Waste Cassava Tuber Fibers as an Immobilization Carrier of *Saccharomyces cerevisiae* for Ethanol Production

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Waste cassava tuber fibers (wCTF), derived from the ethanolic fermentation of cassava tubers, have potential use as anatural adsorption immobilization carrier. Ethanol fermentation was conducted using 15% (w/v) glucose-containing mediumat 40 °C for 48 h by *Saccharomyces cerevisiae* G6-2-2 (1.3 x 10^{10}cells). Ethanol concentration produced by free, wCTF (1.2 g dry weight) adsorbed, wCTFadsorbed-calcium alginate entrapped,and calcium alginate entrapped cellswere 42.10 ± 0.61, 67.35 ± 0.53, 52.10 ± 0.40, and 46.45 ± 0.18 g/L (0.34, 0.45, 0.35, and 0.31 g ethanol/g reducing sugar), respectively. The wCTF adsorbed cells produced a maximum ethanol yield of 82.15 ± 0.48 g/L (0.43 g ethanol/g total sugar) from molasses (20% w/v initial total sugar) after 48 h, compared to 74 g/L to 76 g/L and 48 h to 100 h for the free suspension cells. The increase in ethanol produced by the wCTF adsorbed cells compared to free cells reflected that the cells were protected from environmental stresses and received amino nitrogen from the wCTF that supported growth and ethanol tolerance.

Keywords: Waste cassava tuber fiber; Natural immobilization carrier;Immobilization Ethanol

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INTRODUCTION

The key fundamental raw materials used for ethanol production in Thailand are molasses and cassava tuber (tapioca root) (Department of Alternative Energy Development and Efficiency Thailand 2015). The advantage of molasses over cassava tuber is that the former can be fermented into ethanol without prior saccharification. Although molasses is a low-priced fermentable sugar resource, it has high demand inseveral industries that may result in a shortage of supply (Balat and Balat 2009). If there is a molasses shortage, the ethanol industry would be required to shift to using cassava tubers as an alternative substrate (Sriroth *et al*. 2010). After cassava tubers are fermented, the liquid contains insoluble cassava tuber fibers (CTF) as the waste product. Waste CTF (wCTF) is removed from the fermentation liquid before ethanol distillation and is then disposed by landfilling.

Ethanol is produced from agricultural resources by saccharification of the polysaccharides, followed by fermentation with microorganisms, especially yeast, in the

form of free cells (suspension) or immobilized cells (Behera *et al*. 2010; Yan *et al*. 2012; Bouallagui *et al*. 2013). Ethanol fermentation with immobilized cells is typically better (as in a higher ethanol yield and productivity) than that of free cells because the immobilized cells are protected from environmental stresses, such as acidity, osmosis, CO_2, and degenerative substances produced from fermentation, including furfural and acetic acid (Ingledew 1999; Bai *et al*. 2008; Tesfaw and Assefa 2014). In addition, high cell density are loaded. This results in an increase of ethanol tolerance, ethanol production and decrease in ethanol production time (Tian *et al.* 2015).

Popular methods of immobilizing cells for ethanol fermentation include calcium alginate entrapment and natural adsorption because the techniques are simple and conduct under mild condition (Razmovoski and Vučurović 2011; Zheng *et al*. 2012; Tian and Chen 2016). When using entrapment, yeast cells inoculated are trapped within a gel matrix and they are maintained throughout fermentation; conversely, the cells trapped inside the calcium alginate beads may suffer from a mass transfer limitation of nutrients and O_2, as well as the removal of the CO_2 and ethanol produced (Phisalaphong *et al*. 2007; Yu *et al*. 2007; Zhu 2007). In natural adsorption, cells are naturally adsorbed on a carrier by physical or chemical adsorption, vander Waals force, ionic force, or electrostatic attraction between negative charge of cell wall and positive charge of carrier (Verica*et al.* 2010; Genisheva *et al.* 2014). The growth of yeast cells is not notably affected, and the new active yeast cells can be adsorbed by the carrier when older dead cells are washed off (Iqbal and Saeed 2005; Bai *et al*. 2008). On the contrary, there is an inability to maintain a high number of immobilized cells from the beginning to the end of the fermentation process (Phisalaphong *et al*. 2007; Yu *et al*. 2007; Geisheva *et al*. 2011).A combination of alginate entrapment and natural adsorption caused the structure and mass transfer of alginate gel bead to change, which increased the ethanol yield. As yet, only limited studies have reported on the combination of entrapment and adsorption immobilization (Phisalaphong *et al*. 2007; Razmovski and Vučurović 2011; Alting and Zhaoping 2015; Tian *et al.* 2015). The objective of this research was to evaluate the efficiency of wCTF as an immobilization carrier of *Saccharomyces cerevisiae*through wCTF natural adsorption, and combination of the natural adsorption and calcium alginate entrapment for the improvement of ethanol production.

EXPERIMENTAL

Materials

The wCTF used in this study was collected from the Saptip Co. Ltd.,Ethanol Fuel Production Plant, located in Lophburi province, Thailand, and was kept at -20 °C. The wCTF was thawed at room temperature prior to use. Molasses was obtained from the Angvian Industry Co. Ltd. in Nakhon Ratchasima province, Thailand and kept at 4 °C until use. The molasses was diluted to 20% (w/v) total sugar and clarified by centrifugation at 4 °C and 8000 rpm for 5 min with harvesting of the supernatant for the preparation of the molasses medium.

The total sugar content was analyzed by the phenol sulfuric acid method (Dubois *et al*. 1956). *Saccharomyces cerevisiae* G6-2-2 was isolated from Khonburi sugar PLC, Nakhon Ratchasima province, Thailand, at 40 °C. The maximum ethanol production level was 59.81 g/L (0.40 g ethanol/g glucose) from a 15% (w/v) glucose containing medium at 40 °C for 48 h.

Methods

Preparation of the wCTF for immobilization

The thawed wCTF was washed with running water for 30 min and then dried at 80 °C to a constant weight. The dried wCTF was sieved to screen for 450 µm to 850 µm sized particles and sterilized by autoclaving at 121 °C and100 kPa for 15 min.

Determination of water absorption index (WAI) of the wCTF

The WAI (grams of water absorbed per gram of wCTF dry weight) indicated the quantity of water absorption by wCTF, and was determined according to Anderson *et al*. (1969). The dried wCTF (1.25 g) was suspended in 15 mL of distilled water, stirred for 10 min, and centrifuged (4 °C, 8,000 rpm, and 10 min). The wCTF pellet was harvested, weighed, and the WAI of the wCTF was calculated as the grams of wet weight/grams of dry weight.

Preparation of S. cerevisiae inoculum

A single colony of *S. cerevisiae* G6-2-2 was inoculated in 50 mL of fermentation medium (15% (w/v) glucose, 0.9% (w/v) peptone, 0.6% (w/v) yeast extract, pH 5.0) in a 250 mL flask and incubated at 40 °C with shaking at 200 rpm for 24 h. The obtained culture was inoculated into a new fermentation medium at an initial optical density (OD) at 660 nm of 0.05 and incubated at the same condition until the late log phase. The culture was centrifuged (4 °C, 8000 rpm, and 5 min) to precipitate the cells, which were then suspended in 5 mL of 0.9% (w/v) NaCl.Cellnumber in the cell suspension was counted under light microscope using a haemocytometer and used as inoculum.The inoculum was $1.37x10^{10}$ cells/5mL.

Preparation of wCTF adsorbed S. cerevisiae for cell immobilization

The *S. cerevisiae* G6-2-2 inoculumwas mixed with 1.2 g wCTF (dry weight), and maintained at room temperature for 30 min. After filtration, non-immobilized cells in filtrate were determined by hemocytometer (Table 1).

No. cells immobilized = No. cells inoculated – No. non-immobilized cells (or cells in filtrate)

Subsequently, the number of yeast cells inoculated (1×10^9 to 3.93×10^{10}cells/5 mL) and the amount of wCTF loaded (0.6 g to 2.4 g) were independently and sequentially varied to investigate the optimal dose for ethanol production.

Preparation of calcium alginate entrapped S. cerevisiae and wCTF adsorbed-calcium alginate entrapped S. cerevisiae

The *S. cerevisiae* G6-2-2 inoculum was mixed with 5 mL of 0.9% (w/v) NaCl or 5 mL of a wCTF suspension (1.2 g wCTF (dry weight) in 0.9% (w/v) NaCl) and kept at room temperature for 30 min. Then, it was further mixed with 10 mL of 2.0% (w/v) sodium alginate (pH 7.0) and dispensed at 86 ± 3 µL/ drop (cell-sodium alginate mixture) or 116 ± 4 µL/drop (cell-wCTF-sodium alginate mixture) into 250 mL of 100 mM $CaCl_2$ with gentle stirring at room temperature using a 10-mL glass dropper. The resultant calcium alginate beads were left to harden for 15 min, harvested by filtration, and washed three times with 0.9% (w/v) NaCl (200 mL). All steps were performed aseptically.

The yeast cell number in the filtrate and in the washing NaCl solution were determined under light microscopy using a hemocytometer, and the derived sum of the yeast cell number in the filtrate and washing NaCl solution was defined as the non-immobilized cell number. The number of cell immobilized was calculated as above.

Ethanol production

The *S. cerevisiae* G6-2-2 suspension and immobilized cells were inoculated into 50 mL of molasses medium (20% (w/v)total sugar, 1.3% (w/v) $(NH_4)_2SO_4$, 0.27% (w/v) KH_2PO_4, 0.05% (w/v) $MgSO_4.7H_2O$, pH 5.0) in a 100-mL Duran bottle and incubated at 40 °C in an oxygen limited condition with 100 rpm agitation. The oxygen limited condition was performed by tightly closing the screw cap of the Duran bottle. Supernatant obtained after centrifugation (4° C, 8000 rpm, 10 min) was analyzed forethanol by gas chromatography(Hewlett-Packard, HP5890 series, USA) with a flame ionization detector at 150 °C using a Porapak QS (cabowax 20 M) column (2 m x 0.32 m) at an oven temperature of 175 °C. Helium at a flow rate of 35 mL/min was used as the carrier gas (Jutakanoke *et al*. 2012).

Comparison of the different immobilization systems on the ethanol production level

The free or immobilized *S. cerevisiae* G6-2-2 cells (wCTF-adsorbed, wCTF adsorbed-calcium alginate entrapped, and calcium alginate entrapped), at a final dose of 1.3 x 10^{10} cells, were inoculated into the fermentation medium and fermented to ethanol. Likewise, thefree and wCTF adsorbed cells were inoculated into molasses medium and fermented to ethanol.

Characterization of the wCFT

The wCTF was prepared by soaking in 3.5% glutaraldehyde for 1 h, and dried by sequentially soaking in 30, 50, 70, 95, and 100% ethanol using a critical point dryer (Polaron Range CPD7501, England). The dried wCTF was treated with gold and characterized by scanning electron microscope (SEM; JEOL JSM-5410-LV, Japan)

Statistical analysis

The data were analyzed using an analysis of variance (ANOVA) and significance was accepted at $P< 0.05$. All statistical analyses were performed using the SPSS 17.0 software(SPSS Inc., USA).

Table 1. Number of Immobilized *S. cerevisiae* Cells

Carrier	Number of cells inoculated (× 10^{10})	Number of immobilized cells (× 10^{10})	Number of non-immobilized cells (× 10^{8})
wCTF	1.37 ± 1.27	1.31 ± 2.27	5.54 ± 2.27
wCTF-Ca-alginate	1.37 ± 1.27	1.33 ± 0.70	3.55 ± 0.70
Ca-alginate	1.37 ± 1.27	1.34 ± 0. 33	2.90 ± 0.33

wCTF: waste cassava tuber fibers 1.2 g dry weight of wCTFs were used
Data are shown as the mean ± standard deviation, derived from 3 independent replicates

RESULTS AND DISCUSSION

The WAI of wCTF

The wCTF had a WAI value of 5.93 ± 0.22 g water/g wCTF (dry weight), which indicated that it was likely to be suitable for use as a cell immobilization carrier (Orzua *et al*. 2009; Razmovski and Vučurović 2011). In comparison, the WAI value of wCTF was higher than that previously reported for pecan nut shell, pistachio shell, wheat bran, apple pomace, bean residue, creosote bush leaves (Orzua*et al*. 2009), sotol fiber, corn cobs, and candelilla stalks (Flores-Maltos *et al*. 2014), which have all been reported as agro-industrial wastes that are suitable for use as cell immobilizationcarriers.

Optimal Yeast Cell Number and wCTF Loading Level for Natural Adsorption

Different quantities of *S. cerevisiae* cells (1.0 x 10^9, 6.1 x 10^9, 1.31 x 10^{10}, 2.65 x 10^{10}, and 3.93 x 10^{10}) were immobilized in 1.2 g (dry weight) of wCTF. The wCTF adsorbed cells were inoculated into 50 mL of fermentation medium and the ethanol production level was assayed. The inoculum at 1.31 x 10^{10} cells yielded the highest ethanol production (67.35 ± 0.4 g/L) after 48 h (Fig. 1a). However, when the wCTF loading was decreased to 0.6 g (dry weight) or increased to 2.4 g (dry weight) the ethanol production level decreased (Fig. 1b). This result agree well with Tian *et al.* (2015).Therefore, 1.31 x 10^{10} of*S. cerevisiae* G6-2-2 cells were immobilized in 1.2 g wCTF forfuture experiments.

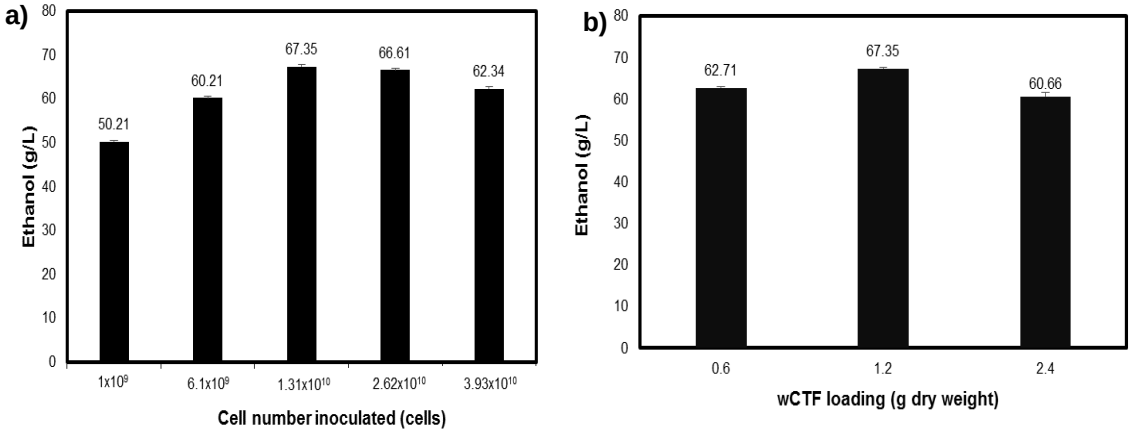

Fig. 1. Effect of the a) immobilized *S. cerevisiae* cells (1.2 g of wCTF), and b) wCTF loading level (1.31 x 10^{10} *S. cerevisiae* G6-2-2 cells) for ethanol production in a 15% (w/v) glucose containing medium at 40 °C for 48 h. Data represent the mean ± standard deviation (0.9), derived from three independent replicates

Comparison of the Immobilization System on the Ethanol Production Level

The free and immobilized *S. cerevisiae* G6-2-2 cells (1.31 x 10^{10}) were investigated for efficient ethanol production. The wCTF-adsorbed cells produced the highest ethanol level (67.35 ± 0.4 g/L) after 48 h. This result was in agreement with Singh *et al*. (2013), who reported that the highest ethanol level was produced by cells immobilized on pretreated sugarcane bagasse compared with calcium alginate entrapped cells or free cells. Yeast cells immobilized by the adsorption technique were free to contact the fermentation medium; therefore, their growth was not effected by mass transfer limitations. Meanwhile, the washed out (dead) cells could be replaced by new

cells (Braschler *et al*. 2005; Bai *et al*. 2008). The porous structure of wCTF increased the mass transfer of wCTF adsorbed-calcium alginate beads, which improved the growth of wCTF adsorbed-calcium alginate entrapped cells. This is because cell biomass and ethanol are co-produced by the ethanolic fermentation-based metabolism of *S. cerevisiae*. Therefore, the wCTF adsorbed-calcium alginate entrapped cells yielded a slightly higher quantity of ethanol than the calcium alginate entrapped cells (Fig. 2).

However, other co-products, such as CO_2 and other degenerative products,created a higher stress level on wCTF adsorbed-calcium alginate cells than thecells adsorbed onto wCTF. Nevertheless, the mass transfer limitationwould likely be the main drawback of the alginate entrapped cells (Kumakura *et al*. 1992; Groboillot *et al*. 1994; Iqbal and Saeed 2005). Several high porosity materials have been applied to overcome this drawback (Yu *et al*. 2010). In addition, several studies have reported that a combination of entrapment and adsorption/immobilization agents, such as alginate and loofa sponge (Phisalaphong *et al*. 2007), alginate and maize stem ground tissue (Razmovski and Vučurović 2011), alginate and delignified sawdust (Alting and Zhaoping 2015) and alginate and pretreated corn stalk (Tian *et al.* 2015) can enhance the gel strength and the mass transfer inside the gel carrier.

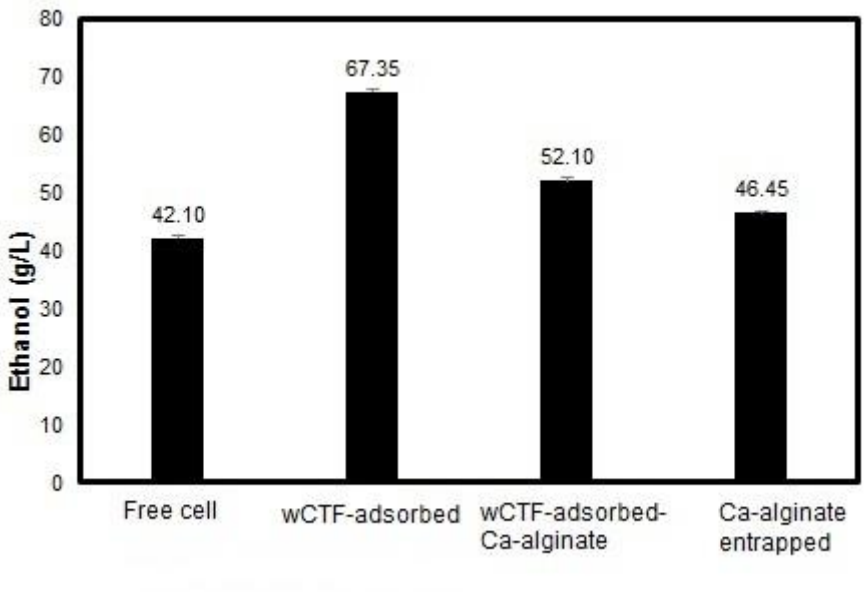

Fig. 2. Effect of the immobilization method on the ethanol production. Free (suspension) *S. cerevisiae* G6-2-2 cells (1.31 x 10^{10}) or those immobilized onto 1.2 g (dry weight) of wCTF and calcium alginate entrapment were employed for the fermentation of 15% (w/v) glucose-containing medium at 40 °C for 48 h. Data represent the mean ± standard deviation (0.6), derived from 3 independent replicates

Comparison of Ethanol Production from Molasses by Free and wCTF-Adsorbed Cells

As shown in Fig. 3, wCTF-adsorbed yeast cells produced a maximum ethanol concentration after 48 h, which was 10.95% higher than that of free cells. The SEM images of wCTF before fermentation showed a rough surface and porous structure (Fig. 4a), including the presence of attached residual yeast cells from the prior commercial fermentation of cassava tubers to ethanol (Fig.4b). After fermentation, the number of yeast cells attached to the porous structure of wCTF increased (Fig. 4c). Analysis of the

molasses medium and that containing 5% (w/v) wCTF showed that the molasses medium containing wCTF had a markedly higher content of several amino acids, including an over two-fold higher level of glutamic acid, histidine, lysine, serine, and tyrosine (Table 2). Natural carriers have been recognized as low cost materials with lower mass transfer limitations than entrapment carriers, while some agricultural-based immobilization carriers also provide extra nutrients. For example, maize-stem ground tissue not only protected cells from different stresses during ethanol fermentation, but also provided extra nutrients, such as protein and soluble salt, which promoted ethanol production from molasses by *S. cerevisiae* (Razmovski and Vučurović 2011).

The use of brewer's spent grains as a natural carrier in molasses-based ethanol production provided extra nutrients to the system and resulted in an increased ethanol yield (Kopsahelis *et al*. 2007). Albers *et al*. (1996) reported that the growth rate and ethanol yield of *S. cerevisiae* in fermentation medium,containing an amino acids mixture as the nitrogen source,was higher than that of ammonium salt medium. In addition, the thermo-tolerance of yeast was enhanced by the supplementation of soybean flour (Balakumar and Arasaratnam 2012).

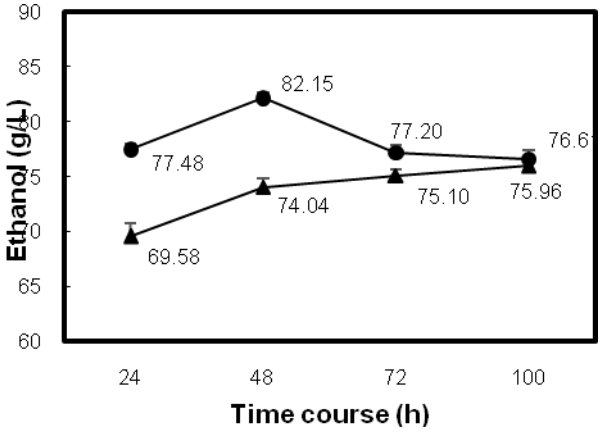

Fig. 3. Ethanol production by *S. cerevisiae* G6-2-2 from molasses medium (20% (w/v) sugar) as (▲) free cells or (●) wCTF-adsorbed cells. Fermentation wasat 40 °C for 48 h. Data represent the mean ± standard deviation (1), derived from 3 independent replicates

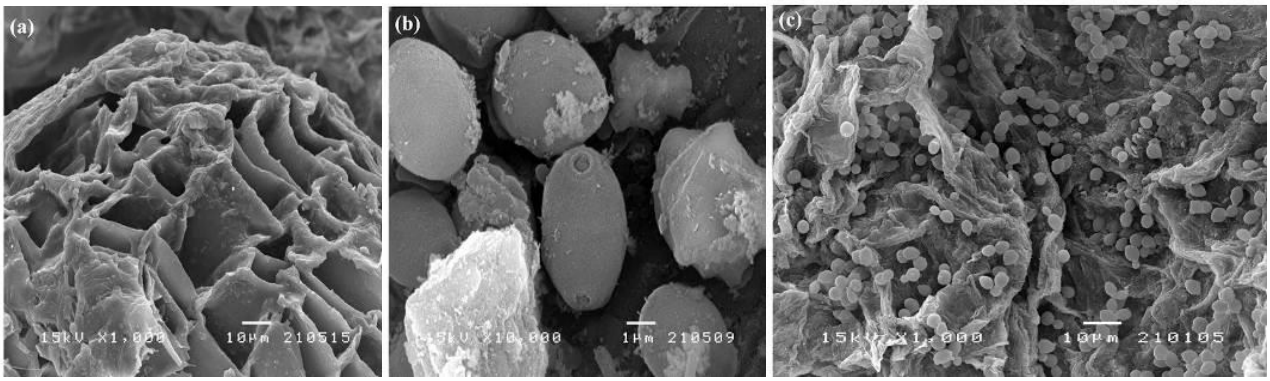

Fig. 4. Scanning electron micrographs of wCTF: a and b) before and c) after fermentation. a) The porous structure of the wCTF with attached residual yeast cells (1000X magnification), b) the budding scar of the attached cell (10,000X magnification), andc) the increased cell number attached to wCTF after fermentation (1000X magnification). Images are representative of at minimum of 3 fields of view per sample and 2 independent samples

Table 2. Comparison of the Amino Acid Composition of the Molasses Medium With or Without 5% (w/v) Waste Cassava Tuber Fibers (wCTF)

Amino acid (mg/100 mL)	Molassesmedium (mg/mL) *	Molasses medium + 5% (w/v) wCTF (mg/mL) *
Alanine	164	163
Arginine	ND	ND
Aspartic Acid	832	524
Cystine	ND	ND
Glutamic Acid	283	340
Glycine	51.5	100
Histidine	13.3	66.0
Hydroxylysine	ND	ND
Hydroxyproline	ND	ND
Isoleucine	59.4	44.1
Leucine	55.4	93.3
Lysine	18.6	112
Methionine	96.6	57.2
Phenylalanine	33.6	51.6
Proline	60.6	65.8
Serine	49.0	171
Threonine	47.7	57
Tryptophan	ND	ND
Tyrosine	122	274
Valine	113	88.2

ND; not detectable.
*Analysed at the ALS Laboratory group (Thailand) Co. Ltd.

CONCLUSIONS

1. The ethanol production level of wCTF adsorbed-calcium alginateentrapped *S. cerevisiae*cellswas 12.16% higher than those of calcium alginate entrapped *S. cerevisiae*cells.
2. But the ethanol production level of wCTF adsorbed *S. cerevisiae* cells was 22.64% higher than those ofthe wCTF adsorbed-calcium alginate entrapped *S. cerevisiae* cells.
3. The ethanol production level of the wCTF adsorbed *S. cerevisiae*cells was 59.9% higher than those of*S. cerevisiae* free cells**.**
4. It is likely that the wCTF provided an exogenous amino nitrogen supply that promoted an increased ethanol yield.

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