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Full Length Research Paper

Prediction of significant factors in the production of ethanol by ragi tapai co-culture using Taguchi methodology

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Ethanol production by co-culture of ragi tapai and *Saccharomyces cerevisiae* from unhydrolyzed cassava starch without addition of enzymes was conducted in a 2 L batch fermentor. Taguchi's method with orthogonal array of L8 was applied in design of experiment (DOE) and the results were analyzed using MINITAB v14 software. Seven factors: Nitrogen-phosphorus-potassium (NPK), urea, fermentation temperature, ragi tapai concentration, *S. cerevisiae* concentration, agitation and co-culturing time were varied at two levels for each factor. The significant factors for the production of ethanol were determined by setting S/N ratio to "larger-is-better" for high yield ethanol and "smaller-is-better" for low yield of ethanol byproducts. The optimum values obtained for each factor were similar to each other. Both have optimum factors of: Urea at 0.8 w/w%, dry ragi tapai and *S. cerevisiae* concentrations each at 10 w/w%, co-culturing time at 3 h gap, NPK at 0.09 w/w% and agitation speed at 100 rpm. The fermentation temperature for high ethanol yield was 35℃, whereas for the byproducts was 30℃. From the validation experiment conducted at 30℃ in 10 L fermentor, the ethanol concentration obtained was 68.00 g/L, while all byproducts concentrations were below 9.00 g/L at the end of the fermentation.

Key words: Co-culture fermentation, ethanol, Taguchi's experimental design, ragi tapai, Saccharomyces cerevisiae.

INTRODUCTION

Ethanol fermentation from starches normally requires three-stage processes namely: Liquefaction, saccharification and fermentation. Alternative to these conventional multistage processes is by direct fermentation of raw starch using amylolytic fungus co-culture with yeast, that is, *Saccharomyces cerevisiae*. The amylolytic fungi are typically found in ragi tapai, the latter is a dry-starter culture usually used to make tapai or tape' in Asian countries.

Study on the direct fermentation of the unhydrolyzed cassava starch using dry starter of ragi tapai and co-culture with *S. cerevisiae* by Azmi et al. (2010) showed that the glucose produced was proportional to the starch concentration investigated but not for ethanol production. The investigators also reported that the yield for co-culture was 48% higher for ragi monoculture. The study concluded that *S. cerevisiae* utilized glucose immediately to produce ethanol before it could accumulate and correspondingly inhibit the fermentation process by osmotic pressure on the cells which is one of the distinct advantages that co-culture or direct fermentation can offer.

Another direct fermentation using co-culture in liquefied starch (using α-amylase only) to produce ethanol was carried out and reported to be effective when compared with the two-step bioconversion process (Verma et al., 2000). Other similar studies by Abouzied and Reddy (1986, 1987), Hyun and Zeikus (1985) and Piršelová et al. (1993) also indicated that better fermentation yields

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Table 1. Selected variables and assigned levels.

Variable	Constituent	Level	
		1 (low)	2 (high)
A	NPK (w/w %)	0.06	0.09
B	Urea (w/w %)	0.5	0.8
C	Temperature (°C)	30	35
D	Ragi tapai (w/w %)	5	10
E	*S. cerevisiae* (w/w %)	5	10
F	Agitation (rpm)	50	100
G	Co-culture time (h)	1	3

were obtained as compared to the mono-cultures.

Taguchi method is a quality engineering method which utilizes a modified and standardized form of design of experiment (DOE) (Roy, 2001). This method focuses on applying engineering techniques to processes. Many factors can be simultaneously optimized and a lot of quantitative information can be extracted by running minimal number of experimental trials. In so doing, the effects of certain factors on a response can be studied under an economical and practical condition (Wang and Wan, 2009).

The main objective of this study was to determine the significant parameters for ethanol production via co-culture fermentation of unhydrolyzed cassava starch in a batch reactor without the employment of enzyme. Furthermore, Taguchi's methodology was used by setting the S/N ratio to "larger-is-better" for high yield of ethanol and "smaller-is-better" for low yield of ethanol byproducts with the aid of Minitab v14.

MATERIALS AND METHODS

Microorganisms and culture conditions

The commercialized ragi tapai was obtained from a local market at Kuala Lumpur and the industrial yeast, *S. cerevisiae* was from Indonesian Bioenergy Foundation. The culture medium containing distilled water with NPK and urea at different concentrations are stated in Table 1. The medium was autoclaved at 121°C for 15 min. The dry starter and yeasts were then placed into the sterilized medium and incubated at 37°C and 200 rpm for 25 to 30 min before inoculation.

Batch fermentation

Cassava or tapioca flour used in the experiment was obtained from a local market at Kuala Lumpur. Ten percent of cassava flour was mixed for 5 min in preheated water (60°C) in a sterile 2 and 10 L jacketed Biostat® B-DCU fermentor, separately. After 5 min, the temperature of the cassava flour mixture was raised to 70°C and mixed for 1 h. At the end of 1 h, in the 2 L fermentor, the temperature and agitation speed were set at different values of parameters using Taguchi method as shown in Table 1. The mixture in the fermentor that was inoculated with ragi tapai and *S. cerevisiae* at different concentration and inoculation time are also presented in Table 1. All experiments in 2 L fermentor were run for 78 h and the optimum condition obtained from the fermentor was applied in a 10 L fermentor to validate the process with extended duration of 11 days (264 h).

Analytical method

The samples taken from the fermentation were centrifuged at 8000 rpm for 30 min and the supernatant was analyzed using IC-Pak™ Ion Exclusion column with SH-1011P pre-column on an HPLC (WATERS) equipped with a refractive index detector at 30°C to determine the concentration of ethanol, glucose and byproducts. The column was eluted at 60°C with 0.5 mM sulfuric acid at 1.0 ml min^{-1}.

Design of the experiment

Table 1 lists seven selected factors with two different levels assigned for each factor. A standard orthogonal array of L8 (2^7) was selected to examine this system. All the runs in this work involved the corresponding combination of levels to which the factors in the experiment were set. The experimental results were analyzed to extract independently the main effects of the factors. Calculations of the signal-to-noise (S/N) ratio were based on "larger-is-better" and "smaller-is-better" as presented in Equation (1) and (2), respectively:

$$\frac{S}{N} = -10\log\left(\frac{\Sigma\left(1/y_i^2\right)}{n}\right) \quad (1)$$

$$\frac{S}{N} = -10\log\left(\frac{\Sigma y_i^2}{n}\right) \quad (2)$$

The analysis of variance was performed in MINITAB v14 to determine the statistically significant factors.

RESULTS AND DISCUSSION

The ethanol concentration of the fermentation process is shown in Figure 1. At the end of 78 h fermentation, highest ethanol concentration obtained was 41.20 g/L from run 1 and the concentration obtained from run 3 at 15.30 g/L was the lowest. Figures 2 and 3 show the concentration profiles of maltoheptaose and glucose, respectively. Maltoheptaose is consistently detected by

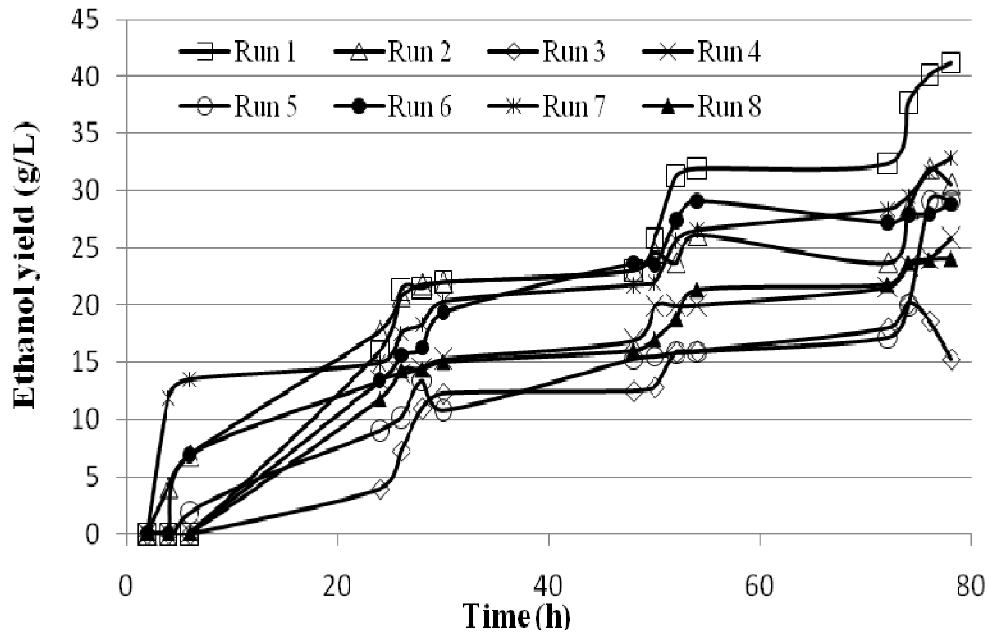

Figure 1. Ethanol concentration from different runs.

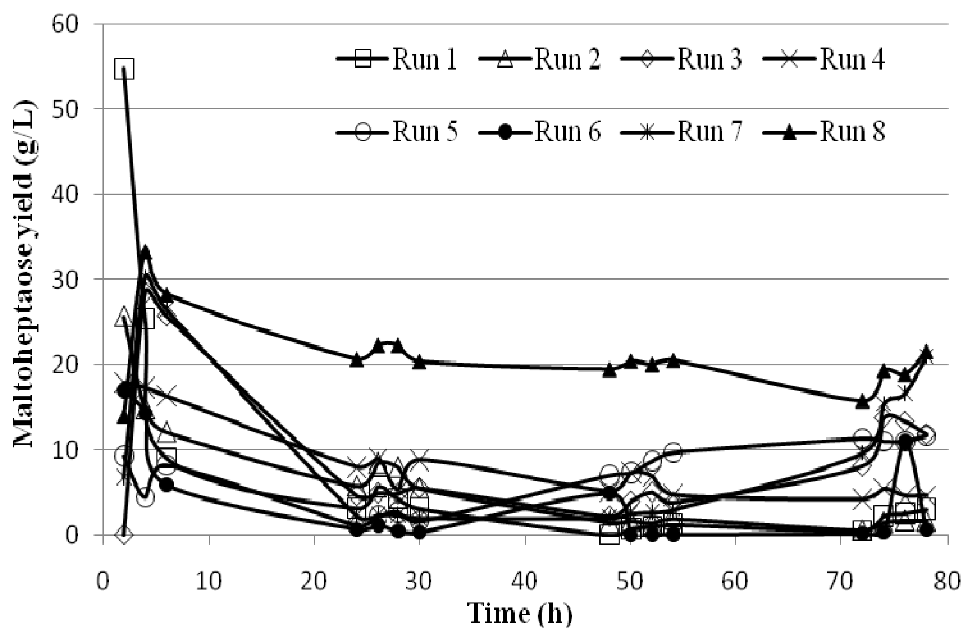

Figure 2. Maltoheptaose concentration from different runs.

HPLC, whereas other types of oligosaccharides detected are not as prominent and consistent. Thus, among the oligosaccharides detected, only maltoheptaose was considered in this experiment.

Maltoheptaose and glucose are the intermediate products consistently produced during fermentation from hydrolysis of starch which are clearly shown in Figures 2 and 3. The productions of both the intermediate products fluctuated particularly in the case of glucose. The profile of glucose concentrations implied that the saccharide was released and at the same time, it was consumed to produce ethanol at different production and consumption rates. This is in agreement with the report of Azmi et al. (2010) where accumulation of glucose concentration was observed in the ragi tapai monoculture fermentation in which no yeast was in the medium to digest the glucose into ethanol efficiently. In the co-culture fermentation of ragi tapai and *S. cerevisiae*, the glucose and maltohep-

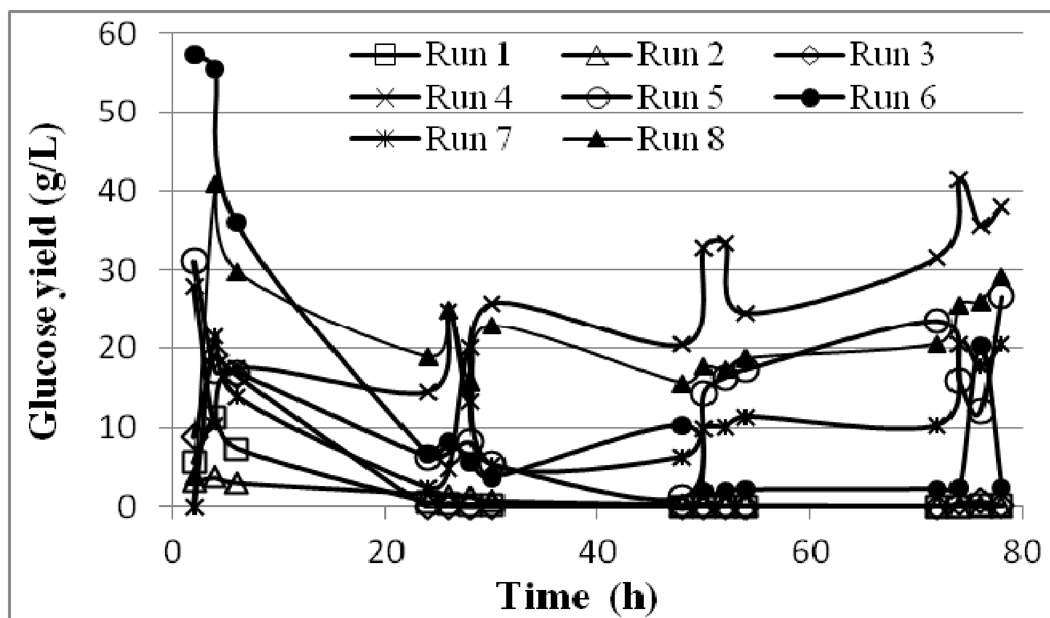

Figure 3. Glucose concentrations from different runs.

taose productions and consumptions were simultaneously and constantly taking place, resulting in the fluctuation of both concentration profiles. This implies that the amylolytic activity was enhanced when low glucose concentration in the medium had continuously replenished in the co-culture fermentation. Moreover, it also reduces the inhibitory effect of lower saccharides and the osmotic stress on cells (Bai et al., 2008). Hence, co-culture fermentation can prevent inhibition effect and reduce osmotic stress on cells, attributing it to the continuous glucose releases and consumption throughout the process.

From the ethanol concentration profiles (Figure 1), it is obvious that the ethanol concentration has not reached its equilibrium and can be increased further if the fermentation is prolonged. Some runs showing high maltoheptaose (Figure 2) and glucose (Figure 3) at the end of 78 h fermentation further confirmed that the fermentation time can be extended to increase the ethanol yield. On the other hand, it is difficult to determine the significant factors that affect the ethanol production and its byproducts, thus a statistical method was employed to analyze the rank and significance of the factors.

Despite some literatures that reported that pH has significant effect on the fermentation process, it was not reported in this study due to highly viscous media which created difficulty for pH control. Thus, the initial pH was set at pH 5.7 ± 0.25 for each experiment. The same applies to dissolved oxygen tension (pO_2) which was set at about 50% of oxygen at the beginning of each experiment. The experimental design matrix and the responses in terms of ethanol and its byproducts are respectively shown in Tables 2 and 3.

Maltoheptaose and glucose are byproducts produced prior to production of ethanol. Their presences imply that starch initially might have been degraded to maltoheptaose by ragi tapai and subsequently to glucose. Once the enriched media was inoculated with *S. cerevisiae*, glucose will be converted into ethanol. This work is aimed at maximizing the utilization of the two byproducts to enhance the ethanol yield. Besides that glycerol, lactic acid and acetic acid are the byproducts that accompanied ethanol or produced after the production of ethanol. These byproducts are the undesired outputs which would reduce the ethanol yield and therefore are desired to be minimized as much as possible. In view of that to analyze the Taguchi design of these byproducts, “the smaller is better” was selected for the signal to noise (S/N) ratio. Meanwhile, “the larger is better” was selected for ethanol yield.

Taguchi's signal-to-noise ratios serve as objective functions for optimization, aid in data analysis and prediction of optimum results. It has been employed in this work to study the most ideal condition for producing ethanol S/N ratio. The response for signal to noise ratios of the factors at the assigned level on maximum ethanol yield (larger-is-better) is summarized in Table 4 and Figure 4. As for minimum byproducts, the data are tabulated in Table 5 and Figure 5. Delta statistics in Tables 4 and 5 measure the size of the effects by taking the difference between the highest average and lowest average value for each response characteristic. Based on the delta value, the factors were ranked from the greatest to the least effect.

Figures 4 and 5 show the responses of main effect plot of S/N ratio for larger-is-better for ethanol product and

Table 2. Taguchi's experimental design matrix.

Run	Factor						
	NPK	**Urea**	**Temperature**	**Ragi**	***S. cerevisiae***	**Agitation**	**Time**
1	2	2	1	2	1	1	2
2	2	2	1	1	2	2	1
3	1	1	1	1	1	1	1
4	2	1	2	2	1	2	1
5	1	2	2	1	1	2	2
6	1	1	1	2	2	2	2
7	1	2	2	2	2	1	1
8	2	1	2	1	2	1	2

NPK, Nitrogen: Phosphorus: Potassium.

Table 3. The ethanol production and the byproducts.

Run	Responses (g/L)					
	Ethanol	**Glucose**	**Glycerol**	**Lactic acid**	**Acetic acid**	**Maltoheptaose**
1	41.20	0.13	2.05	0.00	0.02	2.99
2	31.93	0.00	1.54	2.88	0.00	2.26
3	15.30	0.00	0.31	0.00	0.00	11.82
4	25.88	38.00	1.71	0.00	0.00	0.46
5	29.11	26.66	2.19	0.11	0.00	11.65
6	28.88	2.46	0.45	2.16	0.00	0.65
7	32.86	20.53	1.56	0.76	0.02	21.00
8	24.05	29.19	0.92	0.00	0.00	21.52

Table 4. Response table for S/N ratios for "larger-is-better".

Factor	Level 1	Level 2	Delta	Rank
NPK	28.13	29.57	1.44	4
Urea	27.20	30.50	3.30	1
Temperature	27.82	28.87	0.05	7
Ragi tapai	27.67	30.03	2.36	2
S. cerevisiae	28.38	29.31	0.93	5
Agitation	28.49	29.21	0.72	6
Co-culturing time	28.09	29.60	1.51	3

Table 5. Response table for S/N ratios for "smaller-is-better".

Factor	Level 1	Level 2	Delta	Rank
NPK	-15.679	-14.502	1.177	7
Urea	-16.710	-13.471	3.239	2
Temperature	-6.803	-23.378	16.575	1
Ragi tapai	-16.492	-13.689	2.803	4
S. cerevisiae	-16.398	-13.783	2.615	5
Agitation	-16.313	-13.868	2.445	6
Co-culturing time	-16.613	-13.568	3.046	3

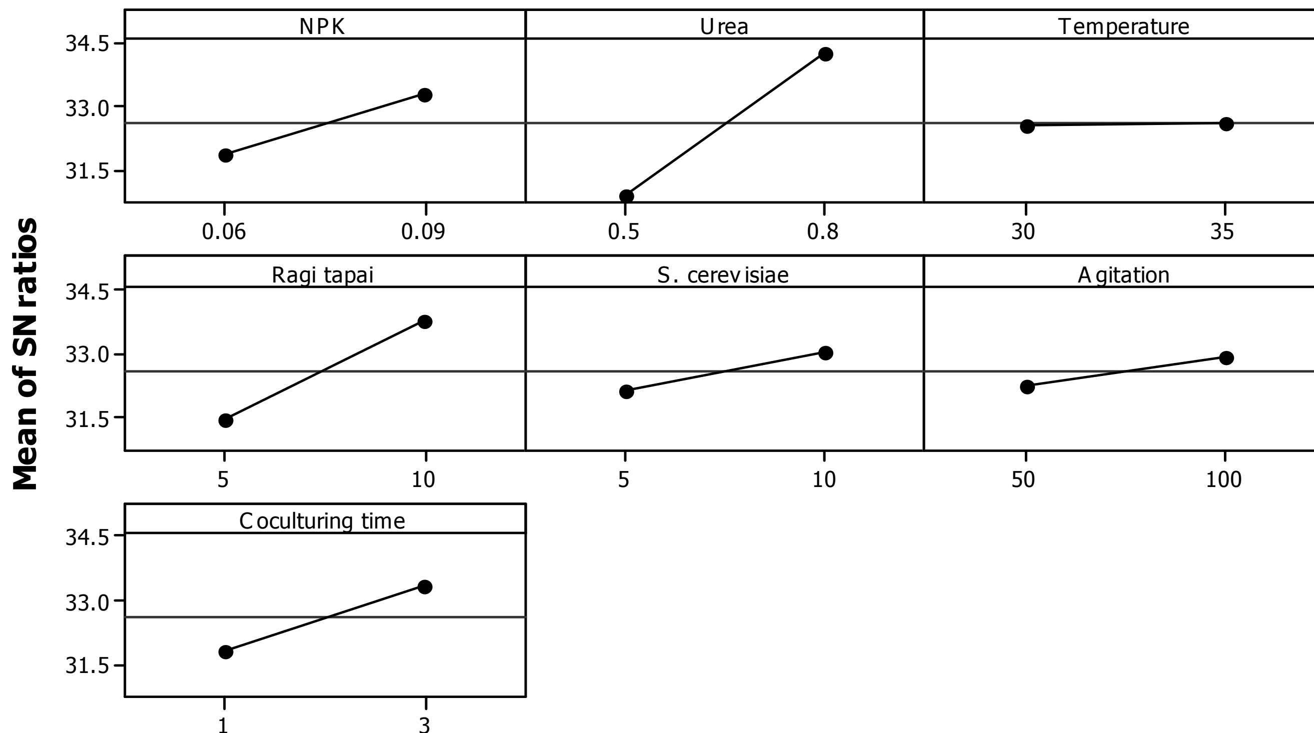

Figure 4. Response plot of S/N ratio (larger-is-better) for ethanol production.

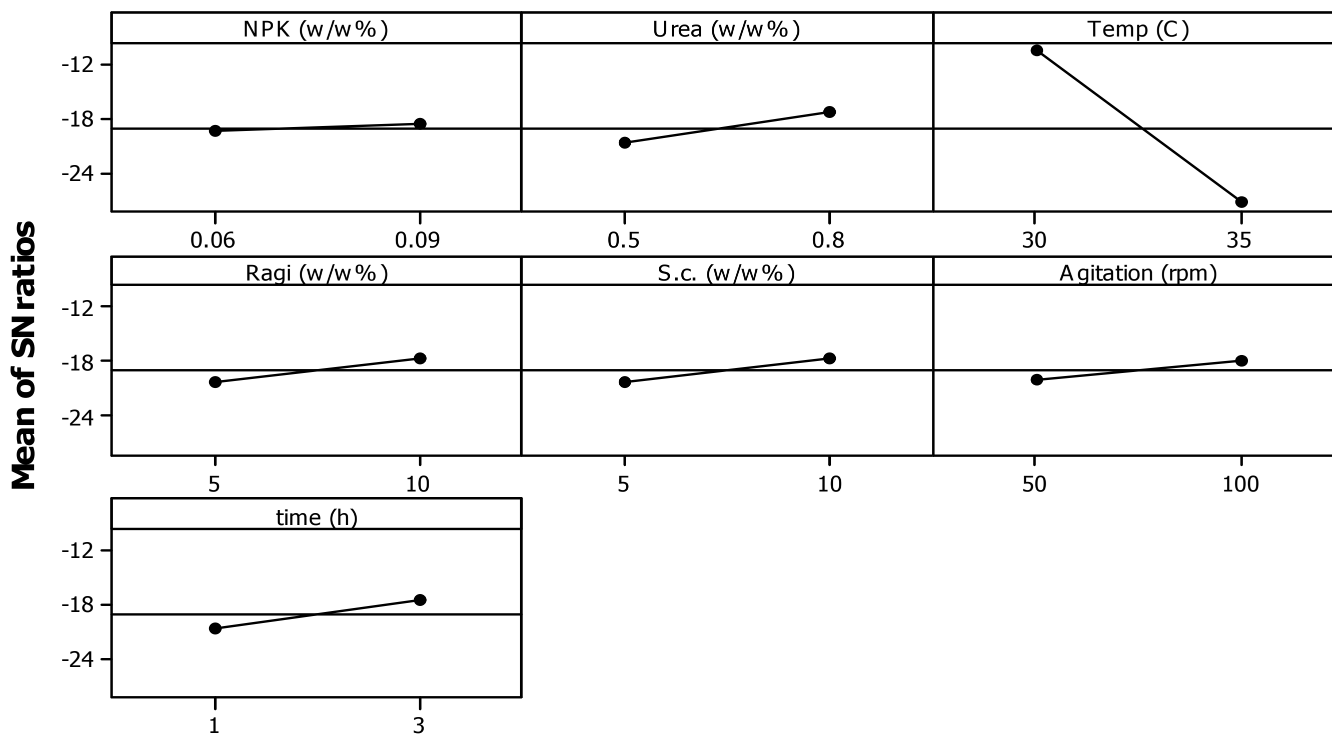

Figure 5. Response plot of S/N ratio (smaller-is-better) for ethanol byproducts.

Table 6. The optimal configuration for ethanol production in the order of significance.

Rank	Larger-is-better		Smaller-is-better	
	Constituent	**Level**	**Constituent**	**Level**
1	Urea	0.8 w/w%	Temperature	30°C
2	Ragi tapai	10 w/w%	Urea	0.8 w/w%
3	Cocult. time	3 h	Agitation	100 rpm
4	NPK	0.09 w/w%	Ragi tapai	10 w/w%
5	*S. cerevisiae*	10 w/w%	*S. cerevisiae*	10 w/w%
6	Agitation	100 rpm	Cocult. time	3 h
7	Temperature	35°C	NPK	0.09 w/w%

smaller-is-better for byproducts, respectively. The greater the slope inclines, the larger the ratio and the bigger the signal as compared to the noise. Therefore, to determine the optimal configuration, selection of level with large S/N ratio is required. As a result, the optimal configuration and significance of the rank based on the two response tables and graphs, were summarized in Table 6 to demonstrate the significant level of the responses.

Table 6 shows that urea is the most significant factor for high yield of ethanol. Urea provides excellent nitrogen source which is essential for growth (Wong et al., 2008; Manginot et al., 1997). Adding nitrogen source to fermentation media at early stage of fermentation increases the cell population and the fermentation rate, subsequently lowering the fermentation time (Manginot et al., 1997; Thomas and Ingledew, 1990). Studies also showed that different nitrogen sources have different efficiency of fermentation. In this study, a small amount of nitrogen-phosphorus-potassium (NPK) was supplied to the fermentation medium. At the applied quantity, the effect is incomparable with urea as the nutrient. However, from the rank of significant factors, it shows that nutrients (from both urea and NPK) play major roles in producing higher ethanol yield and can be further investigated.

Table 6 also shows that ragi is the second most significant factor in producing ethanol. At 70°C (cooking temperature of starch), the starch was softened for further degradation by ragi as the mixture is a good source of amylolytic microbes (Kuriyama et al., 1997). The softened cooked starch is mainly degraded into oligosaccharides and glucose for *S. cerevisiae* to be converted into ethanol. Compressed air was supplied at the beginning of the experiment and about 50% of dissolved oxygen tension was provided for fungus by ragi; followed by an anaerobic environment for *S. cerevisiae* to effectively convert glucose into ethanol. Since there was no aeration involved, no gas dispersion from agitation was required in this work.

The third significant ranked factor is the co-culture time. The time with 3 h gap between inoculation of ragi at zero hour and *S. cerevisiae* at 3 h later, suggested that the availability of oligosaccharides and glucose in the medium as carbon source for *S. cerevisiae* is essential to boost the ethanol production and to increase the ethanol yield.

Eventhough the rank of significance is not in the same order (Table 6), the optimum values for both levels in "larger-is-better" for high ethanol yield and "smaller-is-better" for low byproducts yield are in agreement with each other except for fermentation temperature. This is because the prefered temperature to produce high yield of ethanol is at 35°C, whereas to reduce byproducts, the temperature should be operated at 30°C. However, for high ethanol yield, the temperature is of least significant and can be disregarded. Conversely, in the minimization of the ethanol byproducts, temperature is the most significant contributing factor. Taking these two points into consideration, the preferable fermentation temperature is 30°C. To confirm this, predicted Taguchi results are available in Minitab v14, the S/N ratios and their means are tabulated in Table 7 based on the optimal configuration presented in Table 6.

Table 7 shows that the S/N ratio and mean values for the optimized fermentation parameter at temperature of 35°C (favors ethanol production) is slightly different as compared to those at 30°C (favors minimum production of byproducts). Therefore, it can be concluded that 30°C is suitable to be adopted in the fermentation process.

From the optimum parameters presented in Table 7, a validation experiment in 10 L fermentor was conducted at 30°C for 11 days (264 h). The ethanol and its byproducts concentrations are plotted in Figure 6. Due to the bigger fermenter with 8 folds volume increment, slower responses were expected. The validation experiment gave 68.00 g/L of ethanol which was 65.05% higher than the maximum ethanol production (41.20 g/L) in 1 L fermentor. At the end of fermentation, all byproducts concentrations were below 1.50 g/L, except for lactic acid at 9.00 g/L. Acetic acid was not detected at all (not shown) in the course of the fermentation.

Conclusion

Taguchi's DOE was successfully applied to test the relative importance of seven factors identified in this study. The optimum values for each factor were in agreement with each other except for the fermentation

Table 7. Predicted Taguchi's result.

Constituent	Larger-is-better	Smaller-is-better
NPK (w/w%)	0.09	0.09
Urea (w/w%)	0.8	0.8
Temperature (℃)	35	30
Ragi (w/w%)	10	10
S .cerevisiae (w/w%)	10	10
Agitation (rpm)	100	100
Co-culture time (h)	3	3
S/N ratio	34.0009	33.9490
Mean	42.0003	43.3525

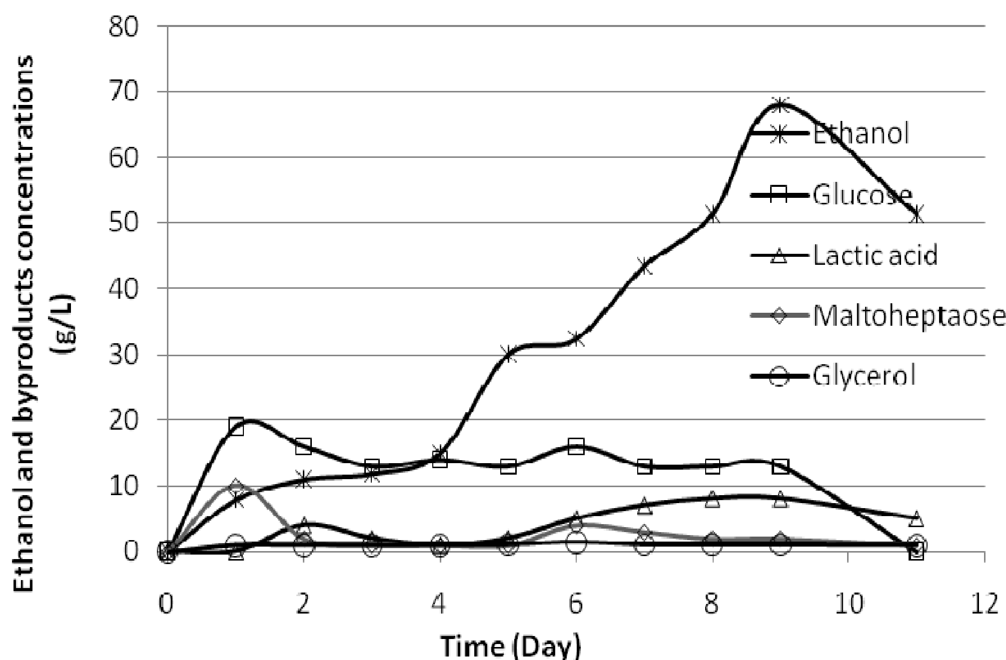

Figure 6. Ethanol and byproducts concentration profile in 10 L fermentor.

temperature. For "larger-is-better", the temperature is 35℃, while for "smaller-is-better" it is 30℃.

Since the fermentation temperature for "smaller-is-better" at 30℃ is more significant, it was recommended for the validation experiment. Using predicted Taguchi result at this fermentation temperature, an increase in ethanol concentration was obtained which gave rise to a 65.05% of ethanol yield, while all other byproducts concentrations were below 9.00 g/L at the end of fermentation.

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