Research Article

Understanding and Efficiently Manipulating Environmental Stress Caused by Metal Ions to Improve Ethanol Fermentation

Boontiwa Ninchan*, Chollada Sirisatesuwon and Kittipong Rattanaporn
Department of Biotechnology, Faculty of Agro-Industry, Kasetsart University, Bangkok, Thailand

Klanarong Sriroth
Department of Biotechnology, Faculty of Agro-Industry, Kasetsart University, Bangkok, Thailand
Mitr Phol Sugar Corp., Ltd, Bangkok, Thailand

* Corresponding author. E-mail: boontiwa.ni@ku.th DOI: 10.14416/j.asep.2021.06.004
Received: 6 January 2021; Revised: 22 March 2021; Accepted: 30 April 2021; Published online: 9 June 2021

Abstract
The inconsistent quality of molasses directly influences ethanol production, particularly due to contamination by metal ions that causes severe problems and reduces production efficiency. This research focused on calcium (Ca^{2+}), potassium (K^{+}), and magnesium (Mg^{2+}) ions that are common in molasses. The key objective was to understand clearly the effect of ions on ethanol fermentation and *Saccharomyces cerevisiae* performance. Individual ions and ion mixtures were studied in sucrose solution and in molasses. The results showed that severe stress could be ordered as Ca^{2+}>K^{+}>Mg^{2+}, respectively, and the adverse effect was greater when the ion concentration increased. Ca^{2+} was a strong inhibitor while trace amounts of Mg^{2+} produced a positive effect. To achieve the greatest efficiency in ethanol production using molasses in the substrate preparation, Ca^{2+} should not exceed 0.18% (w/w) prior to fermentation and the final sugar concentration should be 20–25% (w/v), as adjusting the addition of sucrose will result in a suitable yeast medium. Pretreatment and dilution were the best practices for ion removal, with Ca^{2+} being clearly decreased. Furthermore, determination of the composition and ion concentration in molasses is essential to initial steps that must be routinely applied to ensure that the knowledge gained and the efficient techniques investigated can be used to improve ethanol production.

Keywords: Calcium, Ethanol, Magnesium, Molasses, Potassium

1 Introduction

One of the key factors driving the global economy is petroleum-based fuels. The demand is continuously increasing together with the growth of the manufacturing industry and the need for more transportation fuel. This also applies in Thailand. The demand and supply of energy and the challenge of solving this major problem in Thailand have been addressed using a case study [1]. Thailand is the fastest growing country in Southeast Asia and is a hub for continental ASEAN members [2], [3]. There is increased demand for transportation fuel that currently must be imported due to inadequate domestic production. Thus, one sustainable solution to support the increased demand for energy in Thailand is to produce alternative energy as "bioethanol" to substitute for petroleum fuels [1], [4]. Currently, Thailand ranks 6th in terms of global ethanol production and 7th in terms of global consumption [4]. The target in 2031 for ethanol consumption set by the Ministry of Energy in Thailand is 7.03 million liters/day, with increases in 2019 (4.46 million liters/day) and 2020 (5.08 million liters/day) [5]. Ethanol now constitutes 10, 20, and 85% of the gasohol mixtures marketed as E10, E20, and E85, respectively, and is in high demand for some applications in the food, beverage, cosmetics, and mechanical industries.

Nowadays, feedstocks for ethanol production in

Thailand is usually sourced from low-cost agricultural residues and primarily from sugar-based crops such as blackstrap molasses and sugarcane juice from sugar mills, and as cassava starch and cassava chips from the starch industry [6]. In 2020, there were 27 ethanol factories with a total capacity of 6.125 million liters/day grouped in Central and Northeastern Thailand using different raw materials for ethanol fermentation [3], [7]. Of the daily total, 11 factories produced from molasses, 9 factories from cassava, 6 factories from mixed substrates of molasses-cassava, and 1 factory from sugarcane juice [5]. The demand for ethanol consumption is continuously increasing, so there is continued high demand for feedstock for ethanol production. Generally, cassava-based products in the forms of cassava starch and cassava chips can be used as food and feed, so there are limits on this raw material source for ethanol fermentation. Similarly, sugarcane juice is usually the principal raw material for sugar manufacturing. Sometimes, when the price of raw sugar decreases, sugarcane juice is used as the substrate for ethanol production. However, the continuous growth of the sugar industry suggests that sugarcane juice will be required increasingly as raw material for sugar production. Consequently, molasses is the main raw material for ethanol production.

Molasses is the major by-product of sugar manufacturing and is rich in organic compounds such as sugars (sucrose, glucose, and fructose), amino acids, pigments, and wax [8], while the inorganic components include calcium, potassium, magnesium, sodium, aluminum, copper, manganese, iron, phosphorus, and zinc [9]–[11]. In particular, the presence of ions of inorganic compounds such as calcium (Ca^{2+}), potassium (K^{+}), and magnesium (Mg^{2+}) are mostly found in molasses at concentration ranges of 0.5–1.2% (w/v), 2.4–5.19% (w/v), and 0.29–0.61% (w/v), respectively [12], [13], with the ratio of Ca^{2+} to K^{+} to Mg^{2+} being approximately 1 : 5 : 0.5 [14]. The types and quantities of inorganic compounds or metal ions present in molasses depend on many parameters including the sugarcane variety, fertilizer contamination during cane cultivation, and from the use of some ions in sugar manufacturing such as in the clarification process. Inconsistent quality of molasses makes ethanol production much less efficient and more cost-ineffective [15]. Some metal ions in molasses probably affect parameters associated with yeast fermentation including in ethanol productivity such as biomass growth, the kinetics of enzyme activity, and the sugar consumption of yeast, [6], [15]. Some ions have a positive effect while some have a negative effect [15]–[17]. Furthermore, the concentration of compounds may limit efficient ethanol fermentation [15]. Many literature reviews have reported that calcium causes a severe reduction in ethanol production [12], [15], [18], [19]. Chotineeranat *et al.* [15] reported that the rate of fermentation and ethanol yield was decreased with the yield declining by 25.76% at 0.72% w/v of calcium ion in the sucrose solution as substrate. In addition, the effect was more pronounced when the calcium ion concentration increased [6], [15], [18]. Thus, the molasses composition is a priority regarding both the quality and quantity of metal contamination in the raw materials for ethanol production.

The current research focused on Ca^{2+}, K^{+}, and Mg^{2+} ions as they are mainly found in molasses. These ions were investigated for their effects on the efficiency of ethanol fermentation by *S. cerevisiae* and the influence of these ions on yeast performance. Ions (Ca^{2+}, K^{+}, and Mg^{2+}) and ion mixtures (Ca^{2+}+K^{+}, Ca^{2+}+Mg^{2+}, K^{+}+Mg^{2+}, and Ca^{2+}+K^{+}+Mg^{2+}) were studied as contaminants in a basic substrate model (sucrose solution) to more clearly understand the processes. Furthermore, metal ion contamination was also studied in a complex real substrate (molasses). In addition, good manufacturing practice was investigated for molasses preparation using pretreatment with acid and dilution prior to yeast fermentation. This included the determination of the minimum concentrations of remaining ions which had the least effect on ethanol production. The results were expected to inform and be useful to the ethanol industry.

2 Materials and Methods

2.1 *Effect of metal ions on ethanol fermentation using sucrose solution as a basic substrate model*

2.1.1 Inoculum preparation

The commercial dry active yeast, *S. cerevisiae*, was supplied from Angel Yeast Co., Ltd. (China). *S. cerevisiae* was activated before fermentation by dissolving in sterile distilled water and incubating at 32 °C in a shaker incubator (vs-8480SFN, Vision Scientific Co., Ltd., Korea) at 150 rpm for 30 min.

2.1.2 S. cerevisiae fermentation in sucrose solution

The first experiment studied the effect of each metal ion (Ca^{2+}, K^{+}, and Mg^{2+}) on fermentative parameters: specific growth rate (μ), substrate consumption (Q_s), productivity (Q_p), sugar utilization (sucrose, glucose, and fructose contents), and ethanol concentration. The final concentrations of metal ions in culture medium for Ca^{2+} (in the form $CaCl_2.2H_2O$), K^{+} (in the form KCl), and Mg^{2+} (in the form $MgCl_2.12H_2O$) were 0.07, 0.20, and 0.60% (w/v), respectively. All chemicals were analytical grade and were supplied from Sigma-Aldrich Pte Ltd (Singapore).

The second experiment studied the effect of ion mixtures of Ca^{2+}+K^{+}, Ca^{2+}+Mg^{2+}, K^{+}+Mg^{2+}, and Ca^{2+}+K^{+}+Mg^{2+} in sucrose culture medium as possible contaminants. The ratio of Ca^{2+} to K^{+} to Mg^{2+} present in molasses is approximately 1:5:0.5 as previously mentioned, so 0.20% (w/v) Ca^{2+}, 0.60% (w/v) K^{+}, and 0.07% (w/v) Mg^{2+} were selected as the final concentrations added to the sucrose culture medium. The experiments using mixed ions were prepared as detailed in Table1.

Table 1: Ratio of final concentrations of ion mixtures in sucrose medium under *S. cerevisiae* fermentation

Metal Ions	Final Concentrations (all as % w/v)
Ca^{2+} : K^{+}	0.20 : 0.60
Ca^{2+} : Mg^{2+}	0.20 : 0.07
K^{+} : Mg^{2+}	0.60 : 0.07
Ca^{2+} : K^{+} : Mg^{2+}	0.20 : 0.60 : 0.07

The initial concentration of sucrose solution as culture medium was 200 g/L made up to 300 mL in a 500 mL Erlenmeyer flask. Yeast extract (10 g/L) was added into culture medium containing the relevant metal ion or ion mixture. Then, this was adjusted to pH 4.5 with 1N HCl based on pH meter readings prior to sterilization at 121℃ for 15 min. Then, 0.35 g/L of activated *S. cerevisiae* was cultivated in sterilized sucrose medium at 32 °C and 120 rpm for 72 h. in a shaker incubator (vs-8480SFN, Vision Scientific Co., Ltd, Korea) using two replicates for each culture medium. The medium samples were collected at 0, 2, 4, 6, 24, 30, 48, 54, and 72 h. during fermentation for analysis of the fermentative parameters as detailed previously. In addition, the efficiency of ethanol production was calculated. Three replicates of measurements of fermentative parameters were analyzed and compared with and without metal ions (the control) in culture medium.

2.2 *Effect of metal ions on ethanol fermentation using molasses as complex substrate*

Molasses was obtained from the Saraburi Sugar Factory (Saraburi, Thailand). The compositional analysis of the molasses is shown in Table 2.

Table 2: Composition of molasses

Component	Concentration
Total soluble solid*	78.4°Brix
Sucrose**	36.39% (w/w)
Glucose**	5.13% (w/w)
Fructose**	4.57% (w/w)
Sulphate ash***	10.16% (w/w)
Calcium ion (Ca^{2+})***	1.00% (w/w)
Potassium ion (K^{+})***	1.90% (w/w)
Magnesium ion (Mg^{2+})***	0.38% (w/w)

*AOAC method (AOAC 2000)
**high performance liquid chromatography [6], [20].
***ICP-MS/OES method from Central Laboratory (Thailand) Company Limited

2.2.1 Molasses pretreatment under acidic conditions

The molasses sample was adjusted to pH 4.0 with 0.5 M sulfuric acid (H_2SO_4), then heated at 95 °C for 30 min. Each sample was left overnight at room temperature. The precipitates were removed using filtration while the supernatant, called pretreated molasses, was subsequently used as the substrate for yeast fermentation.

2.2.2 *S. cerevisiae* fermentation in molasses substrate

The pretreated molasses was diluted by adjusting the total soluble solid content to 40–50°Brix (total sugars content as 20–25°Brix) with distilled water. The composition of the ions in the diluted pretreated molasses was analyzed. The diluted pretreated molasses was used as culture medium in 500 mL Erlenmeyer flasks containing 350 mL as a shake flask at the laboratory scale. At the pilot scale, culture medium was used with 3.5 L and 35 L in 5 L, and 50 L fermentors, respectively. Then, the fermentors were supplemented with 2.25 g/L urea as a nitrogen source for yeast fermentation. All culture media were adjusted to pH 4.5 with 1N HCl and then measured using a pH meter prior to sterilization

at 121 °C for 15 min. Then, 0.35 g/L of activated *S. cerevisiae* was cultivated in sterilized culture media at 32 °C and 120 rpm for 72 h. Two replicates for each culture medium were determined. The medium samples were collected at 0, 2, 4, 6, 24, 30, 48, 54, and 72 h for analysis of the fermentation parameters as was performed for the sucrose medium. The efficiency of ethanol fermentation in diluted molasses with and without pretreatment was compared only in the shake flask experiment.

2.3 *Analysis of fermentative parameters*

2.3.1 Cell growth

Cell growth was determined using a hemocytometer to calculate the specific growth rate [6].

2.3.2 High-performance liquid chromatography analysis

The sugar contents (sucrose, glucose, and fructose) and ethanol was determined using high-performance liquid chromatography (HPLC) [6], [20], [21]. The chromatographic system consisted of an LC-10ADVP binary pump (Shimadzu Corporation, Japan) and a refractive index detector (RID-10A, Shimadzu Corporation, Japan). The sample injection volume was 20 μL. The sugar contents were analyzed using a VertiSep™ Sugar CMP column (7.8 × 300 mm, 8 um diameter; Vertical Chromatography Co. Ltd., Thailand) at 80 °C, with a mobile phase of deionized water and a flow rate of 0.4 mL/min. Ethanol concentrations were analyzed using an Aminex HPX-87H (Bio-Rad Laboratories, Inc., USA) at 40 °C, with a mobile phase of 0.50 mm H_2SO_4 and a flow rate at 0.60 mL/min. The medium samples were passed through a 0.45 μm cellulose acetate membrane filter and then 20 μL of the filtered samples were subjected to HPLC. Throughout the experimental work, data were collected and integrated using the LC Solution software (Shimadzu, Japan). The standards (sucrose, glucose, and fructose) were obtained from Merck KGaA (Germany).

2.4 *Statistical analysis*

The Statgraphics XVII-X64 software program was used to conduct LSD multiple range testing and significance was tested at the $p < 0.01$ and $p < 0.05$ levels.

3 Results and Discussion

3.1 *Effect of metal ions on ethanol fermentation using sucrose solution as basic substrate model*

The results of inducing environmental stress with metal ions during ethanol production in the 20% (w/v) sucrose medium are provided in Table 3, including the control (no stress) results for the measured fermentation parameters. The percentage yield reduction is also shown in Table 3 and the efficiency of ethanol production is shown in Figure 1. Under normal conditions with no ions stress, *S. cerevisiae* yeast produced 8.26% (w/v) ethanol concentration with specific growth rate (μ), substrate consumption (Q_s), productivity (Q_p) values of 0.24 h^{-1}, 4.57 g/L/h, and 1.71 g/L/h, respectively, and the efficiency of production of 75.54% (Figure 1). The culture medium containing metal ions had a negative effect by reducing ethanol fermentation (Table 3 and Figure 1).

The key objective in the study of each ion (Ca^{2+}, K^+, and Mg^{2+}) in the basic substrate model (sucrose solution) was to identify how each ion influenced ethanol fermentation. The results showed that the Ca^{2+} ion had a greater effect than the other ions and the adverse effect of Ca^{2+} was greater as the Ca^{2+}concentration increased (Table 3). The yield reduction under Ca^{2+} contamination decreased significantly by 13.56, 28.57, and 61.66% at 0.07, 0.20, and 0.60% (w/v), respectively. Substrate consumption (Q_s) as shown in Table 3 and utilization of sugars (sucrose, glucose, and fructose), as shown in Figure 2, were distinctive parameters that were severely affected under stress by the Ca^{2+}ion because Ca^{2+} acted as an invertase inhibitor [6], [15], [18]. Normally, sugar utilization during yeast fermentation commences with sucrose being hydrolyzed by invertase into its monosaccharides as glucose and fructose which then becomes available as carbon sources for cell metabolism. Contaminated metal ions of Ca^{2+} in the culture medium inhibited invertase activity [18] that affected sucrose conversion resulting in less sucrose utilization and less consumption of glucose and fructose by yeast cells, resulting in high values for the remaining sugar content in the culture medium [Figure 2(a)]. Less sugar consumption was also related to less growth of *S. cerevisiae* cells (Table 3) resulting in reduced efficiency of ethanol production (Figure 1). At a low ion concentration of 0.07% (w/v) of K^+ and

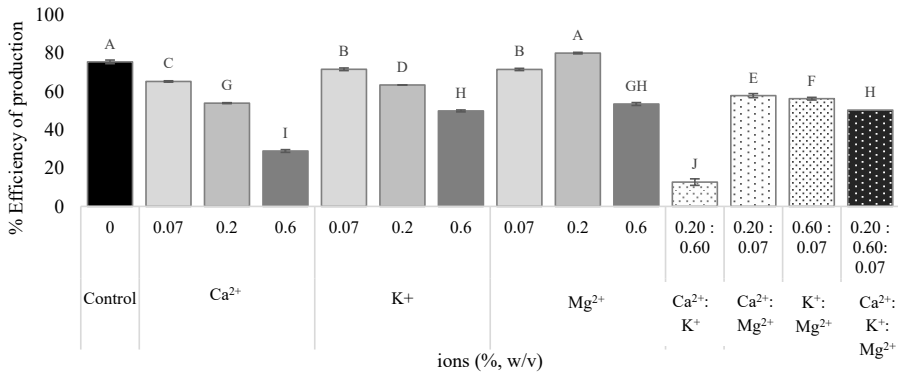

Figure 1: Percentage efficiency of ethanol production (E_f) using sucrose substrate under stress and no stress from metal ions (reported as percentage of experimental to theoretical yield of ethanol).

Remark: Uppercase letters represent significant ($p < 0.05$) differences

$$E_f\ (\%) = \left[\frac{\Delta\text{Ethanol (g/L)}}{\Delta\text{Total sugar as invert (g/L)}}\right]\times\left[\frac{100}{51.11}\right]\times 100$$

Table 3: Fermentative parameters of *S. cerevisiae* fermentation using sucrose substrate under environmental stress and no stress from metal ions

Type of Ion	[Ions], (%, w/v)	Specific Growth Rate μ (h^{-1})	Substrate Consumption Q_s (g/L/h)	Productivity Q_p (g/L/h)	Ethanol Concentration (% w/v)	Yield Reduction (%) (*)
Control	0.00	0.24 ± 0.07^{A}	4.57 ± 0.34^{AB}	1.71 ± 0.23^{A}	8.26 ± 1.07^{A}	0.00 ± 0.00^{K}
Each ion						
Ca^{2+}	0.07	0.17 ± 0.02^{AB}	3.97 ± 0.04^{CDE}	1.58 ± 0.03^{AB}	7.62 ± 0.18^{ABCD}	13.56 ± 0.47^{I}
	0.20	0.12 ± 0.04^{ABC}	3.57 ± 0.10^{DE}	1.25 ± 0.12^{BC}	6.04 ± 0.52^{BCD}	28.57 ± 0.55^{E}
	0.60	0.06 ± 0.03^{BC}	2.50 ± 0.14^{G}	0.67 ± 0.04^{D}	3.25 ± 0.18^{E}	61.66 ± 0.94^{B}
K^{+}	0.07	0.20 ± 0.03^{AB}	4.39 ± 0.15^{BC}	1.70 ± 0.03^{A}	8.17 ± 0.03^{AB}	5.14 ± 0.23^{J}
	0.20	0.15 ± 0.02^{ABC}	3.90 ± 0.18^{CDE}	1.55 ± 0.19^{AB}	7.49 ± 1.31^{ABCD}	16.00 ± 0.51^{H}
	0.60	0.12 ± 0.02^{ABC}	3.52 ± 0.20^{E}	1.22 ± 0.01^{BC}	5.94 ± 0.03^{CD}	33.93 ± 0.66^{C}
Mg^{2+}	0.07	0.19 ± 0.01^{AB}	4.22 ± 0.14^{BC}	1.69 ± 0.21^{A}	8.10 ± 1.39^{ABC}	5.24 ± 0.45^{J}
	0.20	0.20 ± 0.03^{AB}	4.89 ± 0.18^{A}	1.76 ± 0.01^{A}	8.46 ± 0.03^{A}	0.00 ± 0.01^{K}
	0.60	0.11 ± 0.10^{ABC}	3.05 ± 0.06^{F}	1.15 ± 0.05^{C}	5.57 ± 0.34^{D}	29.16 ± 0.61^{DE}
Mixed ions						
Ca^{2+}: K^{+}	0.20:0.60	0.01 ± 0.00^{C}	1.48 ± 0.10^{H}	0.31 ± 0.02^{E}	1.51 ± 0.08^{E}	84.15 ± 1.50^{A}
Ca^{2+}:Mg^{2+}	0.20:0.07	0.12 ± 0.06^{ABC}	4.11 ± 0.13^{CB}	1.43 ± 0.13^{ABC}	6.87 ± 0.60^{ABCD}	20.74 ± 0.99^{G}
K^{+}: Mg^{2+}	0.60:0.07	0.14 ± 0.03^{ABC}	4.10 ± 0.05^{CB}	1.41 ± 0.04^{ABC}	6.78 ± 0.20^{ABCD}	23.39 ± 0.64^{F}
Ca^{2+}:K^{+}: Mg^{2+}	0.20:0.60:0.07	0.12 ± 0.02^{ABC}	4.07 ± 0.07^{BCD}	1.26 ± 0.06^{BC}	6.06 ± 0.31^{ABCD}	31.31 ± 0.87^{D}

Remark: Uppercase superscripts in same column represent significant ($p < 0.05$) differences

* Reported as percentage of difference between control experiment (no stress) and treatment (ions stress) to control experiment.

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

Mg^{2+}, the decreases in ethanol concentration and production efficiency was not significantly different to the control. When the ion concentration increased to 0.20% (w/v), the ethanol concentration and production efficiency under K^{+} stress clearly declined; in contrast, there was an increase in Mg^{2+} stress compared with the control. Mg^{2+} acted as the co-factor of invertase that easily hydrolyzed sucrose to glucose and fructose [12] so that these were depleted at 30 h. and 48 h. fermentation time, respectively [Figure 2(c)]. Mg^{2+} produced

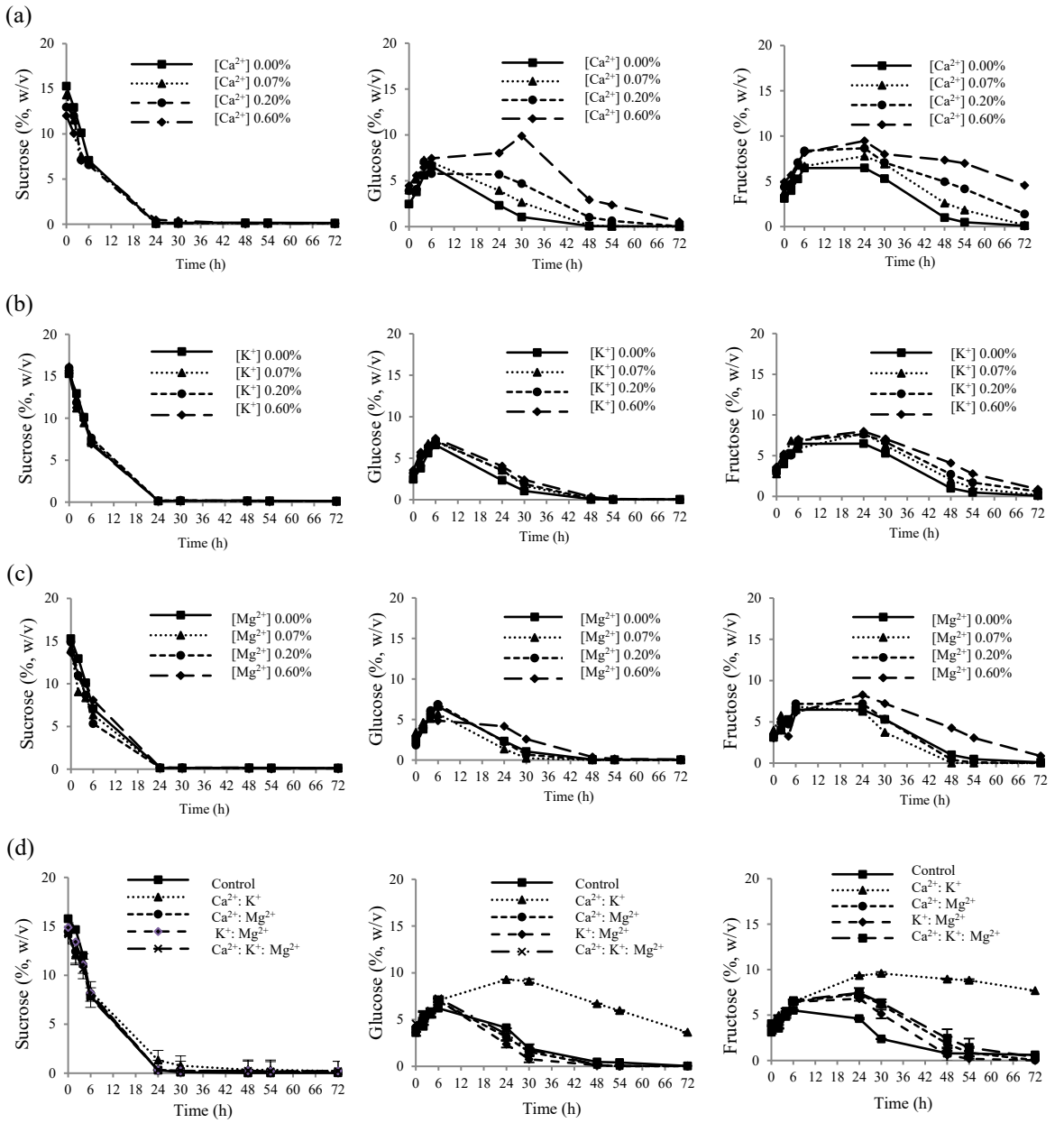

Figure 2: Change in sugar utilization (sucrose, glucose, and fructose) during *S. cerevisiae* fermentation in 20% (w/v) sucrose solution for 72 h with metal ion contamination by: (a) Ca^{2+}, (b) K^{+}, (c) Mg^{2+}, and (d) mixed ions.

showed high growth of *S. cerevisiae* cells [Figure 3(c)] and resulted in a slight increase in the ethanol content (Table 3), while the ethanol efficiency was also quite high (Figure 1). The increase in the Mg^{2+} concentration had a positive effect; however, when the accumulated Mg^{2+} concentration reached 0.60% (w/v), it negatively affected substrate inhibition resulting in reduced ethanol production [6], [12]. Under a severely high the concentration of 0.60% (w/v) K^{+} and Mg^{2+}, the yield was reduced by 33.93% and 29.16%, respectively (Table 3).

The environmental stress of added ion mixtures of Ca^{2+}+K^{+}, Ca^{2+}+Mg^{2+}, K^{+}+Mg^{2+}, and Ca^{2+}+K^{+}+Mg^{2+}

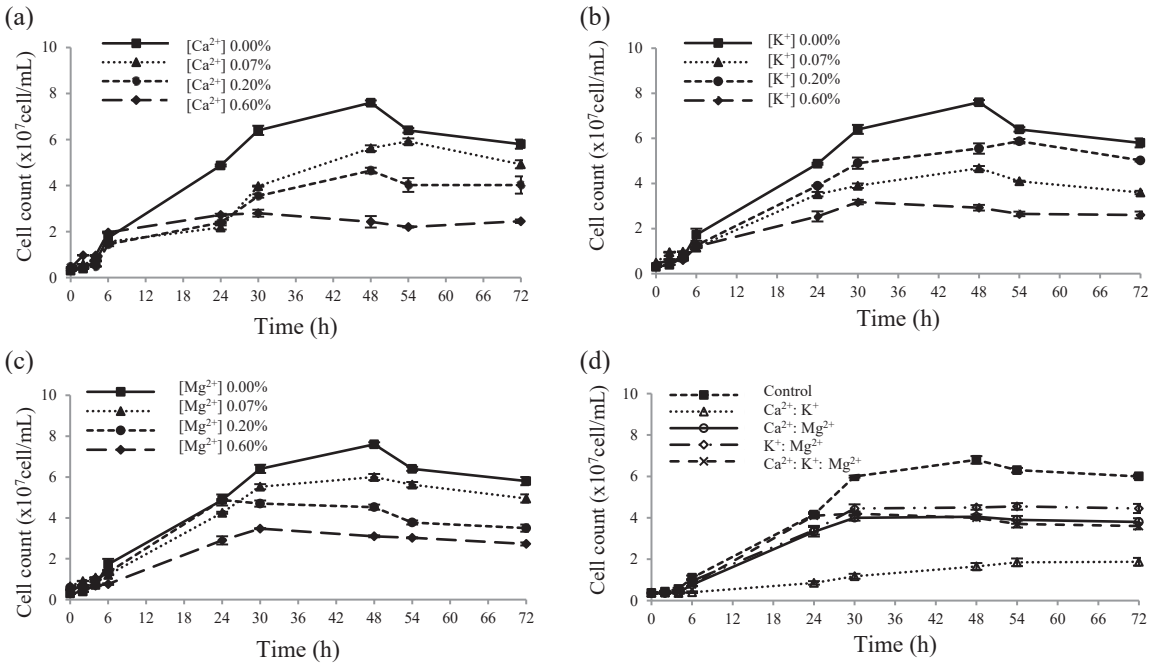

Figure 3: *S. cerevisiae* growth during ethanol fermentation in 20% (w/v) sucrose solution for 72 h under metal ions stress by: (a) Ca^{2+}, (b) K^{+}, (c) Mg^{2+}, and (d) mixed ions.

was detrimental. The Ca^{2+} and K^{+} mixtures showed the lowest ethanol concentration (1.51% w/v; Table 3), and efficiency of production (12.70%; Figure 1) resulting in a yield decrease to 84.15% and all fermentative parameters also significantly declined (Table 3). Furthermore, under severe stress of Ca^{2+}+K^{+}, sugar utilization was clearly negatively affected, resulting in large amounts of glucose and fructose left in the sucrose medium, suggesting that the yeast cells had utilized less sugar compared to the other mixed conditions [Figure 2(d)]. The results of less cell growth and lower efficiency of ethanol production under Ca^{2+}+K^{+} are shown in Table 3 and Figure 1, respectively. An interesting result was that the addition of a small amount of Mg^{2+} at 0.07% (w/v) in the substrate contaminated with mixed ions (Ca^{2+}+Mg^{2+}, K^{+}+Mg^{2+}) increased all fermentative parameters compared to the mixture of Ca^{2+}+K^{+}, including the ethanol content, which increased 6.78–6.87% (Table 3). The efficiency values for ethanol production of Ca^{2+}+Mg^{2+} and K^{+}+Mg^{2+} were 57.90% and 56.30%, respectively, and clearly higher than with the combination of the two strong inhibitors of Ca^{2+}+K^{+} (12.70%) as shown in Figure 1. The contamination with all the ions together (Ca^{2+}+K^{+}+Mg^{2+}) resulted in production efficiency that was 50.34% lower than for Ca^{2+}+Mg^{2+} and K^{+}+Mg^{2+} and the control (Figure 1). The severe stress induced by all ions had greater efficiency than from the high stress from Ca^{2+}+K^{+} as shown in Figure 1 and Figure 4. All ion mixtures reduced the ethanol concentration to 31.31% compared to the control.

In fact, Mg^{2+} and K^{+} ions in the millimolar concentration range are classified as bulk elements and are generally required by growing yeast cells [22]–[24]. However, Ca^{2+} ion requirements for yeast growth are very low, with metals being required in the micromolar range being classified as trace elements [6], [18], [24]. Mg^{2+} ion availability is crucial in the pathways of carbohydrate catabolism and glycolytic enzymes and especially for ethanolic fermentation [23], [24]. An excess of some trace elements (such as Ca^{2+}) has been reported to be toxic to yeast cells [6], [22].

3.2 *Effect of metal ions on ethanol fermentation using molasses as complex substrate*

The initial investigation considered the impact of contamination using single ions and their mixtures

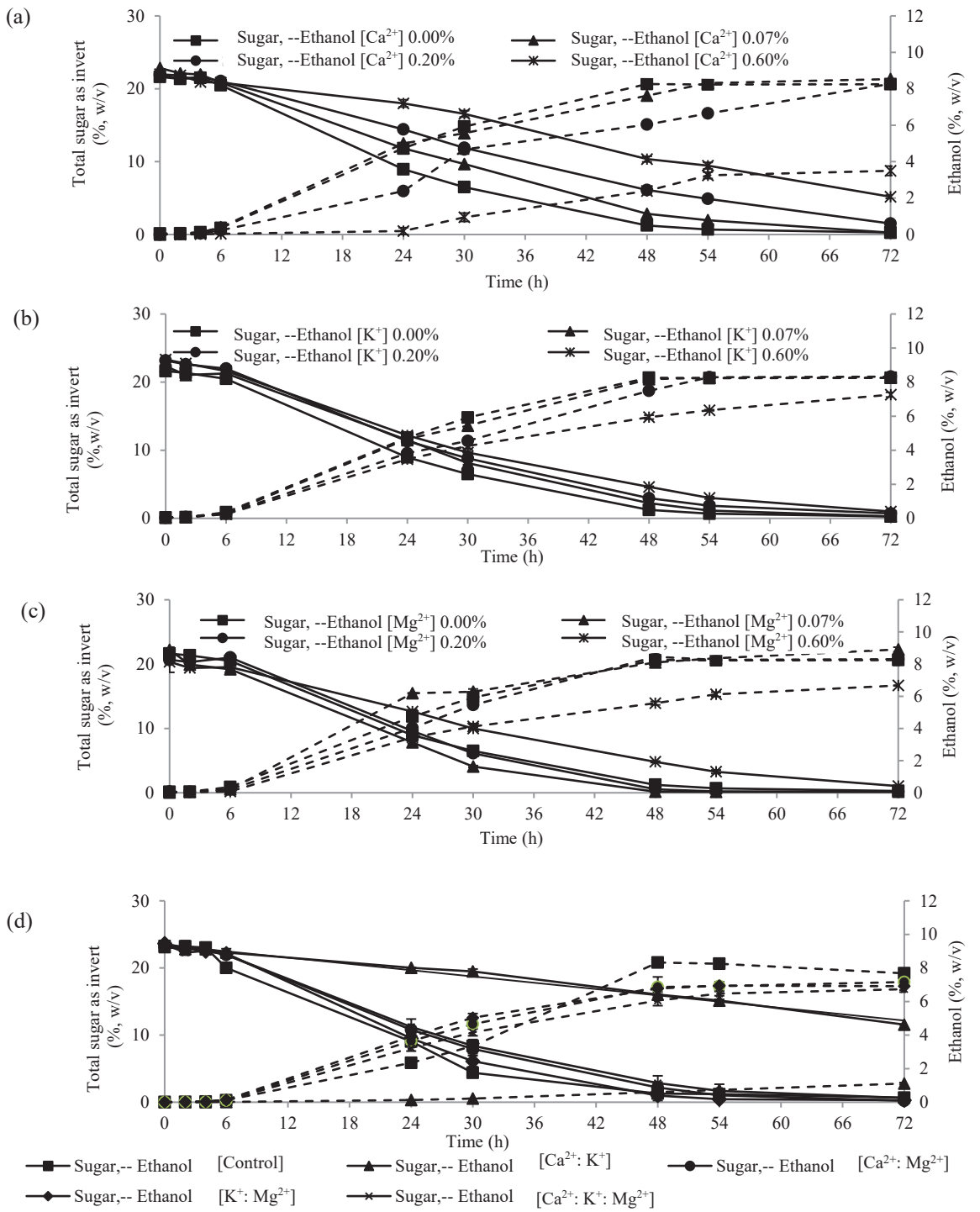

Figure 4: Change in total sugar as invert and ethanol concentration during *S. cerevisiae* fermentation in 20% (w/v) sucrose solution for 72 h under metal ions stress by: (a) Ca^{2+}, (b) K^{+}, (c) Mg^{2+}, and (d) mixed ions.

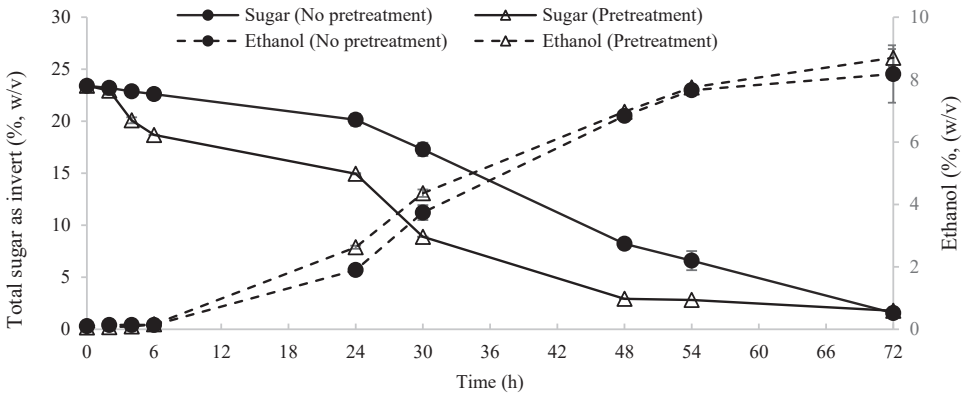

Figure 5: Change in total sugar as invert and ethanol production during *S. cerevisiae* fermentation using diluted molasses without and with molasses pretreatment at laboratory scale.

in a basic sucrose substrate model as a single carbon source and the addition of some essential nutrients as a nitrogen source for *S. cerevisiae* growth. Understanding could be gained on the results of ethanol production and yeast performance. Subsequently, molasses as real yeast substrate was investigated. It is a complex the substrate with many individual components (Table 2). Prior to ethanol fermentation, the pretreated molasses was diluted to 40°Brix so that total sugars remained at approximately 20–25°Brix as a suitable initial sugar concentration for yeast fermentation. The ion compositions with and without molasses pretreatment including dilution is shown in Table 4. There were significant reductions in the Ca^{2+}, K^{+}, and Mg^{2+} concentrations of 82, 56, and 63%, respectively, due to the co-processes of pretreatment and dilution. The dilution the process produced significant but lower decreases of Ca^{2+}, K^{+}, and Mg^{2+} of 58, 54, and 63%, respectively. As expected, pretreatment and dilution were efficient processes for the elimination of some ions, especially Ca^{2+}, which clearly decreased in content (Table 4).

Table 5 and Figure 5 show the results of *S. cerevisiae* fermentation at the laboratory scale in a shake flask using diluted molasses with and without molasses pretreatment. Without pretreatment of the diluted molasses, the *S. cereviaise* cells produced 8.18% (w/v) ethanol concentration, whereas the pretreatment of the diluted molasses, produced significantly greater ethanol production of 8.70% (w/v) (Table 5). The previous results in the sucrose substrate model showed that the Ca^{2+} ion significantly affected sugar utilization. Similarly, reducing the Ca^{2+} ion in the pretreated diluted molasses resulted in significantly higher substrate consumption (Q_s) (Table 5) as the yeast cells hydrolyzed and consumed sugars easily. Table 4 implies that the Mg^{2+} ion was reduced by dilution but was not eliminated by the acidic condition at the same concentration with and without pretreatment. From Table 3, the previous study under mixed ions in sucrose solution found that Mg^{2+} showed greater values for all the fermentative parameters under mixed ions with a small amount at 0.07% (w/v), while the mixture of Ca^{2+} and K^{+} was a severe condition that had the lowest values of substrate consumption and all fermentative parameters. The higher concentration of 0.14% (w/w) Mg^{2+} definitely provided positive support for fermentative parameters, especially sugar consumption by the yeast cells with and without pretreatment in diluted molasses (Table 5). Subsequently, there was increased efficiency in ethanol production.

The main objective of scaling fermentation was to study the efficiency of ethanol production and all

Table 4: Concentration of metal ions of molasses during molasses preparation for *S. cerevisiae* substrate

Metal Ion	Metal Ion Concentration (% w/w)		
	Before Dilution (78.4 °Brix*)	After Dilution (40°Brix*)	
		Without Pretreatment	With Pretreatment
Ca^{2+}	1.00	0.42	0.18
K^{+}	1.90	0.86	0.83
Mg^{2+}	0.38	0.14	0.14

Remark: * Content of total soluble solid in molasses
Analyzed using ICP-MS/OES method from Central Laboratory (Thailand) Company Limited

Table 5: Fermentative parameters of *S. cerevisiae* fermentation during 72 h using diluted molasses without and with molasses pretreatment at laboratory scale

Substrate (diluted molasses)	Specific growth rate μ (h^{-1})	Substrate consumption Q_s (g/L/h)	Productivity Q_p (g/L/h)	Yield (g/g)	Ethanol concentration (% w/v)	Efficiency (%)
Without pretreatment	0.16 ± 0.03^{A}	3.45 ± 0.10^{B}	1.12 ± 0.09^{A}	0.37 ± 0.03^{A}	8.18 ± 0.11^{B}	72.32 ± 0.15^{B}
With pretreatment	0.16 ± 0.05^{A}	3.87 ± 0.09^{A}	1.20 ± 0.01^{A}	0.40 ± 0.07^{A}	8.70 ± 0.13^{A}	78.19 ± 0.18^{A}

Remark: Uppercase superscripts in same column represent significant ($p < 0.05$) differences

Table 6: Fermentative parameters of *S. cerevisiae* fermentation during 72 h using pretreated diluted molasses at laboratory and pilot scales

Treatment		Specific growth rate μ (h^{-1})	Substrate consumption Q_s (g/L/h)	Productivity Q_p (g/L/h)	Yield (g/g)	Ethanol concentration (% w/v)	Efficiency (%)
Laboratory scale	Shake flask	0.16 ± 0.01^{A}	3.87 ± 0.10^{A}	1.20 ± 0.03^{A}	0.40 ± 0.04^{A}	8.70 ± 0.05^{A}	78.19 ± 0.10^{A}
Pilot scale (Fermentor)	5 L	0.17 ± 0.05^{A}	4.02 ± 0.05^{B}	1.26 ± 0.01^{A}	0.40 ± 0.02^{A}	9.15 ± 0.14^{B}	78.24 ± 0.57^{A}
	50 L	0.18 ± 0.04^{A}	4.15 ± 0.06^{B}	1.25 ± 0.01^{A}	0.40 ± 0.01^{A}	9.05 ± 0.07^{B}	79.24 ± 0.27^{A}

Remark: Uppercase superscripts in same column represent significant ($p < 0.05$) differences

fermentation parameters under molasses pretreatment as the substrate was introduced on a bigger scale. Laboratory and pilot scales were compared using pilot scales of 5 L and 50 L. The hypothesis of this study was that the results of fermentative parameters and the efficiency production for the 5 L and 50 L scales should not be significantly different. It was expected that all the results could provide guidelines for the performance on an industrial scale.

The efficiency of the 5 L and 50 L scales were similar because all treatments were applied and set at the same conditions, namely, working volume of substrate (70% of total volume), volume of 10% (v/v) *S. cerevisiae* inoculum, the content of nitrogen source, and conditions of fermentation (Table 6.) In fact, the rate of energy consumption for 50 L was much greater than for 5 L, especially during sterilization.

4 Conclusions

Contamination by ions in a culture medium for *S. cerevisiae* fermentation had a definite negative effect on ethanol production. The order of stress by the ions was $Ca^{2+}>K^{+}>Mg^{2+}$, respectively, with the adverse effect more pronounced as the ion concentration increased. Consequently, pretreatment and dilution were the best practices to prepare suitable molasses substrate for yeast fermentation that also reduced the concentrations of the ions, particularly Ca^{2+}, in the pretreatment process. Furthermore, the molasses composition should be initially analyzed and the concentrations of ions should also be routinely monitored after each step, so that the remaining concentrations are known, especially the Ca^{2+} ion concentration. Optimizing the molasses substrate prior to yeast fermentation required a Ca^{2+} concentration not exceeding 0.18% (w/w) to obtain 8.7–9.2% (w/v) ethanol concentration. Where the Ca^{2+} ion concentration did not exceed this level, the acceptable ions concentration is achieved for the final culture medium. Furthermore, the sugar concentration as a carbon source for cell growth as an important factor in yeast fermentation should be in the range of 20–25% (w/v), so that if necessary, additional sucrose could be added to achieve the required sugar concentration in the yeast substrate. This knowledge and the development of efficient techniques could improve ethanol production and should be of use in the ethanol industry.

References

[1] S. Amornvivat, P. Hoontrakul, A. Tamprasirt, L. Larpchevasit, T. Homchampa, S. Khantachavana, and C. Charoenlad, *Insight: Bioenergy and the Road to Sustainable Growth*. Bangkok, Thailand: Siam Commercial Bank Public Company Limited, 2016.

[2] W. Hicks, "Thailand among ASEAN stars," 2019.

[Online]. Available: https://www.bangkokpost.com/business/1713796/thailand-among-asean-stars

[3] ASEAN UP, "Overview of business in Thailand," 2017. [Online]. Available: https://aseanup.com/business-thailand/

[4] N. Tunpaiboon, "Thailand industry outlook 2019–2021: Ethanol industry," 2019. [Online]. Available: https://www.krungsri.com/bank/getmedia/0c42d6fd-18d7-41c193696dded234800/IO_Ethanol_190710_EN_ EX.aspx

[5] Office of Cane and Sugar Board, "Report of ethanol production," Office of Cane and Sugar Board, Bangkok, Thailand, 2020.

[6] P. Saothong, B. Ninchan, K. Sriroth, K. Rattanaporn, and W. Vanichsriratana, "Kinetics of *Saccharomyces cerevisiae* fermentation under metal ions stress during ethanol production," *Walailak Journal of Science and Technology (WJST)*, vol. 18, no. 6, 2021, doi: 10.48048/wjst.2021.9133.

[7] Department of Alternative Energy Development and Efficiency, Ministry of Energy, "Ethanol factories," 2020. [Online]. Available: https://www.dede.go.th/more_news.php?cid=82&filename=index

[8] M. A. Clarke, "Syrups," in *Encyclopedia of Food Sciences and Nutrition*, 2nd ed. 2003, pp. 5711–5717.

[9] G. N. Abdel-Rahman, N. R. A. Nassar, Y. A. Heikal, M. A. M. Abou-Donia, M. M. Naguib, and M. Fadel, "Effect of different treatments on heavy metal concentration in sugar cane molasses," *International Journal of Agricultural and Biosystems Engineering*, vol. 10, no. 1, pp. 43–48, 2016, doi: 10.5281/zenodo.1125003.

[10] D. Teclua, G. Tivcheva, M. Laingb, and M. Wallis, "Determination of the elemental composition of molasses and its suitability as carbon source for growth of sulphate-reducing bacteria," *Journal of Hazardous Materials*, vol. 161, no. 2–3, pp. 1157–1165, 2009, doi: 10.1016/j.jhazmat.2008.04.120.

[11] Y. Yilmaz, I. Celik, and F. Isik, "Mineral composition and total phenolic content of pomegranate molasses," *Journal of Food, Agriculture & Environment*, vol. 5, no. 3–4, pp. 102–104, 2017, doi: 10.1234/4.2007.1048.

[12] K. Takeshige and K. Ouchi, "Factors affecting the ethanol productivity of yeast in molasses," *Journal of Fermentation and Bioengineering*, vol. 79, no. 5, pp. 449–452, 1995, doi: 10.1016/0922-338X(95)91260-C.

[13] S. A. Mohamed, A. A. A. Said, A. N. A. Zohri, H. A. Tawfek, and A.A. El-Samman, "Organic and inorganic constituent analysis of cane molasses and its affect on microbial fermentation industries," in *International Conference on: World Perspective for Sugar Crops as Food and Energy Supplies*, 2009, pp. 1–16.

[14] S. S. Munsamy, "Investigation into the high ash content in molasses at Nakambala, Zambia," in *the 86th Annual Congress of the South African Sugar Technologists' Association (SASTA 2013)*, 2013, pp. 67–73.

[15] S. Chotineeranat, R. Wansuksri, K. Piyachomkwan, P. Chatakanonda, P. Weerathaworn, and K. Sriroth, "Effect of calcium ions on ethanol production from molasses by *Saccharomyces cerevisiae*," *Sugar Tech*, vol.12, no. 2, pp. 120–124, 2010.

[16] H. Alexandre, V. Ansanay-Galeote, S. Dequin, and B. Blondin, "Global gene expression during short-term ethanol stress in *Saccharomyces cerevisiae*," *FEBS Letters*, vol. 498, no. 1, pp. 98–103, 2001, doi:10.1016/s0014-5793(01)02503-0.

[17] J. Bose, O. Babourina, and Z. Rengel, "Role of magnesium in alleviation of aluminium toxicity in plants," *Journal of Experimental Botany*, vol. 62, no. 7, pp. 2251–2264, 2011, doi: 10.1093/jxb/erq456.

[18] C. Sirisatesuwon, B. Ninchan, and K. Sriroth, "Effects of inhibitors on kinetic properties of invertase from *Saccharomyces cerevisiae*," *Sugar Tech*, vol. 22, no. 2, pp. 274–283, 2020, doi: 10.1007/s12355-019-00757-2.

[19] S. Kulshrestha, P. Tyagi, V. Sindhi, and K. S. Yadavilli, "Invertase and its applications – A brie review," *Journal of Pharmacy Research*, vol. 7, no. 9, pp. 792–797, 2013, doi:10.1016/j.jopr.2013.07.014.

[20] Vertical Chromatography Co. Ltd., "VertiSep™ sugar HPLC columns," 2013, [Online]. Available: http://www.vertichrom.com/pdf/hplc_sugar.pdf

[21] Bio-rad Laboratory, "Chromatograpgy: Aminex® HPLC Columns," 2012. [Online]. Available: https://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_6333.pdf

[22] H. O. Udeh and T. E. Kgatla, "Role of magnesium ions

on yeast performance during very high gravity fermentation," *Journal of Brewing and Distilling*, vol. 4, no. 2, pp. 19–45, 2013, doi:10.5897/JBD 2013.0041.

[23] G. M. Walker, "The roles of magnesium in biotechnology," *Critical Reviews in Biotechnology*, vol. 14, no. 4, pp. 311–354, 1994, doi: 10.3109/ 07388559409063643.

[24] G. M. Walker, R. D. Nicola, S. Anthony, and R. Learmonth, "Yeast-metal interactions: impact on brewing and distilling fermentations," presented at *Institute of Brewing & Distilling Asia Pacific Section 2006 Convention*, Hobart, Australia, Mar. 19–24, 2006.