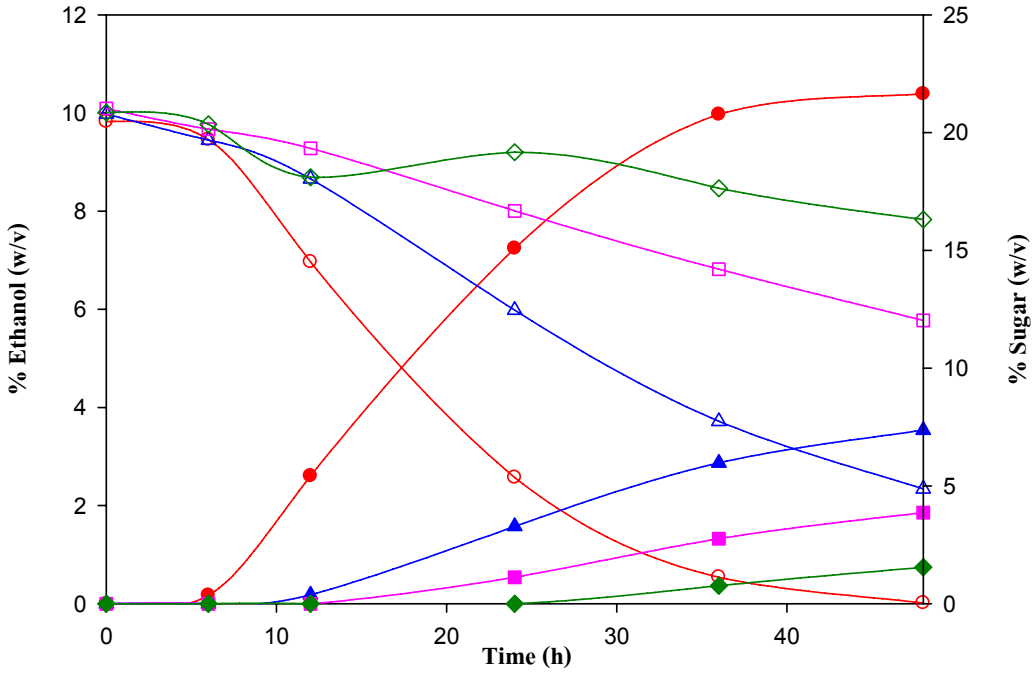

Fig. 1—Changes of ethanol and sucrose concentrations during fermentation of sucrose (20% w/v) having different calcium concentrations (0–2.16% w/v of Ca^{2+}) by *Saccharomyces cerevisiae*. (Open symbols represent sugar contents and filled symbols represent ethanol content at different calcium concentrations; ○,● 0%; Δ, ▲ 0.72%; □, ■ 1.44% and ◊, ♦2.16% Ca^{2+}).

Table 1—Yield (g /g initial sugar as invert) and % fermentation efficiency of ethanol production from sucrose solutions (20% w/v) containing different calcium concentrations.

Calcium concentrations (% w/v)	Initial sugar concentrations, as inverts (% w/v)	Ethanol concentrations (% w/v)	Yield (g/g initial sugar as invert)	% Fermentation efficiency[(a)]	% Reduction[(b)]
0	20.46	10.39	0.508	99.38	–
0.72	20.79	7.37	0.354	69.37	30.22
1.44	20.73	3.66	0.177	34.55	65.24
2.16	20.12	1.41	0.070	13.71	86.20

[(a)] reported as a percentage of experimental to theoretical yields of ethanol
[(b)] reported as the percentage of the difference between the control experiment (without calcium) and the treatment (with calcium) to the control experiment.

$$\% \text{ Reduction} = \frac{(\text{Ethanol yield}_{control}) - (\text{Ethanol yield}_{treatment})}{(\text{Ethanol yield}_{control})} \times 100$$

To further investigate that, sucrose solutions were converted to invert sugars by external invertase in the presence of calcium. Regardless of the incubation temperatures at 55°C, an optimum temperature of this enzyme or 32°C, an optimum temperature for *Saccharomyces cerevisiae* yeast, the invertase enzymes were inhibited by calcium as the amount of reducing sugars was lower (Figure 2).

At 55°C, the effect of calcium was less as compared to that at 32°C. At a low calcium concentration (0.18% w/v), the activity reduction of invertase enzymes was 1.84 and 17.29% at 55 and 32°C, respectively, as compared to the control treatment, i.e. no calcium addition.

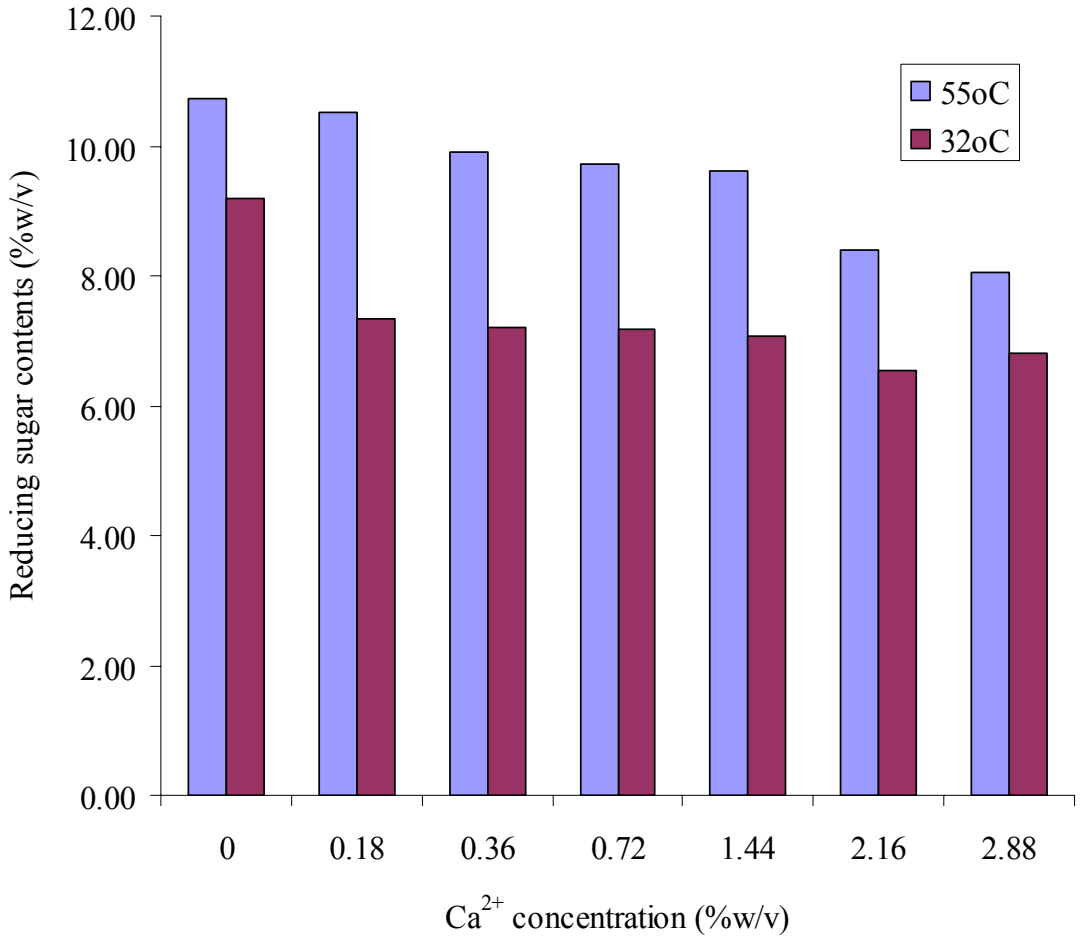

Fig. 2—Effect of calcium (Ca^{2+}) on the hydrolytic activity of invertase enzymes (5.84 mg/mL at pH 4.5) when incubated with sucrose solutions (10% w/v) containing different calcium concentrations at 32 and 55°C for 1 h.

The effects of calcium, at low concentrations (0–0.72% w/v Ca^{2+}), on ethanol productivity were further investigated by fermenting sucrose, glucose and fructose with *Saccharomyces cerevisiae* yeasts at 32°C without controlled conditions to simulate the actual industrial process.

Without calcium ions, the fermentation efficiencies of all sugars were highest, yet the fermentation efficiency of sucrose was lower than the previous trial which was achieved under controlled conditions in a fermentor.

Nevertheless, the results demonstrated the reduction of ethanol productivity during fermentation of sucrose, glucose and fructose by *Saccharomyces cerevisiae* yeasts when calcium ions were added (Table 2).

The degrees of fermentation efficiency reduction were different. It was likely that fermentation of glucose was least affected by calcium.

At the same level of calcium, fermenting sucrose seemed to be the most affected by calcium (% yield reductions were 25.16, 13.72 and 20.18% for sucrose, glucose and fructose fermentation in the presence of 0.72% w/v Ca^{2+}, respectively.

Table 2—Yield (g /g initial sugar as invert) and % fermentation efficiency of ethanol production from glucose and fructose solutions (20% w/v) containing different calcium concentrations.

Sugar	Calcium concentrations (% w/v)	Initial sugar concentrations, as inverts (% w/v)	Ethanol concentrations (% w/v)	Productivity g/L/h)	Yield (g/g initial sugar as invert)	% Fermentation efficiency [(a)]	% Reduction [(b)]
Sucrose	0	20.26	8.71	1.81	0.43	84.11	0.00
	0.18	20.26	8.32	1.73	0.41	80.35	4.46
	0.36	20.26	8.11	1.69	0.40	78.34	6.86
	0.72	20.26	6.46	1.35	0.32	62.44	25.16
Glucose	0	17.93	7.90	2.19	0.44	86.20	0.00
	0.18	17.93	7.74	2.15	0.43	84.50	1.97
	0.36	17.93	7.40	2.06	0.41	80.80	6.26
	0.72	17.93	6.81	1.42	0.38	74.37	13.72
Fructose	0	19.06	8.15	2.26	0.43	83.70	0.00
	0.18	19.06	7.79	1.62	0.41	81.23	0.28
	0.36	19.06	6.94	1.45	0.36	71.95	11.16
	0.72	19.06	6.24	1.30	0.33	64.00	20.18

[(a)] reported as a percentage of experimental to theoretical yields of ethanol

[(b)] reported as the percentage of the difference between the control experiment (without calcium) and the treatment (with calcium) to the control experiment.

$$\% \text{ Reduction} = \frac{(\text{Ethanol yield}_{control}) - (\text{Ethanol yield}_{treatment})}{(\text{Ethanol yield}_{control})} \times 100$$

The composition of molasses is presented in Table 3. Calcium can affect the production efficiency of ethanol from molasses as it acts as an inhibitor of invertase enzymes, which are very crucial for breaking sucrose to glucose and fructose.

This causes a slow reaction rate during the limiting step of molasses fermentation. In addition to the inhibition effect, the metal ion can be toxic to the yeasts and influence the ionic strength and pH of the medium.

Table 3—Qualities of molasses.

Compositions/parameters	Value
Sucrose (% by weight)	32.60
Glucose (% by weight)	4.90
Fructose (% by weight)	7.40
Calcium (% by weight)	0.66
Conductivity ash (% by weight)	10.18
Sulfated ash (% by weight)	10.21
Total soluble solids (°Brix)	85.20
pH	5.16

Some techniques are introduced to minimise this problem such as the addition of external invertase which is not cost-effective and the formation of insoluble complexes of metal ions with some chemicals, e.g. ferrocyanide and EDTA (Oderinde *et al*., 1986). The pretreatment of molasses with sulfuric acid is also applied in order to remove calcium ions.

In this work, blackstrap molasses containing 0.66% w/v Ca^{2+} was used for ethanol production with the acid pretreatment. As expected, a slight improvement on ethanol production from pretreated molasses was observed (Figure 3).

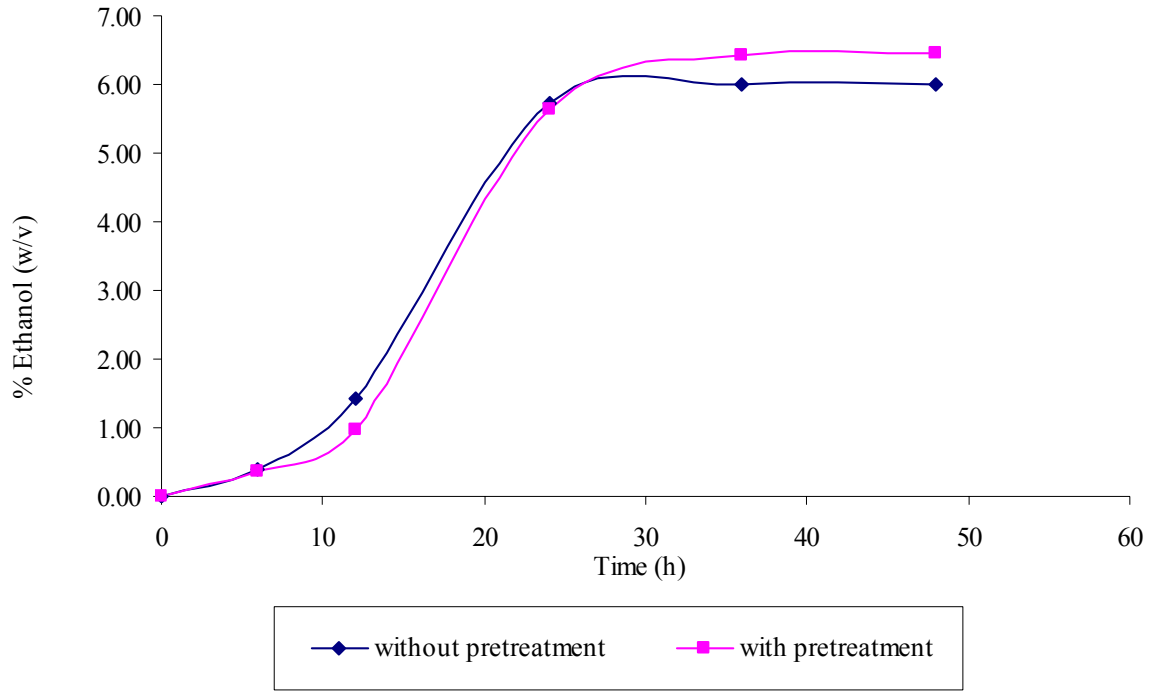

Fig. 3—Changes of ethanol concentrations during fermentation of pretreated molasses (25°Brix) by *Saccharomyces cerevisiae*.

Conclusion

In the cane sugar process, molasses can be adulterated with calcium ions, leading to its inferior quality and for being used as the feedstock for ethanol production by yeast fermentation.

Calcium ions can act as an invertase inhibitor which inhibits the inversion of sucrose to invert sugars. Furthermore, the fermentation of invert sugars can be adversely affected by calcium.

It is, therefore, critical for ethanol factories to analyse the quality of molasses prior to use. An efficient technique for eliminating calcium should be further developed for industrial uses.

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EFFET DES IONS DE CALCIUM SUR LA PRODUCTION DE L'ÉTHANOL À PARTIR DE LA MÉLASSE PAR LES *SACCHAROMYCES CEREVISIAE*

Par

SUNEE CHOTINEERANAT[1], RUNGTIVA WANSUKSRI[1], KUAKOON PIYACHOMKWAN[1], PATHAMA CHATAKANONDA[2], PIPAT VEERATHAWORN[3] et KLANARONG SRIROTH[1,4]

[1]*Cassava and Starch Technology Research Unit, National Center for Genetic Engineering and Biotechnology, Pathumthani, Thailande*
[2]*Kasetsart Agricultural and Agro-Industrial Product Improvement Institute, Kasetsart University, Bangkok, Thailande*
[3]*Mitr Phol Sugarcane Research Centre Co., Ltd*
[3]*Department of Biotechnology, Kasetsart University, Bangkok, Thailande*
aapkrs@ku.ac.th

MOTS-CLÉS: Mélasse, Éthanol, Calcium, *Saccharomyces cerevisiae*, Invertase.

Résumé

UN DES PRODUITS de base le plus largement utilisé pour la fermentation de l'alcool de bouche ou de biocarburant est la mélasse 'blackstrap', un sous-produit de la production de sucre à partir de la canne à sucre. La qualité irrégulière de la mélasse, fréquemment issue de la production industrielle, fait cependant que la production d'éthanol soit moins efficace en quantité de produit obtenu et en coût. En outre des sucres fermentescibles réducteurs (saccharose, glucose et fructose) présents dans la mélasse, le calcium se trouve également dans la plage de 10 à 20% des cendres carbonatées totales (15% en poids). Lors de la fabrication de sucre, le calcium est généralement appliqué sous forme de chaux, CaO, au cours de la défécation, ce qui entraîne parfois un chaulage en excès si la qualité de jus est mauvaise. Dans cette étude, le rôle des résidus de calcium (0–0.72% m/v de Ca^{2+} sous la forme de chlorure de calcium) sur l'efficacité de la fermentation par levure a été évalué à l'aide des solutions modèles de saccharose, de glucose et de fructose (20% p/v). Les résultats suggèrent un effet néfaste des ions de calcium sur les performances des levures qui étaient en relation avec la concentration. Une légère baisse de taux de fermentation et des rendements de l'éthanol étaient évidents lorsque le calcium était présent à 0.18% m/v Ca^{2+} dans toutes les solutions de sucre. Cet effet a été plus prononcé lorsque la concentration en ions de calcium a augmenté. À 0.72% m/v de Ca^{2+}, les taux de fermentation et les rendements d'éthanol de tous les sucres ont été considérablement réduit (les rendements en éthanol ont diminué de 14 à 25% par rapport à l'échantillon de contrôle, c'est-à-dire aucun ion de calcium ajouté). À une très forte concentration de Ca^{2+} (2.16% m/v), la fermentation de saccharose par la levure a été presque totalement inhibée. Cela peut être, en partie, en raison de l'effet d'inhibition des enzymes invertases pour la conversion de saccharose en sucres invertis, une étape limitative de la fermentation de saccharose en éthanol par la levure. Le prétraitement des mélasses par acide et la chaleur avant la fermentation a été ensuite introduit pour éliminer le calcium, ce qui a amélioré l'efficacité de fermentation.

EFECTO DE LOS IONES CALCIO EN LA PRODUCCIÓN DE ETANOL A PARTIR DE MELAZAS POR *SACCHAROMYCES CEREVISIAE*

Por

SUNEE CHOTINEERANAT[1], RUNGTIVA WANSUKSRI[1],
KUAKOON PIYACHOMKWAN[1], PATHAMA CHATAKANONDA[2],
PIPAT VEERATHAWORN[3]and KLANARONG SRIROTH[1,4]
[1]*Cassava and Starch Technology Research Unit,*
National Center for Genetic Engineering and Biotechnology, Pathumthani, Thailand
[2]*Kasetsart Agricultural and Agro-Industrial Product Improvement Institute,*
Kasetsart University, Bangkok, Thailand
[3]*Mitr Phol Sugarcane Research Centre Co., Ltd*
[3]*Department of Biotechnology, Kasetsart University, Bangkok, Thailand*
aapkrs@ku.ac.th

PALABRAS CLAVE: melazas, Etanol, Calcio,
***Saccharomyces cerevisiae*, Invertasa.**

Resumen

UNA DE LAS materias primas más ampliamente utilizadas para la fermentación del etanol potable y combustible son las mieles finales de caña (mieles C), un subproducto de la producción de azúcar de caña. La inconsistente calidad de las mieles en la producción industrial, hacen, sin embargo, la producción de etanol mucho menos eficiente y efectiva económicamente. Además de los azúcares fermentables (sacarosa, glucosa, fructosa) presente en las melazas, también el calcio se encuentra en un rango de 10–20% de la ceniza total carbonada (15% en peso). En el proceso azucarero el calcio se aplica usualmente como lechada, CaO, durante la clarificación, lo que resulta en una sobre alcalización si la calidad del jugo es pobre. En este estudio se evalúa el papel de los residuos de calcio (0–0.72% w/v de Ca^{2+} en forma de cloruro de calcio). Los resultados sugieren un efecto depresor de los iones calcio sobre el comportamiento de la levadura que era dependiente de la concentración. Resultó evidente un ligero crecimiento en la velocidad de fermentación y en el rendimiento de etanol cuando el calcio estaba presente a 0.18%w/v Ca^{2+} en todas las soluciones de azúcar. Este efecto fue más pronunciado cuando la concentración de iones calcio se elevaba. A valores de 0,72% w/v Ca^{2+}, las velocidades de fermentación y los rendimientos de etanol en base a todos los azúcares caían considerablemente (el rendimiento de etanol decrecía 14–25% en relación con las muestras de control que no tenían calcio). A muy altas concentraciones de Ca^{2+} (2.16% w/v) , la fermentación de azúcares por las levaduras fue casi absolutamente inhibida. Esto puede deberse, en parte, al efecto inhibidor de la enzima invertasa en la conversión de sacarosa en azúcares invertidos, un paso limitante en la fermentación alcohólica de los azúcares por levaduras. El pretratamiento de las melazas con ácido y calor, previa a la fermentación, se aplicó para remover el calcio, lo que mejoró la eficiencia de fermentación.