RESEARCH ARTICLE
doi: 10.2306/scienceasia1513-1874.2019.45.003

ScienceAsia 45 (2019): **3–9**

Comparison of yeast extract prepared by autolysis or steam explosion as a cheap nutrient supplement for very high gravity ethanol fermentation of cassava starch

Thanaporn Palasak[a], **Sarintip Sooksai**[b], **Ancharida Savarajara**[c,*]

[a] Biotechnology Program, Faculty of Science, Chulalongkorn University, Bangkok 10330 Thailand
[b] The Institute of Biotechnology and Genetic Engineering, Chulalongkorn University, Bangkok 10330 Thailand
[c] Department of Microbiology, Faculty of Science, Chulalongkorn University, Bangkok 10330 Thailand

*Corresponding author, e-mail: sanchari@chula.ac.th

Received 23 Mar 2018
Accepted 27 Feb 2019

ABSTRACT: Very high gravity fermentation of saccharified cassava starch containing 280 g/l reducing sugar to ethanol by the osmotolerant *Saccharomyces cerevisiae* G2-3-2 strain required supplementation with inorganic nutrients (KH_2PO_4, $(NH_4)_2HPO_4$, and $MgSO_4 \cdot 7H_2O$ and yeast extract (YE) to maximize the ethanol production level and rate. Comparison of the YE prepared by the autolysis of spent brewer's yeast at either 50 °C for 24 h (A-YE) or steam explosion at 200 °C for 20 min (SE-YE), as a replacement for commercial yeast extract (C-YE) at 7.5 g/l, revealed that A-YE gave a slightly higher ethanol concentration (113.85 g/l) than the SE-YE (107.81 g/l), and was comparable to that obtained with the C-YE (113.14 g/l). At the optimal concentration of A-YE supplementation (5.23 g/l), the ethanol concentration in the ferment reached 115.77 g/l at 24 h, an ethanol productivity of 4.82 g/l/h.

KEYWORDS: *Saccharomyces cerevisiae*, osmotolerant, spent brewer's yeast

INTRODUCTION

Bioethanol is a promising alternative energy that is renewable and relatively environment-friendly, where several starch crops, such as corn, wheat and cassava, have already been commercially exploited for bioethanol production in several countries[1]. A higher starch yield per hectare and all year availability due to flexibility in planting and harvesting of cassava are its major advantages over other crops. Cassava chip is one of the two main raw materials for ethanol production in Thailand. However, as prepared it is mostly mixed with sand and stone, causing mechanical damage to production equipment. Although the price of cassava starch is higher than cassava chip, the cost of the two raw materials are comparatively on the same level when considering the maintenance and repair expenditures of the production equipment due to the wear and tear caused by sand and stone from cassava chips.

To increase the productivity and cost effectiveness of bioethanol production, many process improvements, including very high gravity (VHG) technology, have been studied. Overall, VHG is defined as the preparation and fermentation to completion of a solution containing more than 270 g/l total sugar[2]. Ethanolic fermentation from a very high sugar concentration has many advantages from an industrial point of view, resulting in reduced costs because of the lower energy consumption[3].

However, these fermentations are rarely fast and complete due to the induced physiological changes in the fermenting microbial cells. The high sugar concentration in the fermentation media causes an increased osmotic pressure that has a damaging effect on yeast cells and leads to an increased level of cell lysis and reduced viability, and so a decreased ethanol production[4]. Nevertheless, these effects can be relieved to some extent by supplementation of the media with appropriate nutrients[1,5,6].

Saccharomyces cerevisiae can ferment a higher concentration of sugar when all the required nutrients are provided in sufficient amounts[7]. Several investigators have observed that yeast extract (YE), ammonium ions, urea, and calcium and magnesium ions have protective effects on either the growth

and fermentation or the viability of *S. cerevisiae*, and so stimulate the fermentation rate and ethanol production[1].

Recently we isolated a high ethanol producing-osmotolerant *S. cerevisiae* strain (G2-3-2) and determined its optimized ethanol production medium (oEPM), in terms of maximal ethanol production rate, as; glucose 280 g/l, Bacto-peptone 5 g/l, YE 7.5 g/l, $(NH_4)_2HPO_4$ 1 g/l, $MgSO_4 \cdot 7\,H_2O$ 1.5 g/l, KH_2PO_4 3 g/l, $CaCl_2 \cdot 2\,H_2O$ 1 g/l, $MnSO_4 \cdot 4\,H_2O$ 0.5 g/l, $ZnSO_4 \cdot 7\,H_2O$ 0.2 g/l, pH 5.0[8]. To produce ethanol from saccharified cassava starch (SCS) by VHG technology using *S. cerevisiae* G2-3-2 on industrial scale, the required nutrient supplementation had to be reinvestigated. The SCS is composed of not only fermentable sugars but also some level of nitrogen, and major and minor trace elements required for the growth and ethanol fermentation of *S. cerevisiae*. Furthermore, YE and peptone are not economically viable supplements in industrial fermentation due to their high cost.

In this study, the required nutrient supplementation for the VHG ethanol fermentation of SCS was investigated. As a cheap and readily available source, YE was prepared from spent brewer's yeast, a rich source of amino acids, vitamins, minerals and fatty acids for promoting the growth of yeast and ethanol fermentation[4], by cell autolysis (A-YE) and steam explosion (SE-YE) to compare the effect of intracellular lysate and whole cell decomposition, respectively, on the improvement of VHG ethanol production.

MATERIALS AND METHODS

Cassava starch and enzymes

Cassava starch, a product of E.T.C. International Trading Co., Ltd. (Nonthaburi, Thailand) under the Thai fish brand, was used in this study. The α-amylase (Spezyme alpha; 13 775 AAU/g) and glucoamylase (GC 147; 580 TGAU/g) (Genencor, Danisco US Inc., USA) used in this study were a gift from the Thai Alcohol Public Co., Ltd., Nakhonpathom, Thailand.

Saccharification of cassava starch

Cassava starch (15 g) was suspended in distilled water (50 ml), adjusted to pH 5.8 and then liquefied by 26.39 AAU/g α-amylase at 85 °C for 3 h. The resultant gelatinized starch solution was adjusted to pH 4.5 and saccharified by 16.45 TGAU/g glucoamylase at 60 °C for 30 min. After clarification of the SCS solution by centrifugation (7871*g*, 4 °C, 10 min) the resultant clear syrup was analysed for its reducing sugar content[9], while inorganic nutrients and trace elements were analysed by Food Research and Testing Laboratory, Faculty of Science, Chulalongkorn University based on AOAC standard method[10,11]. The dose of α-amylase and glucoamylase used in the above liquefaction and saccharification steps were selected from preliminary experiments (26.39–42.22 AAU/g and 4.11–28.79 TGAU/g, respectively) designed to obtain a clear syrup containing 280 g/l reducing sugar.

Microorganism and inoculum preparation

The osmotolerant *S. cerevisiae* G2-3-2 strain[8] was used in this study. A single colony grown on YPD agar (YE 3 g/l, Bacto-peptone 3 g/l, glucose 100 g/l, agar 20 g/l, pH 5.0) at 30 °C for 48 h was inoculated into 50 ml of modified YM broth (YE 7.5 g/l, Bacto-peptone 5 g/l, malt extract 3 g/l and glucose 150 g/l, pH 5.0) in a 250-ml Erlenmeyer flask and incubated at 30 °C, 200 rpm for 24 h. The resultant culture was transferred into 50 ml of modified YM broth in a 250 ml Arm flask at an initial optical density at 660 nm of 0.05 and incubated at the same condition as above until it reached late log phase (15 h). The late log phase cells were then precipitated by centrifugation (7871*g*, 4 °C, 15 min), resuspended and the cell density determined before use as the inoculum.

Ethanol production medium

The SCS medium (SCSM) composition was the same as the oEPM except for containing 280 g/l glucose equivalent of SCS solution instead of glucose. Each medium was sterilized by autoclaving at 110 °C for 10 min.

Ethanol production

The inoculum was inoculated at an initial yeast cell number of 1×10^9 cells/ml into 35 ml of oEPM or SCSM in a 50-ml Erlenmeyer flask. The inoculated flask was incubated at 30 °C under an oxygen limited condition for 72 h with shaking at 100 rpm allowing CO_2 release by capping the flask with a perforated rubber stopper connected to a U-shape glass tube containing a $CuSO_4$-lock. Thus only the oxygen dissolved in the culture medium and in the air space (ca. 15 ml) above was available. Every 24 h, the resultant cultures (three independent flasks) were then centrifuged (7871*g*, 4 °C, 15 min) and the supernatants were analysed for the ethanol and reducing sugar contents.

Table 1 Composition of the SCS media and their modification.

Medium No.	Trace element[a]	Inorganic nutrient[b]	Complex nutrient	
			C-YE[c]	Peptone[d]
SCSM	+	+	+	+
mSCSM 1	−	+	+	+
mSCSM 2	−	−	+	+
mSCSM 3	−	+	−	−
mSCSM 4	−	−	−	−
mSCSM 5	−	+	+	−
mSCSM 6	−	+	−	+

[a] $CaCl_2 \cdot 2H_2O$ 1 g/l, $MnSO_4 \cdot 4H_2O$ 0.5 g/l, and $ZnSO_4 \cdot 7H_2O$ 0.2 g/l; [b] $(NH_4)_2HPO_4$ 1 g/l, $MgSO_4 \cdot 7H_2O$ 1.5 g/l, and KH_2PO_4 3 g/l; [c] C-YE 7.5 g/l; [d] peptone 5 g/l; +, with; −, without.

Table 2 Some inorganic nutrient and trace element contents in the SCS solution.

Composition	mg/kg
Copper (Cu)*	–
Calcium (Ca)	6.79
Zinc (Zn)	0.07
Manganese (Mn)	0.07
Potassium (K)	94.51
Phosphorus (P)	15.06
Nitrogen (N)	183
Magnesium (Mg)	5.68

* Limit of detection 0.36 mg/kg.

Effect of SCSM composition on VHG ethanol production

In addition to SCSM, the VHG ethanol fermentation was performed in a series of incomplete SCSM that lacked some components (Table 1). The basic modified SCSM (mSCSM) had no added trace elements, due to their potential presence in the SCS. Additional changes included the omission of one or more of the inorganic nutrients (-IN), peptone (-P) and YE (-YE). Cultures were otherwise grown as above (up to 72 h at 30 °C under an oxygen limited condition with CO_2 release and shaking at 100 rpm) to determine the effects of the supplements on the ethanol production level.

Preparation of A-YE and SE-YE

Spent brewer's yeast, obtained from the Pathumthani Brewery Co., Ltd. (Pathumthani, Thailand), was precipitated by centrifugation using a screw decanter continuous centrifuge (Kansai centrifugal separator manufacturing Co., Ltd., Fukushima, Osaka, Japan) at room temperature and 8838g, and washed with 1 M NaOH and then distilled water prior to being stored at −20 °C until used. The washed spent brewer's yeast cells (300 g) were then suspended in distilled water (final 500 ml). For the A-YE, the cell suspension was incubated at 50 °C for 24 h, while for the SE-YE the cell suspension was treated in a high-pressure reactor (Parr Instrument Co., USA) at 200 °C for 20 min[12]. In both cases the post-treatment suspension was centrifuged (4 °C, 11 305g, 10 min) and the resultant supernatant was spray-dried and analysed for total nitrogen[13], vitamins (Pantothenic acid[11], Biotin[14], Thiamine[15], and Pyridoxine[15]) and amino acid contents commercially at ALS Laboratory Group Thailand Co., Ltd.

Comparison between commercial YE (C-YE), A-YE and SE-YE supplements on VHG ethanol production

The VHG ethanol fermentation was performed using the mSCSM(-P) medium (Table 1) except the C-YE was, where stated, replaced with either A-YE or SE-YE to give the same level of nitrogen and protein content as C-YE at 7.5 g/l. The C-YE (Bio Springer Company, France) used in this study was prepared by autolysis.

Analytical and statistical procedures

Yeast cell number was determined by haemocytometer under light microscopy. Ethanol concentration was analysed by gas chromatography[8]. Results are presented as the mean ± standard deviation (SD) from the indicated number of independent replications. The statistical significance between different means was tested using Duncan's new multiple range test (DMRT), performed using the SPSS 16.0. Significance was accepted at the $p < 0.05$ level.

RESULTS AND DISCUSSION

Production of the SCS solution (280 g reducing sugar/l)

The SCS solution, as a clear syrup containing 280 g/l reducing sugar was obtained when cassava starch (15 g) suspended in distilled water (50 ml) was sequentially liquefied and saccharified by 26.39 AAU/g α-amylase and 16.45 TGAU/g glucoamylase (data not shown), respectively. The contents of some of the trace elements and inorganic nutrients in the clear SCS syrup are shown in Table 2.

Table 3 Ethanol production by *S. cerevisiae* G2-3-2 in oEPM medium.

Time (h)	Concentration* (g/l)	Yield (g/g)	Productivity (g/l/h)
24	$92.76^{B} \pm 1.53$	0.33	3.87
48	$106.73^{A} \pm 1.26$	0.38	2.22
72	$108.96^{A} \pm 0.21$	0.39	1.51
96	$105.90^{A} \pm 0.97$	0.38	1.10

* Data are displayed as the mean ± SD and derived from triplicate experiments. Means followed by a different letter are significantly different, $p < 0.05$, by one-way ANOVA and Duncan's multiple range test.

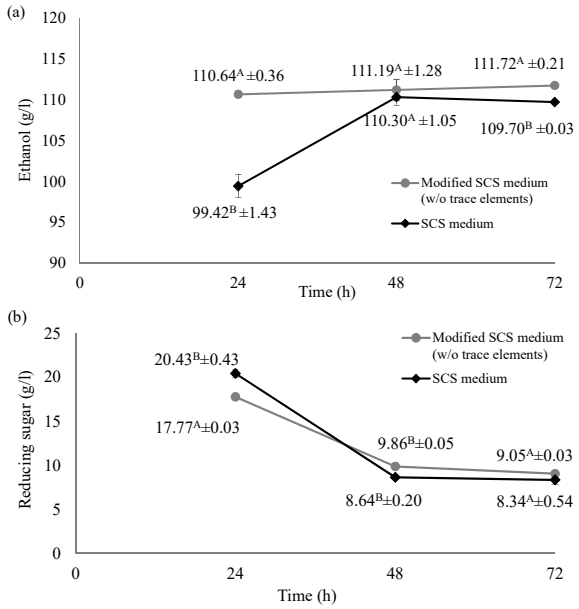

Fig. 1 (a) Ethanol production and (b) residual reducing sugar level in the SCSM (inorganic nutrients, YE, peptone and trace elements) and mSCSM (without the trace elements). The data are displayed as the mean ± SD and derived from triplicate experiments. Means with a different letter are significantly different, $p < 0.05$, one-way ANOVA and DMRT.

Effect of the SCS medium composition on VHG ethanol production

Under the evaluated conditions, *S. cerevisiae* G2-3-2 produced a maximum ethanol concentration (108.96 g/l) at 72 h, with an ethanol yield and productivity of 0.39 g/g reducing sugar and 1.51 g/l/h, respectively, in the oEPM medium. The ethanol production rate decreased with increasing fermentation time, while the ethanol concentration in the ferment increased with fermentation time up to 72 h (Table 3). In SCSM, a slightly higher maximum ethanol concentration (110.30 g/l) was obtained, and at a shorter fermentation time of 48 h (Fig. 1). Furthermore, a higher maximum ethanol concentration (110.64 g/l) was obtained sooner (within 24 h) in the mSCSM, that is when the SCSM was modified by removal of the trace elements supplement (Fig. 1). Hence the effect of the inorganic and complex nutrients (C-YE and peptone) on the VHG ethanol production in the mSCSM was further determined.

Table 4 Effect of medium composition in modified SCS medium (without trace elements) on VHG ethanol production.

Time	Ethanol concentration*(g/l) in mSCSM			
(h)	YE+P+IN	YE+P (-IN)	YE (-P)+IN	P (-YE)+IN
24	$110.6^{A} \pm 0.4$	$100.2^{B} \pm 0.4$	$113.1^{A} \pm 0.9$	$107.2^{B} \pm 1.3$
48	$111.2^{A} \pm 1.3$	$110.8^{A} \pm 1.8$	$113.2^{A} \pm 0.3$	$109.5^{C} \pm 0.2$
72	$111.7^{B} \pm 0.2$	$109.1^{C} \pm 1.3$	ND	ND

* Data are displayed as the mean ± SD and derived from triplicate experiments. Means followed by a different letter are significantly different ($p < 0.05$ by one-way ANOVA and Duncan's multiple range test). +, with and −, without YE; C-YE 7.5 g/l; P 5 g/l; IN, inorganic nutrients; ND, not determined.

When the inorganic nutrients were omitted, a similar maximum ethanol concentration (110.76 g/l) was obtained but after a longer fermentation period (48 h). This likely reflects the insufficient levels of phosphate and magnesium ions in the SCS, as they are necessary for the growth and vitality of yeast, being involved in metabolic and bioenergetics pathways. Magnesium ions are required for the activity of several enzymes [16]. Whereas, removal of the peptone resulted in a slightly larger maximum ethanol concentration (113.14 g/l), which was still obtained within 24 h. The omission of the YE, however, resulted in a decreased maximum ethanol concentration to 109.48 g/l and was obtained at a slower rate, being obtained at 48 h (Table 4). That the mSCSM gave a lower ethanol level than the mSCSM(-P) might result from the higher ammonium content in the ferment. Excessive ammonium ions increase the production of higher alcohols, acetic acid and H_2S, which have a negative effect on ethanol production [1]. From these results, mSCSM(-P) was selected for further investigation of the different YEs.

Table 5 Comparison of the protein and nitrogen contents of the different yeast extracts.

Type of YE	Protein content (%)*	Nitrogen content (%)*
C-YE	$72.83^{A} \pm 0.70$	$11.65^{A} \pm 0.12$
A-YE	$69.84^{B} \pm 0.36$	$11.13^{A} \pm 0.12$
SE-YE	$50.39^{C} \pm 0.37$	$8.06^{B} \pm 0.06$

* Data are displayed as the mean ± SD and derived from triplicate experiments. Means followed by a different letter are significantly different ($p < 0.05$, by one-way ANOVA and Duncan's multiple range test).

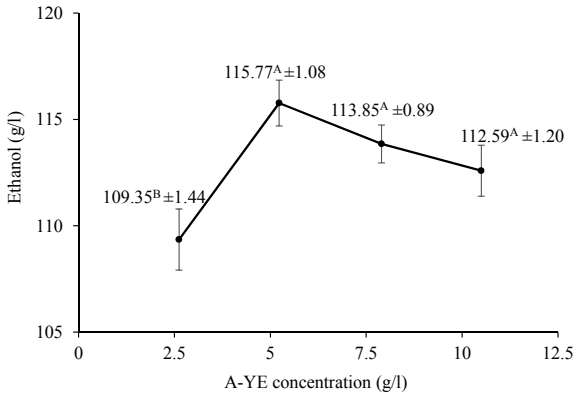

Fig. 2 Effect of the A-YE concentration on the ethanol production in mSCSM(-P) except supplemented with different concentrations of A-YE in place of C-YE. The data are displayed as the mean ± SD and derived from triplicate experiments. Means with a different letter are significantly different ($p < 0.05$, DMRT).

Comparison of the A-YE and SE-YE characteristics

The residual solid yield of SE-YE was 65% lower than that obtained for A-YE, which reflects the larger extent of hydrolysis that occurred at the higher temperature in SE-YE production than in A-YE. Furthermore, the protein and nitrogen contents of the SE-YE were 17% and 38%, respectively, lower than those of A-YE (Table 5). The protein and organic nitrogen in the SE-YE might have been degraded at the very high process temperature (200 °C) of the steam explosion. In contrast, the A-YE had very similar protein and nitrogen contents to the C-YE (Table 5), in accord with their similar production process (autolysis).

Comparison of A-YE and SE-YE as supplements for VHG ethanol production

Each VHG ethanol fermentation was performed in mSCSM(-P) containing the respective YE. For the medium with C-YE, the maximum ethanol concentration (113.14 g/l) was produced after 24 h when the media was supplemented with C-YE at 7.5 g/l (protein and nitrogen contents of 0.55% and 0.09% (w/v), respectively). The replacement of the C-YE with the low-cost A-YE or SE-YE was then evaluated at the same protein and nitrogen level. Supplementation with A-YE (7.9 g/l) gave essentially the

Table 6 Amino acid composition of the different yeast extracts (mg/100 g).

Amino acids	C-YE*	A-YE*	SE-YE*
Alanine	5240	5210	5380
Arginine	7850	7750	4820
Aspartic acid	6980	6830	3160
Cystine	300	436	27
Glutamic acid	12 600	6690	8170
Glycine	3100	3390	3030
Histidine	1340	1400	927
Isoleucine	3680	4110	2450
Leucine	5740	6290	3990
Lysine	5670	5610	2950
Methionine	1520	1620	966
Phenylalanine	3370	3740	2190
Proline	3190	3710	3140
Serine	4350	4310	3160
Threonine	3600	3690	2300
Tryptophan	699	781	293
Tyrosine	1450	3050	2470
Valine	4310	4790	3280

* ALS Laboratory Group Thailand Co., Ltd.

Table 7 Vitamin contents (mg/100 g) in the different yeast extracts.

Vitamin	YE	Vitamin content*,a
Biotin	C-YE	52.70[b]
	A-YE	58.40[b]
	SE-YE	56.70[b]
Thiamine	C-YE	2.19
	A-YE	12.40
	SE-YE	0.43
Pantothenic acid	C-YE	9.72
	A-YE	7.71
	SE-YE	0.57
Pyridoxine	C-YE	1.12
	A-YE	0.90
	SE-YE	ND

* ALS Laboratory Group Thailand Co., Ltd.;
[a] Limit of detection is 0.003 mg/100 g;
[b] the concentration unit is μg/100 g; ND, not determined.

same maximum ethanol concentration (113.85 g/l) as that obtained with the C-YE, while SE-YE gave a lower level of ethanol (107.81 g/l). Thus the A-YE was selected for optimizing the amount of YE supplement by evaluation at 2.62–10.5 g/l. Under these conditions, the mSCSM(-P) supplemented with 5.23 g/l A-YE gave a maximum ethanol concentration of 115.77 g/l after 24 h of fermentation (Fig. 2).

Analysis of the amino acid content of the C-YE, A-YE and SE-YE revealed that the SE-YE contained a lower level of amino acids compared to A-YE, except for alanine and glutamic acid (Table 6). The amino acid content of YE is known to decrease with increasing temperature during the preparation process[12]. In addition, the higher level of some amino acids in A-YE might also be a result of several proteins produced in the autolysis process. Of note, the level of the thiamine, pantothenic acid and pyridoxine, which are important for growth and ethanol fermentation of yeast, were found at a markedly lower concentration in SE-YE than A-YE (Table 7). Of these, thiamine was also significantly lower in C-YE than in A-YE, although still five-fold higher than in SE-YE and so still potentially limiting. This might account for the lower ethanol production by SE-YE. Deficiency of thiamine, pantothenic acid and pyridoxine results in a reduced yeast growth[17]. For example, pyruvate decarboxylase, which catalyses decarboxylation of pyruvate to acetaldehyde and CO_2, requires thiamine[18,19], while the synthesis of coenzyme A and acetyl-CoA requires pantothenic acid[20].

CONCLUSIONS

The VHG ethanol production from SCS (280 g/l reducing sugar) by *S. cerevisiae* G2-3-2 required supplementation of the substrate with inorganic nutrients and YE. The YE prepared by cell autolysis (A-YE) was as effective as the more expensive C-YE, but the SE-YE gave a lower ethanol concentration in the ferment, perhaps reflecting the lower content of essential amino acids and vitamins in SE-YE. Thus the cost of ethanol formation by VHG fermentation of cassava could be made more commercially viable by using locally made A-YE in place of imported C-YE.

Acknowledgements: The authors express sincere appreciation and thanks to the Thai Alcohol Public Company, Thailand for providing the α-amylase and glucoamylase enzymes; Mrs Vasana Tolieng for her excellent assistance in facilitating and guiding the usage of the screw decanter continuous centrifuge; Dr Robert DJ Butcher for his assistance in English proofreading and valuable comments on the manuscript. This study was financially supported by the Thai Government budget (fiscal year 2016).

REFERENCES

1. Laopaiboon L, Nuanpeng S, Srinophakun P, Klanrit P, Laopaiboon P (2009) Ethanol production from sweet sorghum juice using very high gravity technology: Effect of carbon and nitrogen supplementations. *Bioresour Technol* **100**, 4176–82.
2. Bayrock DP, Ingledew WM (2001) Application of multistage continuous fermentation for production of fuel alcohol by very-high-gravity fermentation technology. *J Ind Microbiol Biotechnol* **27**, 87–93.
3. Thomas KC, Hynes SH, Ingledew WM (1996) Practical and theoretical considerations in the production of high concentrations of alcohol by fermentation. *Process Biochem* **31**, 321–31.
4. Kawa-Rygielska J, Pietrzak W (2014) Ethanol fermentation of very high gravity (VHG) maize mashes by *Saccharomyces cerevisiae* with spent brewer's yeast supplementation. *Biomass Bioenergy* **60**, 50–7.
5. Wang FQ, Gao CJ, Yang CY, Xu P (2007) Optimization of an ethanol production medium in very high gravity fermentation. *Biotechnol Lett* **29**, 233–6.
6. D'Amore T, Panchal CJ, Russell I, Stewart GG (1988) Osmotic pressure effects and intracellular accumulation of ethanol in yeast during fermentation. *J Ind Microbiol* **2**, 365–72.
7. Bafrncová P, Smogrovicová D, Sláviková I, Pátková J, Dömény Z (1999) Improvement of very high gravity ethanol fermentation by media supplementation using *Saccharomyces cerevisiae*. *Biotechnol Lett* **21**, 337–41.
8. Hoondee P, Tolieng V, Tanasupawat S, Kitpreechavanich V, Akaracharanya A (2014) Very high gravity ethanol fermentation by the newly isolated osmotolerant *Saccharomyces cerevisiae* isolate G2-3-2. *Chiang Mai J Sci* **41**, 1–16.
9. Somogyi M (1952) Notes on sugar determination. *J Biol Chem* **195**, 19–23.
10. Association of Official Analytical Chemists (2005) *Official Methods of Analysis*, 18 edn, AOAC International.
11. Association of Official Analytical Chemists (2012) *Official Methods of Analysis*, 19 edn, AOAC International.
12. Lamoolphak W, Goto M, Sasaki M, Suphantharika M, Muangnapoh C, Prommuag C, Shotipruk A (2006) Hydrothermal decomposition of yeast cells for production of proteins and amino acids. *J Hazard Mater* **137**, 1643–8.
13. Kjeldahl J (1883) Neue methode zur bestimmung des stickstoffs in organischen Körpern. *Fresenius Zeitschrift Anal Chemie* **22**, 366–82.

14. Angyal G (1996) *US Food and Drug Administration, Methods for the Microbiological Analysis of Selected Nutrients*, AOAC International.
15. Arella F, Lahély S, Bourguignon JB, Hasselmann C (1996) Liquid chromatographic determination of vitamins B1 and B2 in foods a collaborative study. *Food Chem* **56**, 81–6.
16. Udeh HO, Kgatla TE (2013) Role of magnesium ions on yeast performance during very high gravity fermentation. *J Brew Distilling* **4**, 19–45.
17. Nordstrom K (1962) Formation of ethyl acetate in fermentation with brewer's yeast III. *JIB* **68**, 398–407.
18. Dixon M, Webb EG (1964) *Enzymes*, Academic Press, USA.
19. Pronk JT, Ydesteensmay H, Van Dijken JP (1996) *Pyruvate Metabolism in Saccharomyces cerevisiae Yeast*, Wiley, USA.
20. Gutierrez LE (1993) Effect of some vitamins and micronutrient deficiencies on the production of higher alcohols by *Saccharomyces cerevisiae*. *Scientia Agricola* **50**, 484–9.