African Journal of Microbiology Research Vol. 6(4), pp. 704-712, 30 January, 2012
Available online at http://www.academicjournals.org/AJMR
DOI: 10.5897/AJMR11.576
ISSN 1996-0808 ©2012 Academic Journals

Full Length Research Paper

Ethanol production by *Zymomonas mobilis* PTCC 1718 using low cost substrates

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Accepted 19 October, 2011

We report our attempts to increase ethanol production from *Zymomonas mobilis* PTCC 1718, by optimization of the components of growth medium and culture conditions. First, low cost substrates, carbon and nitrogen sources, temperature and initial pH were optimized by one-factor-at-a-time method. Then, nutritional improvement was performed using Taguchi method. Amongst the different selected sources, sucrose and wheat bran have significant effects on ethanol production. The optimum temperature, inoculum-size and pH were 50°C, 12% (v/v) and 5.5, respectively. The optimal concentrations of nutritional components for improved ethanol production were: 2.5 g/L sucrose, 1.5 g/L whey, 0.25 g/L wheat bran extract, 0.5 g/L yeast extract, 0.25 g/L peptone, 0.7 g/L KH_2PO_4 , 0.1 g/L $(NH_4)_2SO_4$ and 0.05 g/L $MgSO_4$. Ethanol production, which was 14.49 g/L (w/v) in the basal medium, increased to 41.67 g/L (w/v) and 86.26 g/L (v/v); about 2.8 and 5.9-fold higher than that in the basal medium, after optimization with one-factor-at-a-time and Taguchi method, respectively. Bioreactor fermentation was carried out in a 15 L stirred fermentor under optimal condition. Maximum ethanol production in 15 L fermentor indicated 91.22 g/L (w/v), about 6.3 fold higher than the basal medium.

Key words: Ethanol, medium components, *Zymomonas mobilis* PTCC 1718, optimization, Taguchi method.

INTRODUCTION

Ethanol production through biotechnological means has acquired considerable interest due to possible utilization of bio-ethanol as an alternative fuel. The rise in prices and environmental problems caused by fossil fuels has contributed to this recent interest from economical and ecological perspectives (Khaw et al., 2007; Patle and Lal, 2008; Yamshita et al., 2008). To make the price of bio-ethanol more competitive, yield of ethanol needs to be improved through optimization of culturing medium. Also, the cost of production could be reduced by using locally available alternative nutrients that meet the microbial requirement (Ruanglek et al., 2006).

Zymomonas mobilis, a gram-negative ethanol producing bacterium, has been of considerable interest in recent years for ethanol production because it gives a near-theoretical yield of ethanol from glucose and fructose. It is an osmo-and ethanol-tolerant bacterium that has shown higher specific rates of glucose uptake and ethanol production via the Entner-Doudoroff pathway under anaerobic conditions (Roger et al., 1982, 1997; Yamshita et al., 2008).

Fermentation technologies utilizing strains of *Z. mobilis* in place of the traditional yeast, have been proposed by a number of authors, as they have been shown to ferment under fully anaerobic conditions with faster specific rates of glucose uptake and ethanol production as well as ethanol yields close to theoretical (Rogers et al., 1979; Doelle et al., 1993; Davis et al., 2006).

Currently, use of low cost substrates as potential sources for ethanol production by *Z. mobilis* has been considered in industry. Some investigators have attempted to study the consequence of using some low,

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cost materials such as thippi, starch cassava, tapioca, starch from feedstock, sago starch, waste starch stream paper sludge and wheat stillage on ethanol production by *Z. mobilis* (Davis et al., 2006; Yamshita et al., 2008; Sonali and Banwari, 2008), but to the best of our knowledge, there is little information concerning the effect of wheat bran, rice bran, whey, potato wastes and barley flour on ethanol production by this strain. The purpose of this study is to optimize medium components and culture conditions to improve ethanol production by *Z. mobilis* PTCC 1718 using one-factor-at-a-time and Taguchi's arrays designs. At first, the effects of various carbon, nitrogen sources and environmental factors were investigated by one-factor-at-a-time method, and then the concentration of medium components was optimized using Taguchi method as a fractional factorial design. Also, the effects of some low cost materials such as wheat bran, rice bran, whey, potato wastes and barley flour on ethanol production were evaluated in some details.

MATERIALS AND METHODS

Di-phenylcarbazide powder and all other materials and media used for optimization study were obtained from Merck Co., Darmstadt, Germany.

Organism

Z. mobilis PTCC 1718 (DSMZ 424) obtained from the Persian Type Culture Collection (PTCC, Tehran, Iran) was used for ethanol production throughout this study.

Medium and culture conditions

Lyophilized *Z. mobilis* was grown in a medium containing 10.0 g/L bacto peptone, 10.0 g/L yeast extract, 20.0 g/L glucose, and 15.0 g/L agar in anaerobic conditions at 30°C. For ethanol production, the bacteria were cultured in a basal medium containing peptone (2.5 g/L), yeast extracts (2.5 g/L), glucose (15.0 g/L), KH_2PO_4 (0.1 g/L), $MgSO_4$ (0.1 g/L), and $(NH_4)_2SO_4$ (0.1 g/L) (pH 4.5). Flask culture experiments were performed in 250 ml-Erlenmeyer flasks containing 50 or 100 ml of media after inoculating with 8% (v/v) of seed culture prepared in the basal medium. Fermentations were carried out in a static state at 37°C.

Pretreatment procedures for low-cost substrates

Pretreatments for rice and wheat bran were performed by mixing with distilled water 10% (w/v) and alpha amylase (α-amylase from *Bacillus amyloliquefacience* E.C. 3.2.1.1 obtained from Sigma-Aldrich). The mixture was incubated at 70°C for 24 h. Then, the mixture was boiled at 100°C for 1 h for deactivation of α-amylase enzyme and the filtrate was used in the medium. For potato extract preparation, sliced potato was mixed with distilled water 30% (w/v) and boiled until thoroughly cooked. The mixture was filtered through cheese-cloth and used as substrate in the medium. Pretreatment of barley flour was made by mixing with distilled water 10% (w/v) and boiled at 100°C for 20 min and the filtrate added in the medium.

Ethanol assay

The ethanol was assayed by dichromate colorimetric method (Williams et al., 1950). The reaction mixture containing 1 ml each of the sample, potassium dichromate 50 g/L and saturated di-phenylcarbazide was heated at 90°C for 5 to 15 min until it turned brown. Then, 1 ml of sodium potassium tartrate (40%) (Rochelle salt) was added for stabilization of the produced color. The absorbance was measured at 575 nm. The concentration of ethanol was calculated from a standard curve covering the concentration range (0.1 to 0.01%) of ethanol.

Optimization of medium components and culture conditions by one-factor-at-a-time method

To find a suitable carbon source for ethanol production by *Z. mobilis* PTCC 1718, different carbon and nitrogen sources including xylose, fructose, rafinose, lactose, manitol, maltose, sucrose and glucose were used at 15 g/L in basal medium. Also, the effect of sucrose at concentrations of 25, 35, 45, 55, 65 and 80 g/L were studied on ethanol production.

The selected nitrogen sources for optimization process were peptone, ammonium nitrate, potassium nitrate, wheat bran, whey, barley flour, rice bran and potato extract. The nitrogen sources were used at 2.5 g/L in basal medium. The effect of wheat bran, whey, peptone and yeast extract at concentrations of 2.5, 5.0, 7.5 and 10 g/L were studied on ethanol production.

To examine the effects of temperature on ethanol production, *Z. mobilis* PTCC 1718 was cultivated at temperatures: 20, 25, 30, 35, 40, 45, 50, 55, 60 and 65°C. In order to study the effects of initial pH on ethanol production, various pH; including 3.5, 4.5, 5.5, 6.5, 7.5 and 8.5 were tried.

Taguchi's array for medium optimization

To examine the interactions among nutritional components of production medium and optimize their concentrations for ethanol production, Taguchi's arrays were used at two steps: at first a L_{12} (8^2) orthogonal array was selected to study the effects of eight 2-level of medium components on ethanol production (Table 2). At the second step, a L_9 (4^3) was used to adjust the concentration of most effective factors (four 3-level) on ethanol production (Table 3). The orthogonal arrays, inner arrays and ANOVA were obtained using Minitab15 software based on Taguchi's method.

Bioreactor fermentation

Batch fermentations were performed in a 15-L stirred fermentor (MBR Co., Switzerland). The following conditions were considered for fermentation process: temperature (50°C), agitation speed (120 rpm), aeration rate (0.5 vvm), working volume (5 L) of optimized medium and initial pH (5.5). The maximum specific growth rates (*μmax*), maximum specific rates of sucrose uptake (*qs*) and ethanol production (*qp*), as well as the yields of ethanol (*Yp/s*) and biomass (*Yx/s*) based on sucrose consumed, were calculated using equations described earlier and pre-programmed in a Microsoft Excel Spreadsheet Program.

RESULTS AND DISCUSSION

As shown in Table 1, among the sources evaluated, the highest ethanol production was obtained in media containing low cost substrates such as wheat bran

Table 1. Effect of carbon and nitrogen sources on ethanol production by *Z. mobilis* PTCC 171 in shake flask culture.

Carbon and nitrogen sources	Ethanol production g/L(w/v)
Xylose	6.20± 0.09
Fructose	7.60± 0.03
Rafinose	8.39±0.05
Lactose	7.64±0.08
Manitol	7.86±0.12
Maltose	7.75±0.06
Sucrose	7.98±0.11
Glucose	5.30±0.15
Ammonium nitrate	11.2±0.13
Potassium nitrate	12.4±0.19
Barley flour	19.4±0.18
Wheat bran	26.9±0.14
Rice bran	19.6±0.07
Potato extract	18.2±0.17
Whey	19.9±0.10
Control(Basal medium with glucose)	8.78±0.16

Fermentations were carried out for 132 h at 37°C with initial pH 4.5. Values are mean ±S.D. of triple determination.

extracts, rice bran extracts, whey and potato extracts. Amongst various monosaccharide and disaccharides, rafinose and sucrose have significant effect on ethanol production. The ethanol production in the medium containing rafinose was higher. We used sucrose due to its low material cost in medium formulation. Amongst different sucrose concentrations, the highest ethanol production was about 15.25 g/L (w/v) in medium containing sucrose at concentration of 25 g/L.

Higher ethanol production was seen in the media containing whey, peptone and yeast extract at concentration of 2.5 g/L. Also, the maximum ethanol yield was observed in medium containing wheat bran at concentration of 5.0 g/L.

The optimal temperature and pH for ethanol production was found to be 50°C and 5.5, respectively. The best results in fermentative xylose conversion: 75% xylose utilization and 76% ethanol yield was observed at pH 10 in conditioned hydrolysate of hemicelluloses (Mohagheghi et al., 2006). Optimum values of temperature 32.4°C and pH: 4.93 for the production of ethanol from sago starch by co-immobilized amyloglucosidase and cells of *Z. mobilis* have been reported (Bandaru et al., 2006).

The effect of different inoculum size, including 4, 8, 12, 16 and 20% (v/v), were studied on ethanol production by *Z. mobilis* PTCC 1718. In a medium optimized for carbon and nitrogen sources, the best inoculum size for ethanol production was 12% (v/v).

To optimize culture medium components, Taguchi's array were used in two stages. In the first stage, Taguchi's array factor and level assignments including the main effects of each factor and interactions are given in Table 2. An L_{12} (8^2) orthogonal array was selected.

The values of level 1 were based on optimized medium determined by one-factor-at-a-time method and each value for level 2 was twice of the level 1. Also, the experimental conditions for each run and ethanol yield are included in Table 2. The mean of main effects of each factor on ethanol production is shown in Figure 1. As shown in Figure 1, the ethanol production is higher for sucrose (A), whey (B), peptone (E) and KH_2PO_4 (F) at level 2 in the medium. There is no significant higher ethanol productions for wheat bran (C) and $(NH_4)_2SO_4$ (G) at levels 1 and 2 in the medium. Also, ethanol production for yeast extract (D) and $MgSO_4$ (G) is higher at level 1 in the medium. The ANOVA for the experimental results obtained by optimal levels of each factor for obtaining higher ethanol production are given in Table 4. Also, the contribution percent and interaction between each medium component are included. The order of factor effects on ethanol production was sucrose>whey>KH2PO4>MgSO4>peptone>wheat bran> (NH4)2SO4 >yeast extract. The results showed that the effects of sucrose and whey are more significant than the other nutrients. According to the obtained results, sucrose showed interactions with whey, wheat bran and yeast extract. Ammonium sulfate had negligible effect on ethanol production by this strain. Results obtained in first Taguchi's array allowed further adjustments of the most

Table 2. Assignment of experimental factors and their results in Taguchi's optimization method first stage.

Level	Factors							
	A, Sucrose (g/L)	B,Whey(g/L)	C, Wheat extract(g/L)	D, Yeast extract(g/L)	E, Peptone(g/L)	F, KH2PO4 (g/L)	G, (NH4)2SO4(g/L)	H, MgSO4(g/L)
1	0.75	0.25	0.25	0.25	0.25	0.1	0.1	0.05
2	1.5	0.5	0.5	0.5	0.5	0.2	0.2	0.1

	Run								
	A	B	C	D	E	F	G	H	Ethanol production g/L(w/v)
1	1	1	1	1	1	1	1	1	16.48±0.12
2	1	1	1	1	1	2	2	2	14.83±0.10
3	1	1	2	2	2	1	1	1	13.25±0.16
4	1	2	1	2	2	1	2	2	35.50±0.08
5	1	2	2	1	2	2	1	2	30.78±0.19
6	1	2	2	2	1	2	2	1	43.25±0.07
7	2	1	2	2	1	1	2	2	20.62±0.14
8	2	1	2	1	2	2	2	1	49.47±0.02
9	2	1	1	2	2	2	1	2	43.59±0.22
10	2	2	2	1	1	1	1	2	51.95±0.31
11	2	2	1	2	1	2	1	1	56.14±0.44
12	2	2	1	1	2	1	2	1	50.72±0.19

Fermentations were carried out for 132 h at 50°C with initial pH 5.5. Values are mean ±S.D. of triple determinations.

effective components on ethanol production. A L_9 (4^3) was performed. Table 3 shows the factor and levels assignments including the main effects of medium components on ethanol production. The main effects plot for ethanol production by each factor is shown in Figure 2. As shown in Figure 2, the ethanol production was higher for sucrose (A) and whey (B) at level 3 in the medium. The effect of KH_2PO_4 (C) and $MgSO_4$ (D) were more significant at level 2 and 1, respectively. The order of factor effects on ethanol production was sucrose>whey >$MgSO_4$>KH_2PO_4. Sucrose showed significant interaction with whey for ethanol production at this stage. So, sucrose and whey could be very useful low cost substrates for ethanol production by *Z. mobilis*. The ANOVA for the experimental results obtained by optimal levels of each factor for obtaining higher ethanol production are given in lower part of Table 4. Also, the contribution percent and interactions between medium components are included in Table 4. The effect of other low cost substrates such as wheat bran, potato extracts, rice bran, and barley flour together with whey and sucrose were studied in optimization process. Table 5 presents a summary of comparative kinetic data for ethanol production including the maximum specific growth rates (*μmax*), maximum specific rates of sucrose uptake (*qs,max*) and ethanol production (*qp,max*), as well as the yields of ethanol (*Yp/s*) and biomass (*Yx/s*) based on sucrose consumption. As shown in the Table 5, higher specific of sucrose uptake and ethanol production were seen in medium containing whey and wheat bran extract as low cost substrates. According to the obtained results, higher specific of sucrose uptake and ethanol production is due to interaction among sucrose, whey and wheat bran in the medium. The maximum specific rates of sucrose uptake and ethanol production determined in this study were 7.8 and 4.2 g/g/h, respectively. The maximum ethanol yield in the medium containing sucrose, whey and wheat bran was 75.35 g/L (w/v) about 5.16-fold higher than the basal medium. The maximum specific rate for glucose uptake of 7.0 g/g/h and ethanol production of 3.4 g/g/h for medium containing hydrolysed wheat flour (glucose equivalent 110 g/L) supplemented

Table 3. Assignment of experimental factors and their results in Taguchi's optimization method second stage.

Level	Factors			
	A, Sucrose (g/L)	B, Whey (g/L)	C, KH_2PO_4 (g/L)	D, $MgSO_4$(g/L)
1	1.5	0.5	0.2	0.05
2	2.0	1.0	0.7	0.1
3	2.5	1.5	0.12	0.15

Run					
	A	B	C	D	Ethanol production g/L(w/v)
1	1	1	1	1	41.38±0.18
2	1	2	2	2	39.76±0.23
3	1	3	3	3	49.01±0.11
4	2	1	2	3	56.91±0.21
5	2	2	3	1	51.79±0.14
6	2	3	1	2	44.87±0.29
7	3	1	3	2	45.27±0.45
8	3	2	1	3	45.03±0.62
9	3	3	2	1	86.07±0.33

Fermentations were carried out for 132 h at 50°C with initial pH 5.5. Values are mean ±S.D. of triple determinations.

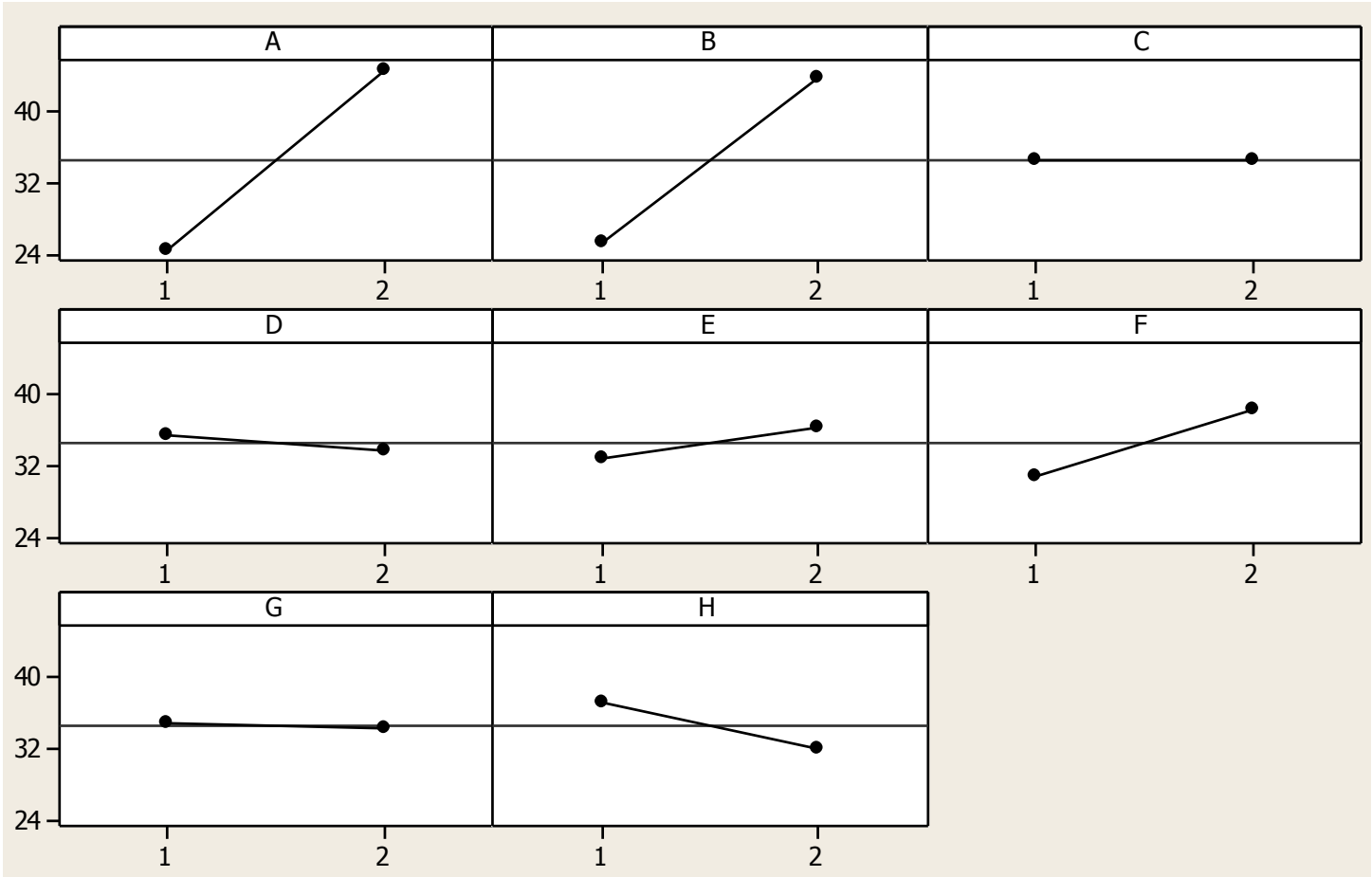

Figure 1. The mean of main effects plot for each level ("1" and "2") medium component; A), Sucrose, B) Whey, C) Wheat bran, D) Yeast extract, E) Peptone, F) KH_2PO_4, G) $(NH_4)_2SO_4$ and H) $MgSO_4$ on ethanol production by *Zymomonas mobilis* PTCC 1718 in first Taguchi array.

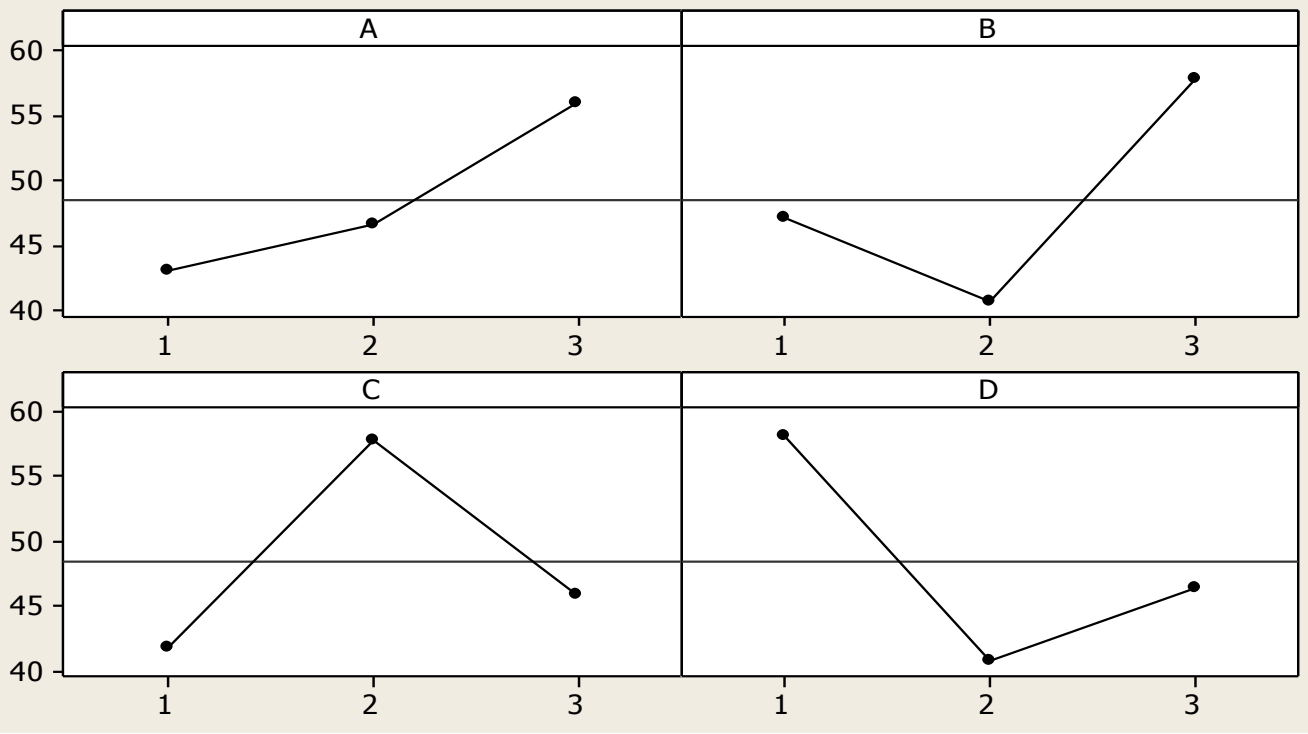

Figure 2. The mean of main effects plot for each level (“1”, “2 and “3”) medium component; A) Sucrose, B) Whey, C) KH_2PO_4 and D) $MgSO_4$ on ethanol production by *Z. mobilis* PTCC 1718 in second Taguchi array.

Table 4. Results of ANOVA and optimal factor levels by Taguchi method (first and second stages).

Method	DOF	Sum of mean square	Mean square	F-value	Contribution percent	Optimum level
First Taguchi						
Sucrose	1	2452.658	2452.658	418.7990	43.505	2
Whey	1	2047.950	2047.950	349.6939	36.326	2
Wheat extract	1	0.003	0.003	0.0005	0.000	1
Yeast extract	1	15.049	15.049	2.5697	0.266	1
Peptone	1	71.605	71.605	12.2268	1.270	2
KH_2PO_4	1	363.240	363.240	62.0244	6.44	2
$(NH_4)_2SO_4$	1	0.933	0.933	0.1593	0.0165	2
$MgSO_4$	1	156.265	156.265	26.6828	2.771	1
A*B	1	107.878	107.878	18.4205	1.913	
A*C	1	195.414	195.414	33.3676	3.466	
A*D	1	226.581	226.581	39.6895	4.019	
Second Taguchi						
Sucrose	1	506.722	506.722	34.99421	16.08	3
Whey	1	344.411	344.411	23.78503	10.93	3
KH_2PO_4	1	48.664	48.664	3.36075	0.00	2
$MgSO_4$	1	412.454	412.454	28.48406	13.09	1
A*B	1	1295.583	1295.583	89.47296	41.13	
A*C	1	453.964	453.964	31.35077	14.41	
A*D	1	136.177	136.177	9.40437	4.32	

Table 5. Summary of the kinetic parameters for the batch fermentation of *Z. mobilis* PTCC.

Parameter	Media			
	Whey + Wheat bran + Sucrose	Whey + Rice bran + Sucrose	Whey + Potato extract + Sucrose	Whey + Barly flour + Sucrose
μmax (L/h)	0.43	0.38	0.33	0.40
qs,max (g/g/h)	6.8	4.3	5.2	3.9
qp,max (g/g/h)	3.4	2.5	2.1	1.8
Overall				
Yp/s (g/g)	0.53	0.44	0.48	0.47
Yx/s (g/g)	0.021	0.032	0.025	0.019
Qp(g/L/h)	3.7	3.2	2.8	3.3

Fermentations were carried out for 48 h at 50°C with initial pH 5.5. Values are mean ±S.D. of triple determinations.

with 1 g/L $(NH_4)_2SO_4$ using *Z. mobilis* ZM4F have been reported (Torres and Barrati, 1988). From studies with saccharified wheat starch, lower maximum specific values of glucose uptake of 2.9 and 1 g/g/h for *Z. mobilis* NRRL14023 and Saccharomyces *cerevisiae* were reported by Wayman et al. (1988). For the various hydrolyzed waste starch (HWS), specific rates of glucose uptake and ethanol production in the ranges of 6.9 to 7.1 and 3.0 to 3.3 g/g/h was reported by Davis et al. (2006). According to the obtained results, whey and wheat bran have significant effect on ethanol production by *Z. mobilis* PTCC 1718.

After optimization process, the effect of omitting peptone and yeast extract in medium formulation was studied. Considerable reduction in ethanol production was observed upon excluding peptone and yeast extract. This finding was in agreement with Othumpangat et al. (1999) who reported that glucose and yeast extract are the key media component that influence ethanol production by *Z. mobilis*. Ethanol production using agro-industrial wastes as low cost-feed stock (thippi) by using *Z. mobilis* and *Candida tropicalis* was studied by Patle and Lal (2008). They found that enzymatic hydrolysis using amylase yielded very high amounts of reducing sucrose, and thus, proved thippi to be a potential substrate for ethanol production. A higher ethanol yield could be obtained with *Candida tropicalis* and *Z. mobilis* mixture during thippi hydrolysate fermentation.

In this study, the optimal concentrations of nutritional components for improved ethanol production were determined to be 25 g/L sucrose, 15 g/L whey, 2.5 g/L wheat bran, 5.0 g/L yeast extract, 2.5 g/L peptone, 7.0 g/L KH_2PO_4, 1.0 g/L $(NH_4)_2SO_4$, and 0.5 g/L $MgSO_4$.

Figure 3 shows time course comparison of ethanol production in basal and optimized media by one-factor-at-a-time and Taguchi methods. In basal medium, maximum ethanol production was obtained after 72 h of incubation. In this study, the improved medium obtained after one-factor-at-a-time and orthogonal array designs showed maximum ethanol production at 48 h of incubation. The ethanol production in optimized medium after one-factor-at-a-time and second stage of Taguchi were found to be 41.67 g/L (w/v) and 86.26 g/L (w/v); about 2.8-fold and 5.9-fold higher than the basal medium, respectively. After we obtained the optimal medium composition by statistical designs, we tested its feasibility in a 15-L stirred tank bioreactor.

Figure 4 shows the typical time courses of cell growth and ethanol production in a 15-L stirred tank bioreactor under optimized medium based on second Taguchi's array using wheat bran and whey as low cost substrates. By using the optimized medium, the maximum ethanol production was about 91.22 g/L (w/v) about 6.3 –fold higher than the basal medium.

Conclusion

The results obtained in this study are useful in scaling up the fermentation processes for ethanol production using low cost substrates such as sucrose, wheat bran and whey. Especially, whey in medium formulation increased production yield and might be a good ingredient considering its ease of use and low material cost in culture medium. Two optimization techniques used in this work can be efficiently applied to other fermentation processes for optimizing growth and production conditions.

ACKNOWLEDGEMENTS

Financial support of this project was provided by Islamic Azad University-Lahijan Branch. The authors thank Miss Katayon Dastan in microbiology research laboratory of Islamic Azad University-Lahijan Branch for her helpful assistance and also acknowledge Dr. Santanu Dasgupta in Department of Cell and Molecular Biology, Biomedical

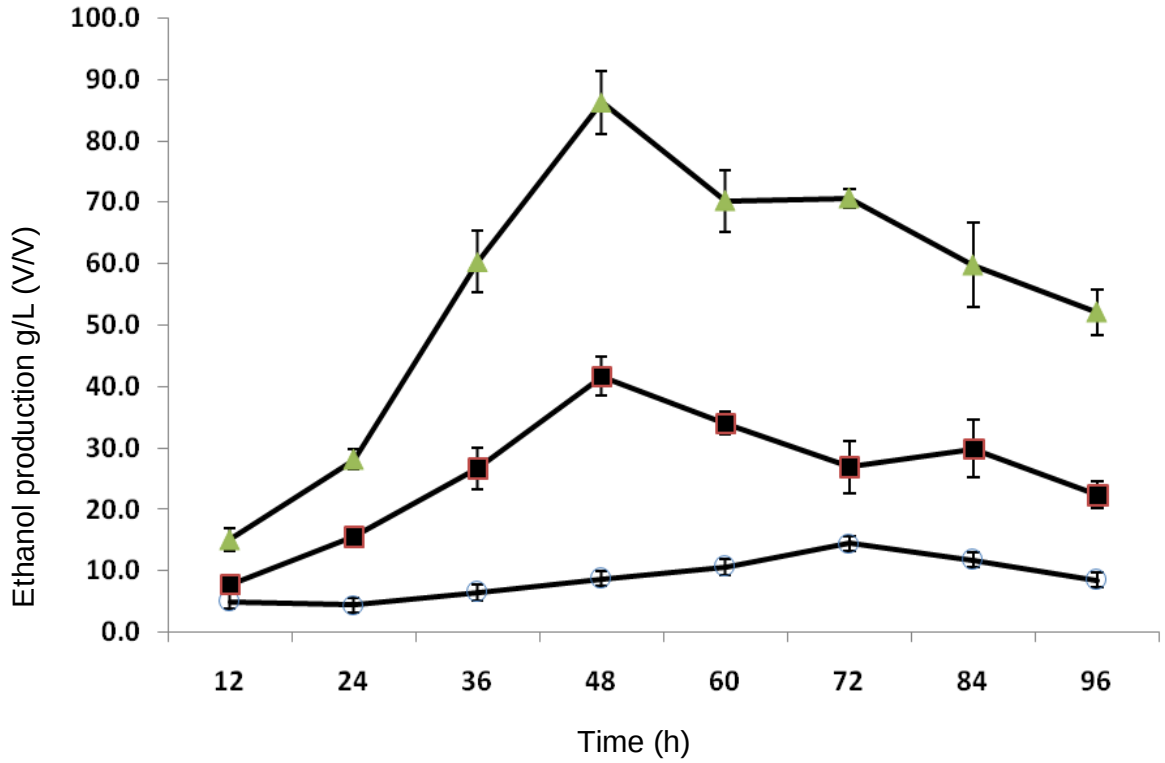

Figure 3. Time course of ethanol production by *Z.mobilis* PTCC 1718 in three growth media; (○) Basal medium, (■) optimum medium based on one-factor-at-a-time medium, (Δ) optimum medium based on second Taguchi arrays design. Experimental data are mean ± S.D. of triple determinations.

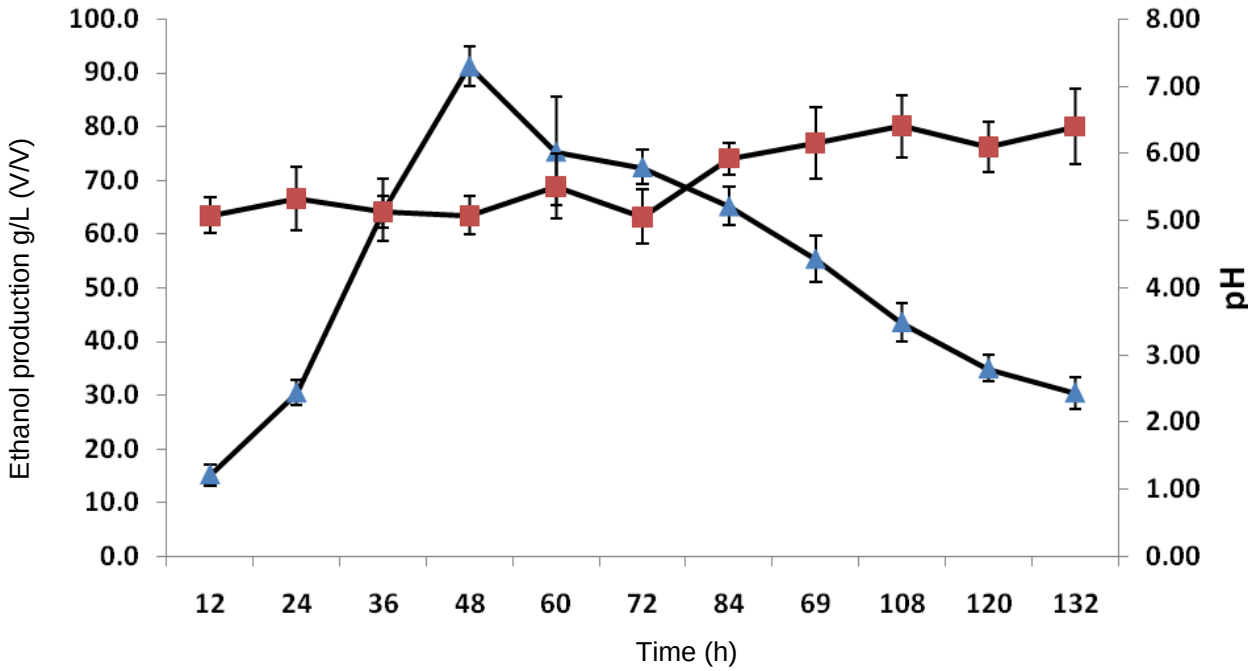

Figure 4. Time course of ethanol production by *Z.mobilis* PTCC 1718 in 15 L stirred- tank bioreactor under optimized medium based on second Taguchi 's array. Fermentations was performed under the following conditions: temperature, 50°C; agitation speed, 120 rpm; aeration rate, 0.5 vvm; working volume, 5 L of optimized medium and initial pH 5.5. Experimental data are mean ± S.D. of triple determinations.

Centre, Uppsala University for critical reading of the manuscript and providing thoughtful suggestions in improving the language and organization.

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