Influence of Calcium Chloride as a Cofactor on SSF of Low-Grade Maize

P. Pinij and S. Chaiklangmuang

***Abstract*—** Ethanol fermentation feedstock based on low-grade maize waste for culture of *Saccharomyces cerevisiae* was presented by Simultaneous saccharification and fermentation (SSF) process. The calcium chloride concentrations (0-10 mM) were investigated both low-grade maize and pure corn flour samples in liquefaction step with α-amylase enzyme. After that, the calcium chloride concentrations were tested in SSF process for ethanol production. In addition, the gluco-amylase enzyme concentration (0-100 U/g) and fermentation time in the range of 0-7 days were taken in order to optimize SSF process. The calcium chloride was used as a cofactor for enzyme stabilizer. It was found that the calcium chloride had the function as a cofactor both liquefaction step and SSF process, the maximum ethanol concentration was achieved at 2.5 mM calcium chloride. The optimized gluco-amylase enzyme concentration was obtained by 75 U/g and fermentation time was presented at 4-5 days.

***Keywords*—** Calcium chloride, Cofactor, Ethanol, Maize, SSF.

I. INTRODUCTION

Nowadays, the energy is one important key for business and industries which is necessary to supply in suitable cost and enough quantity for consumer demand. Algae oil, fuel briquettes, RDF [1]-[3] are an alternative to commonly known biofuel sources. In addition the ethanol is attractive alternative fuel by virtue of low cost and renewable resource. Moreover, it was reduce particular emission from ignition engine. The starch is a high yield feedstock for ethanol production [4]. Inherent starches are pretreated to yield different end products of various industrial applications. However starch material has some limitation such has led to a shortage in food, imbalance in food chain, increased food price and indirect land use. Low-grade maize waste was selected in this work. Moreover, it's the major animal feed residue in the Thailand.

However, the traditional pretreatment methods such as acid hydrolysis and heating are terminated currently because degradation or carbohydrate loss and formation of inhibitory by-products. This problem has been to develop of effective pretreatment method such as enzyme hydrolysis [5], [6]. Enzymes are biological catalysts of biological process to the same any other chemical catalysts, it has many potential advantages in addition to yield, such as economic in term of cost, short hydrolysis time, low corrosion, low toxicity and especially specific of enzyme [7], [8]. In the enzymatic process to complete hydrolysis of highly concentrated raw starch are presented at least two type of enzyme for digesting raw starch granules. First, the starch was hydrolyze by α-amylase enzyme to break down α-1,4 glycosidic bonds into maltose, dextrin and less amounts of glucose in liquefaction step and secondary to present gluco-amylase enzyme to break down α-1,4 and 1,6 glycosidic bonds into glucose in saccharification step [4], [9].

The structural stability of α-amylases depends on various intrinsic (e.g., amino acid sequence) and extrinsic (e.g., solution conditions, such as pH, presence of cofactors, metal ions, etc.) factors [10]. Approximately 70% of all enzymes require cofactors [11]. The cofactor does not change at the end of the reaction and may be considered as an important part of catalyst mechanism. Cofactors distributed into two classes: (a) specific coenzyme, which brings reaction itself of some chemical grouping in organic molecules of somewhat complex structure, and (b) activators, which in many ways carry the enzyme itself into a catalytically active state. When enzyme inactive affected present absence of salt, others are importantly influenced by the nature and concentration of the ions present. Certain ions are absolutely essential for the activity of some enzyme, while others (e.g. Ag^{+}, Hg^{+}, Pb^{++}) are very toxic to nearly all enzyme. Some ions are poisons for some enzyme activators for other; some may even inhibit an enzyme at one concentration and be activators of the same enzyme at another. Some of the effects, particularly those of anions, are fairly unspecific; the enzyme is usually active to some extent without electrolytes, and almost any anion will have an effect. In other cases, particularly with cations, the enzyme is inactive by itself and the requirement for a cation is usually fairly specific; in some cases only particular cation is effective, in other cases two or three different cations can act. The fact that as far as our present knowledge goes the cation effects are much more specific than the anion effects may be partly due to the way in which investigation has been carried out. The affects, like reactions between ions and proteins in general, may be extremely complex, particularly so since, in addition to the purely colloid effects, there may be specific requirements by the active center of an enzyme for particular type of ion. Metal cations have been found to activate one or more enzyme namely Na^{+}, K^{+}, Rb^{+}, Cs^{+}, Mg^{++}, Ca^{++}, Zn^{++}, Cd^{++}, Cr^{+++}, Cu^{++}, Mn^{++}, Fe^{++}, Co^{++}, Ni^{++}, Al^{+++} [12].

Currently, the Simultaneous saccharification and fermentation (SSF) process are worldwide. The SSF process

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was considered as superior to the traditional saccharification and subsequent fermentation in the production of ethanol because of increased the ethanol yields and lower enzyme requirement [13]. In addition, the SSF process can use single reactor and the same temperature for saccharification and fermentation process as a result the reduction of operation cost can be achieved. The complete process time was reduced and the energy for saccharification was saved [14]-[17].

The calcium chloride was used as a cofactor in this study because inexpensive and easy to operate. Effect of calcium chloride was presented on ethanol production from low-grade maize by SSF process.

II. Materials And Methods

2.1Materials and Chemicals

Low-grade maize waste was obtained from the maize-animal feed industry in Thailand. The low-grade maize was mashed in a hammer mill to a particle size of ≤ 2 mm and dried in oven at 80 °C for 24 h. The content of the main components in low-grade maize was determined according to the standard ASTM D3172-89 methods and presented in Table 1. The pure corn flour (PCF) was obtained from McGarrett: Thai Soon Foods Co., Ltd., Thailand and used as a controller. Calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$) of analytical reagent grade was purchased from VWR lab products Pvt. Ltd. The α-amylase and gluco-amylase enzymes produced from *Bacillus licheniformis* of commercial grade were used for starch hydrolysis.

TABLE I
MAIN CHEMICAL COMPOSITION OF LOW-GRADE MAIZE

Composition	Content (% w/w)
Moisture	2.97
Ash	2.27
Volatile matter	80.47
Fixed carbon	14.29

2.2Microorganisms

Fermentation was performed by *Saccharomyces cerevisiae* TISTR 5339 for the inoculum preparation, the 0.3 % v/v *S. cerevisiae* was cultivated in 50 mL of growth medium containing 10 g/L of glucose, 3 g/L of yeast extract, 3 g/L of malt extract and 5g/L of peptone in a 250 ml Erlenmeyer flask. The culture was incubated at 30 °C, 150 rpm for 12 h. Before starting the experiment, the culture medium was sterilized in an autoclave at 121 °C and 1 atm for 15 min.

2.3 Liquefaction

Liquefaction step was carried out in 250 ml media bottles, which contained 25 g low-grade maize (LM) and 100 ml distilled water was sterilized in an autoclave at 121 °C and 1 atm for 15 min. The α-amylase enzyme was added in liquefaction step with an activity of 72 U/g of low-grade maize. The calcium chloride concentration was varied at 0-10 mM. Then the low-grade maize slurry (LMS) was hydrolyzed at 75 °C, 150 rpm for 5 h. in water bath. The similar liquefaction process of PCF was performed. The reducing sugar (RS) content was determined of LMS and pure corn flour slurry (PCFS).

2.4 SSF process

Subsequently, LMS and PCFS were subjected to ethanol fermentation with *Saccharomyces cerevisiae* under anaerobic condition by SSF process. The concentration of gluco-amylase enzyme was varied at 0-100 U/g of LM. Culture media included 0.1% w/v peptone, 0.05 % w/v $MgSO_4$ and 0.3 % v/v *Saccharomyces cerevisiae* was simultaneously added into LMS and PCFS. The fermentation time was 0-7 days and the incubating temperature was 30 °C. After that the RS and ethanol contents in SSF slurries were analyzed.

2.5Analytical methods

The SSF slurry was separated to gain the solid fraction and hydrolysate fraction by filtration. The hydrolysate fraction was determined the RS and ethanol contents by using the DNS (3, 5-dinitro salicylic acid) method, which measured by UV-vis spectrophotometer (hp HEWLETT PACKARD, model 8453) [18] and gas chromatography, Agilent 6890 with FID detector, a capillary column (HP-FFAP HP19091F-112 at 250 °C and flow rate of 2.5mL/min), respectively. All hydrolysate samples were extracted with MIBK for GC technique. The pH of the hydrolysate fraction was measured using pH-meter (ID1000 model). In addition, the data results were subjected to One Way ANOVA using statistical package for social science (SPSS) computer program to find out the significance at $p < 0.05$.

III. Result And Discussion

3.1Effect of calcium chloride to reducing sugar in liquefaction step

The pH and RS contents were measured in LMS and PCFS using calcium chloride concentration of 0-10 mM as a cofactor in liquefaction step as shown in Table 2. From experimental results, the calcium chloride slightly affected on pH value in LMS and PCFS. Nevertheless, LMS got pH value lower than PCFS, this may be due to the fact that in LMS has phenolic acid compound which is more abundant components than PCFS. The phenolic acid compound is a functional group of carboxylic acid that can dissolve water and dissociate to H^+ [19], [20]. The pH optimum of enzyme activity was 6.0-6.5 [21]. Therefore, the enzyme activity in PCFS was activated well than in LMS. As a result, PCFS had RS content more than LMS.

The RS content in PCFS wasn't statistically significant difference using calcium chloride in concentration of 0-10 mM. However, the RS content in LMS was statistically significant difference, the highest RS content was achieved at 5 mM of calcium chloride concentration. At high concentration of calcium chloride led to decrease RS content.

The high concentration of calcium chloride may inhibit α-amylase activity. Inherent of α-amylase terminal chain existing negatively charged, so if in system has over Ca^{2+} concentration, it will bring to capture the activity site of enzyme resulting to decrease enzyme activity. On the other hand, if using low concentration of calcium chloride, it will bring to decrease enzyme activity [22].

Therefore, we were interested in studying the calcium chloride concentration at 1, 2.5 and 5 mM to compare with non-calcium chloride in SSF process toward assumption that the calcium chloride may be have the effect of hydrolysis with gluco-amylase enzyme.

TABLE II
AMOUNT OF REDUCING SUGAR IN LIQUEFACTION STEP FROM LOW-GRADE MAIZE

Concentration of $CaCl_2$ (mM)	LMS		PCFS	
	RS (mg as maltose/L)	pH	RS (mg as maltose/L)	pH
0.0	89,049.24^{b}	5.1	112,310.61^{a}	6.8
1.0	92,707.58$^{a, b}$	5.1	108,226.89^{a}	6.3
2.5	95,431.82^{a,b}	5.0	106,932.76^{a}	6.1
5.0	96,740.15^{a}	5.0	111,681.82^{a}	6.0
7.5	96,703.03^{a}	4.9	113,920.83^{a}	6.0
10.0	90,485.61$^{a, b}$	4.9	106,457.95^{a}	6.0

3.2Effect of calcium chloride to SSF process

After SSF process, RS and ethanol contents were investigated following Fig 1. The experiments showed that the ethanol production depended on the calcium chloride concentration (0, 1, 2.5, 5 mM) with the fermentation time during 0-7 day and using gluco-amylase concentration at 75 U/g. The ethanol concentration increased with increasing the fermentation time from 1 to 5 days according with reducing the RS content. After that the ethanol decreased with further increase in fermentation time because the ethanol can act as an oxidant to stress-mediated cell death read to decreasing elevated ethanol levels [23]-[25]. The maximum ethanol concentration was achieved for 2.5 mM during 4-5 days of fermentation time due to a shift from yeast growth. The ethanol concentration in LMS and PCFS were 3.61±0.24% w/v for 5 days and 3.22±0.16% w/v for 4 days, respectively. The ethanol concentration of LMS was higher than PCFS because the LMS contained minerals supporting yeast to grow. In view of non-using calcium chloride in LMS and PCFS, it occurred the low ethanol concentration. Thereby, the calcium chloride can be a cofactor function in SSF process. Consequently, the calcium chloride concentration at 2.5 mM was confirmatory selected because of the highest ethanol concentration and using low calcium concentration to study the gluco-amylase effect in 3.3.

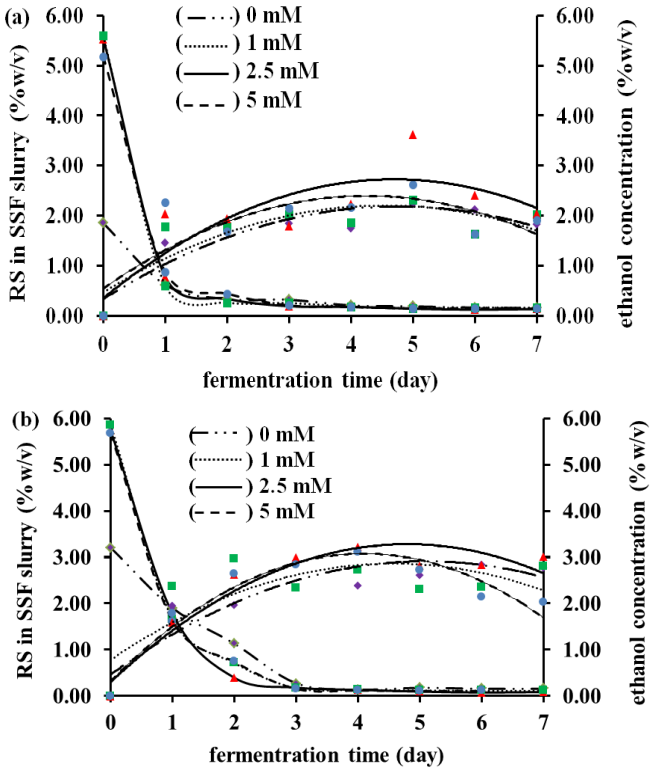

Fig.1 Ethanol concentration and RS in SSF slurry profiles: LMS (a) and PCFS (b)

3.3Effect of gluco-amylase concentration on ethanol production with 2.5 mM calcium chloride

The calcium chloride concentration at 2.5 mM was selected for testing in ethanol fermentation system (SSF process) in fermentation time of 4-5 days, which varied gluco-amylase enzyme (0, 50, 75, 100 U/g) as indicated in Fig 2. The gluco-amylase enzyme hydrolyzed disaccharide into glucose and ethanol which continuously process as a parallel working between gluco-amylase enzyme and yeast. Rasmus et al. [26] reported that the higher gluco-amylase concentration increased the final ethanol concentration. The concentration of ethanol is slightly difference when using gluco-amylase concentration at 50, 75 and 100 U/g. The gluco-amylase concentration at 75 U/g was found to be maximum for ethanol production in LMS and PCFS, which were 3.43±0.21% w/v and 3.70±0.14% w/v, respectively.

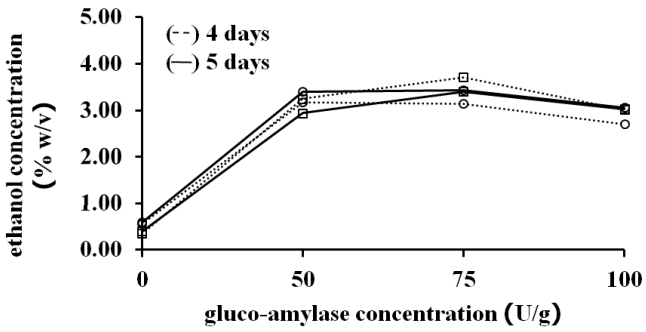

Fig.2 Ethanol concentration with gluco-amylase enzyme concentration profiles: LMS (○) and PCFS (□)

IV. Conclusions

In this work, the influence of calcium chloride as a cofactor was performed on liquefaction step and SSF process using LM and PCF samples. The optimal concentration of calcium chloride was achieved at 2.5 mM in LMS and PCFS with the fermentation time in rang of 4-5 days. The calcium chloride has good function as a cofactor in liquefaction step and SSF process. Using calcium chloride for testing in ethanol fermentation system on gluco-amylase effect, it was found that the optimal concentration of gluco-amylase at 75 U/g provided the maximum ethanol production.

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Reference

[1] S. Boonma, et at., Enhanced Carbon Dioxide Fixation and Bio-Oil Production of a Microalgal Consortium, Clean Soil Air Water. 43 (2015) 761-766. https://doi.org/10.1002/clen.201400171

[2] S. Chaiklangmuang, et at., Development of Fuel Briquettes from Biomass-Lignite Blends, Chiang Mai J. Science. 35 (2008) 43-50.

[3] U. Auprakul, et al., Densified Fuels from Mixed Plastic Wastes and Corn Stover, Advanced Materials Research. 931-932 (2014) 1117-1121. https://doi.org/10.4028/www.scientific.net/AMR.931-932.1117

[4] F. O. Ajibola, M. O. Edema, O. B. Oyewole, Enzymatic production of ethanol from cassava starch using two strains of saccharomyces cerevisiae, Nigerian Food Journal. 30 (2012) 114-121. https://doi.org/10.1016/S0189-7241(15)30044-8

[5] M. W. Ullah, W. A. Khattak, M. Ul-Islam, S. Khan, J. K. Park, Bio-ethanol production through simultaneous saccharification andfermentation using an encapsulated reconstituted cell-freeenzyme system, Biochemical Engineering Journal. 91 (2014) 110-119. https://doi.org/10.1016/j.bej.2014.08.006

[6] G.P. Philippidis, T.K. Smith, C.E. Wyman, Study of the enzymatic hydrolysis ofcellulose for production of fuel ethanol by the simultaneous saccharificationand fermentation process, Biotechnology and Bioengineering. 41 (1993) 846–853 https://doi.org/10.1002/bit.260410903.

[7] M.J. Taherzadeh, K. Karimi, Enzyme-based hydrolysis processes for ethanolfrom lignocellulosic materials: a review, Bioresources 2 (2007) 707–738.

[8] A. Wiseman, Enzyme Biotechnology. Cornwall: 1995.

[9] Y.H. Huang, Y.L. Jin, Y. Fang, Y.H. Li, G.H. Zhang, Y. Xiao, Q. Chen, H. Zhao, Simultaneous saccharification and fermentation (SSF) of nonstarch polysaccharides and starch from fresh tuber of Canna edulis ker at a high solid content for ethanol production, Biomass & Bioenergy 52 (20013) 8-14.

[10] G. Rabbani, E. Ahmad, N. Zaidi, R.H. Khan, Cell Biochemistry and Biophysics 61 (2011) 551–560. https://doi.org/10.1007/s12013-011-9237-x

[11] H. W. Doelle, S. Rokem, M. Berovic, Biotechnology Industrial Biotechnology-Part III. UK: 2009.

[12] W. Gerhartz, Enzymes in Industry Production and Application. Germany: 1990.

[13] D. Dahnum, S. O. Tasum, E. Triwahyuni, M. Nurdin , H. Abimanyu, Comparison of SHF and SSF processes using enzyme and dry yeast for optimization of bioethanol production from empty fruit bunch, Energy Procedia 68 (2015) 107-116. https://doi.org/10.1016/j.egypro.2015.03.238

[14] A. Wingren, M. Galbe, C. Roslander, A. Rudolf, G. Zacchi, Effect of reduction in yeast and enzyme concentrations in a simultaneous-saccharification-and-fermentation-based ethanol process, Applied Biochemistry and Biotechnology 121-124 (2005) 485–499. https://doi.org/10.1385/ABAB:122:1-3:0485

[15] M. V. Sivers, G. Zacchi, Ethanol from lignocellulosic: a review of the economy, Bioresource Technology. 56 (1996) 131–140. https://doi.org/10.1016/0960-8524(96)00018-1

[16] A. Wingren, M. Galbe, G. Zacchi, Techno-economic evaluation of producing ethanol from softwood: comparison of SSF and SHF and identification of bottlenecks, Biotechnology Progress 19 (2003) 1109–1117. https://doi.org/10.1021/bp0340180

[17] M. Linde, E. Jakobsson, M. Galbe, G. Zacchi, Steam pretreatment of dilute H2SO4-impregnated wheat straw and SSF with low yeast and enzyme loadings for bioethanol production, Biomass and bioenergy 32 (2008) 326-332. https://doi.org/10.1016/j.biombioe.2007.09.013

[18] G.L Miller, Use of dinitrosalicylic acid reagent for determination of reducing sugar, Analytical Chemistry. 31 (1959) 426–428. https://doi.org/10.1021/ac60153a026 https://doi.org/10.1021/ac60154a035 https://doi.org/10.1021/ac60147a030 https://doi.org/10.1021/ac60147a607 https://doi.org/10.1021/ac60146a607 https://doi.org/10.1021/ac60149a611 https://doi.org/10.1021/ac60155a008 https://doi.org/10.1021/ac60155a028

[19] Pandey, R., Singh, A., Maurya, S., Singh, U.P., Singh, M., "Phenolic acids in different preparations of Maize (Zea mays) and their role in human health," International Journal of Current Microbiology and Applied Sciences, Vol. 2, June 2013, pp. 84-92.

[20] Mattila, P., Pihlava, J., Hellstrom, J., "Contents of Phenolic Acids, Alkyl- and Alkenylresorcinols, and Avenanthramides in Commercial Grain Products," Journal of Agricultural and Food Chemistry, Vol. 53, September 2005, pp. 8290-8295. https://doi.org/10.1021/jf051437z

[21] Ivanova, V.N., Dobreva, E.P., Emanuilova, E.I., "Purification and characterization of a thermostable alpha-amylase from Bacillus licheniformis," Journal of Biotechnology, Vol. 28, April 1993, pp. 277-289 https://doi.org/10.1016/0168-1656(93)90176-N.

[22] Yadav, J.K., "A differential behavior of α-amylase, in terms of catalytic activity and thermal stability, in response to higher concentration CaCl2," International Journal of Biological Macromolecules, Vol. 89, April 2008, pp. 1056-1059.

[23] Dijken, J., Weusthuis, R., Pronk, J., "Kinetics of growth and sugar consumption in yeasts," Antonie van Leeuwenhoe, Vol. 63, 1993, pp. 343-352. https://doi.org/10.1007/BF00871229

[24] Tahir, A., Aftab, M., "Effect of Cultural Conditions on Ethanol Production by Locally Isolated Saccharomyces Cerevisiae BIO-07," Journal of Applied Pharmacy, Vol. 3, 2010, pp. 72-78.

[25] Kitagaki, H., Araki, Y., Funato, K., "Ethanol-induced death in yeast exhibits features of apoptosis mediated by mitochondrial fission pathway," Federation of European Biochemical Societies Vol. 581, 2007, pp. 2935-2942. https://doi.org/10.1016/j.febslet.2007.05.048

[26] Devantieri, R., Pedersen, S., Olsson, L., "Characterization of very high gravity ethanol fermentation of corn mash. Effect of glucoamylase dosage, pre-saccharifacation and yeast strain," Appl Microbiol Biotechnol Vol. 68, 2005, pp. 622-629. https://doi.org/10.1007/s00253-005-1902-9