ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Optimization of Fermentation Parameters for Bioethanol Production from Waste Glycerol by Microwave Induced Mutant *Escherichia Coli.*

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ABSTRACT

Biodiesel being one of the most promising alternative and renewable biofuels has lately seen rapid increase in its production capacity. Along with that there has also been a great increase in production of the co-product, crude glycerol. More attention is being paid to the utilization of crude glycerol from biodiesel production in order to defray the production cost of biodiesel and to promote biodiesel industrialization on a large scale. Bioconversion of crude glycerol to bioethanol through microbial fermentation is a very promising application of glycerol. The yield of bioethanol from fermentation of glycerol is greatly influenced by various parameters such as temperature, pH, glycerol concentration, organic nutrient concentration, and agitation speed and the bacterial species. The present study was undertaken to investigate optimum parameters for bioethanol production from raw glycerol (biodiesel by-product) by mutant *Escherichia coli* (*E.coli*) (ATCC11505) strain immobilized on chitosan optimized by Taguchi statistical method. The initial parameters were set each at four levels and the orthogonal array layout of L_{16} (4^5) was conducted. The result indicated that the significant controlling parameters for optimizing the operational fermentation were temperature 36°C, medium pH 7.0, initial glycerol concentration (250 g/l), and organic source concentration (5 g/l). The predicted value of bioethanol production under optimized conditions was (117.6 g/l). Immobilized cells are mainly used for economic benefits of continuous production or repeated use in continuous as well as in batch mode.

Keywords: Bioethanol, *Escherichia coli* (ATCC 11105), Fermentation, Crude Glycerol, Chitosan, Immobilization, Optimization, Taguchi design

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ISSN: 0975-8585

INTRODUCTION

The ever increasing dependence on fossil fuels due to rapid growth in automotive industry along with power plants and home furnaces, particularly in large urban areas, has resulted in generation of high levels of pollution during the last few decades. In the last three decades there have been greater awareness and efforts made in order to mitigate climate change and improve energy security by sourcing fuels from renewable source (Balat, 2011). While biofuels currently only contribute a small share of the energy supply to the transport sector, several government and intergovernmental organizations have declared policy targets which can lead to a significant increase in transport biofuel utilization. In the EU, the policy is to achieve energy from renewable sources in the transport sector to at least 10% by 2020 (EC, 2009). All these factors have boosted the production capacity and market values of biodiesel and bioethanol, both of which are the most common biofuels and are gaining lots of attention compared to other categories of biofuels (Deenanath, et al., 2012). Bioethanol remains the most actively pursued biofuel at the industrial level. Conventional crops such as corn and sugarcane are unable to meet the global demand of bioethanol production due to their primary value of food and feed. Lignocellulosic substances such as agricultural wastes are attractive feedstocks for bioethanol production since they are cost effective, renewable and abundant. However, the technology for bioethanol production from agricultural waste has several challenges and limitations such as biomass transport and handling, and efficient pretreatment methods for total delignification of lignocellulosics.

Glycerol is formed as a major byproduct during the production of biodiesel (fatty acid methyl esters) from fats or oil. Approximately 1kg of glycerol is formed for every 10 kg of biodiesel produced. It has been estimated that approximately 4 billion gallons of crude glycerol would be produced by 2016 due to the 37 billion gallons of global biodiesel production (Yang, et al., 2012). Rapid increase in the production of biodiesel has created a glut of glycerol in market, thus, greatly reducing the price of glycerol and hence indirectly effecting the production cost of biodiesel as well (Pagliaro and Rossi, 2010; Ahmed and Papalias, 2010). Apart from the fall in economic/market value of industrial glycerol, crude glycerol surplus presents other challenges. The crude glycerol from the biodiesel by-product cannot be applied in any industrial process without prior purification. The impurities in the crude glycerol are diverse and may require complex and expensive processes to remove. Methanol, one of the impurities in crude glycerol, is known to be a toxic alcohol (Selembo, et al., 2009). The sodium or potassium hydroxide residue of the crude glycerol gives it elevated pH, which may endanger biotic community if crude glycerol is disposed without neutralization. Therefore, crude glycerol must be treated before disposal, hence a rapid, economically and effective purification method plus greater utilization schemes for the glycerol is required (Sarma, et al., 2012). Bioconversion of glycerol into liquid biofuels, green chemicals and bioenergy on the basis of fermentation processes can provide an efficient solution for sustainable management of glycerol, which can improve the economics of biodiesel industries (Lynd, et al., 2005). In this context, glycerol is used as a substitute for common, traditional substrates such as sucrose, glucose and starch (Suhaimi, et al., 2012; Nicol, et al., 2012; Nielsen, et al., 2003). Until recently, the fermentative metabolism of glycerol had been reported in species of the genera *Klebsiella, Citrobacter, Enterobacter, Clostridium, Lactobacillus, Bacillus, Propionibacterium, and Anaerobiospirillum* (Yazdani and Gonzalez, 2007). However, the potential for using these organisms at the industrial level could be limited due to issues that include pathogenicity, the need for strict anaerobic conditions and supplementation with rich nutrients, and unavailability of the genetic tools and physiological knowledge necessary for their effective manipulation. The use of microbes such as *Escherichia coli (E. coli)*, an organism very amenable to industrial applications, could help overcome the aforementioned problems Gonzalez, et al., (2008) had reported anaerobic fermentation of glycerol by *E. coli,* a species that had been long considered as incapable of utilizing glycerol for fermentation, however the yield was very low. The potentials for using *E. coli* at the industrial level could overcome issues such as pathogenicity, requirement of strict anaerobic conditions, and need of supplementation with rich nutrients. A native, nonpathogenic strain of *E. coli,* able to ferment glycerol to useful products under anaerobic condition without the need of genetic engineering tools, have been reported recently and more researches are actively looking at *E.coli* as the fermentative bacteria for bioethanol production (Dharmadi, et al., 2006; Chaudhary, et al., 2011; Suhaimi, et al., 2012; Adnan, et al., 2014; Saifuddin and Refal, 2015).

Recently, we reported of glycerol-utilizing mutant *Escherichia coli*-Microwave (EC-MW) strain for production of bioethanol using low power microwave technique (Saifuddin and Refal, 2014; Saifuddin and Refal, 2015). Several strategies have been developed to increase the concentration, yield and productivity of bioethanol. The immobilized-permeabilized mutant cells system appeared to be effective for promoting the

operational stability and reusability of cells (Saifuddin and Refal, 2015). Given the outstanding aspect of immobilized permeabilized mutant EC-MW strain as mentioned earlier, and to further enhanced production of bioethanol, it is essential to optimize the composition of culture media and process conditions. Optimization of process conditions is one of the most critical stages in the development of an efficient and economic bioprocess. Statistical methodologies involve use of mathematical models for designing fermentation processes and analyzing the process results (Bas, et al., 2007). In conventional methods, numerous experiments have to be carried out to optimize all the parameters (factors) to establish best possible conditions by interrelation of all the parameters. In these methods, studying one variable at a time is cumbersome and uneconomical. Another approach is to use statistical tools and experimental designs. Taguchi methods have been widely used to optimize the reaction variable by devising minimum number of experiments (Byrne and Taguchi, 1987). Taguchi experimental design is a fast and considerable way of optimization conferring remarkable outcome in simultaneous study of many factors Better quality at low cost is the main aim for generation of Taguchi design of experiments (DOE) approaches to maximize robustness of products and processes (Antony, et al., 1998).

In this study, the main objective is to optimize the parameters aspects of bioconversion of crude glycerol (as fermentation substrate) by immobilized permeabilized mutant EC-MW strain. The fermentation parameters were optimized by a well-known statistical method given by Taguchi (Dasu, et al., 2003; Chang, et al., 2006; Sirisansaneeyakul, et al., 2007; Davarey and Pakshirajan, 2010). In addition to statistical analysis of the result, the optimization fermentation parameters platform presented here is a valuable complementary approach described our earlier conversion of technological schemes (Saifuddin and Refal, 2014, 2015), which allows for identifying of underlying physiological variations imposed by different fermentation conditions.

MATERIALS AND METHODS

Bacterial Strain Mutation and Pre-culture Conditions

Microwave irradiation-induced mutation on *Escherichia coli* cells was performed according to the method described previously (Saifuddin and Refal, 2014). Briefly, wild type *E. coli* (ATCC 11105) cells were exposed to microwave irradiation at frequency 2.45 GHz, with output power 180W. The stock cultures were routinely stored in 20% (v/v) of sterile glycerol at -80°C. Prior to the experiment, the bacterial inoculum were propagated anaerobically with Mueller-Hinton broth at (150 rpm at 37°C for 72 hours) (Sigma-Aldrich, USA). The pre-cultured of the active growth phase ($O.D_{600}$=1.3) were centrifuged at (8000 rpm, 10 min). The centrifuged cells (bottom pellet) were washed with 0.9% (w/v) of sodium chloride solution followed by rinsing two times with distilled water to get rid of the growth broth. At the end, the cells mass was suspended in the sterilize sodium chloride and was stored at 4°C for further use.

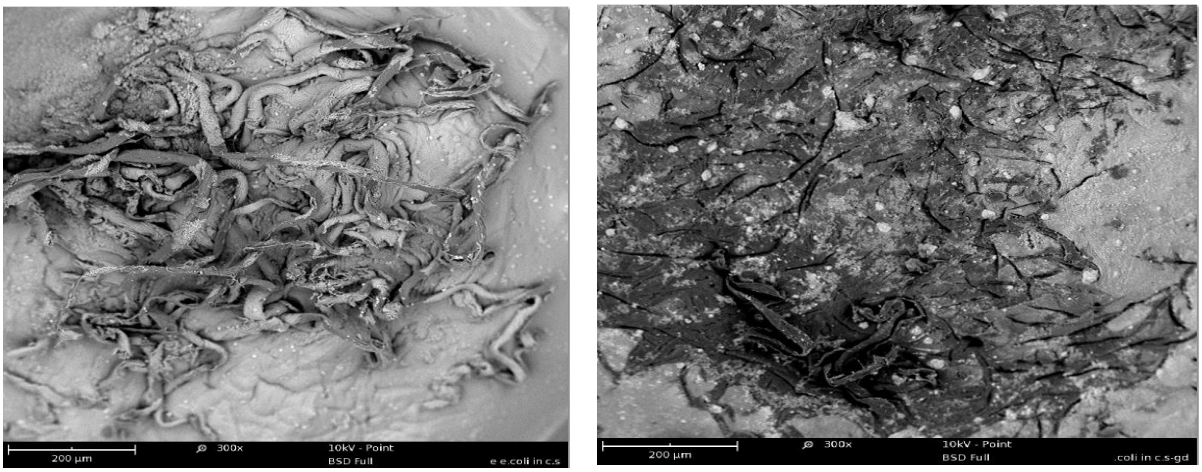
Immobilization of Permeabilized Mutant Escherichia coli on Crossed linked Chitosan

In this study, immobilization process was achieved by following the approach described by Saifuddin and Refal, (2015), which was performed in two steps. In the first step, bacteria strain of (EC-MW) was permeabilized by incubation with N-cetyl-N, N, N-trimethyl ammonium bromide at (0.1% w/v, 45 min, 8000 rpm). Afterward, the resultant cells were added into chitosan solution (3 g chitosan in 100 mL of 2% acetic acid), and stirred for 30 min to get a uniform mixture suspension. This was then followed by dropped wise addition (using hypodermic syringe) of the chitosan suspension solution into gently stirred 2.0 M sodium hydroxide solution for gelled sphere (beads) formation. Finally the wet chitosan beads were cross-linked with 100 mL solution of the glutaraldehyde (25%) using a novel **microwave irradiation** method at (2.45 GHz, 200 W). as described previously. Subsequently, the cross-linking chitosan beads were collected and washed extensively with the distillate water, which were then left in distillate water at 4°C for experiment use.

Analysis of Immobilized mutant E. coli on Chitosan

Surface morphologies of the immobilization of the mutant *E. coli* attached on (a) chitosan beads and (b) cross-linking chitosan (with glutaraldehyde) at 300 x magnifications obtained was confirmed by scanning electron microscopy (SEM) analysis is depicted in figure1 (Saifuddin and Refal, 2015). Bioethanol production in the fermentation broth was monitored by using ethanol assay kit (Megazyme, K-ETOH). While the utilization of glycerol by immobilized *E.coli* cells was determined by using glycerol assay kit (Megazyme, K-GCROL).

ISSN: 0975-8585



(a) **(b)**

Figure 1: Scanning electron microscopy image of chitosan coated mutant bacteria cells (a), and cross-linked chitosan by glutaraldehyde (b). (Reproduced from: Saifuddin and Refal, 2015)

Anaerobic Fermentation of Glycerol Using Mutant E.coli

Crude glycerol samples were collected from Sime Derby Oil Mill, (Carey Island, Klang Malaysia). The as obtained, crude glycerol was a sticky dark brown solution with a high viscosity, and changed to a semi-solid substance on storage. The pre-treatment was done according to the procedure reported by (Saifuddin, et al., 2014) and the purity of the crude glycerol was about 65% (w/v).

The fermentation media for enrichment and cultivation of glycerol fermenting bacteria used in this experiment consist of 1 L of 10X MOPS (**3-N-Morpholino propanesulfonic acid)** buffered minimal media (100 ml 10X MOPS concentrate; 1 ml 1.32 M K_2HPO_4 ; 5 ml 1.90 M NH_4Cl; 1 ml 0.276 M K_2SO_4; 2.5 ml 4 mg/ml thiamine) supplemented with 1% 0.132 mM N_2HPO_4 and 0.1% 1 µmol sodium selenite. The minimal media was filtered using 0.2 µm millipore filter. The use of a more complex medium was deliberately avoided. Glycerol solution containing of partially purified glycerol made up to 1 L with distillate water was added to the minimal media. The final volume of the fermentation media was 2 L and the different starting of glycerol concentration used for Taguchi experimental design are as listed in table 1. The fermentations were carried out under anaerobic condition using 3.5 L flasks (working volume was 2.0 L) supplied with side port for sample withdrawal and the headspace was purged with nitrogen gas in order to maintain strict anaerobic conditions. The fermentation flasks were autoclaved before the fermentation process. According to Taguchi design, orthogonal array of sixteen experiments were used to evaluate the effect of five parameters which showed significant influence on the bioethanol production in four levels (table 1). Experiments were performed according to an experimental plan given in table 2. The concentration of the media was varied according to the experimental plan given in table 2. Subsequently, one hundred grams of cross-linked chitosan beads immobilized with *E. coli* mutant strain (*EC*-MW) were added into fermentation media. All flasks were purged for 4 min with nitrogen gas at the start and after every 24 hours of fermentation to maintain anaerobic conditions. The samples of fermentation broth were withdrawn after every 12 hours up to a period of 4 days by using hypodermic syringe. The syringe needle (not removed) was fitted with a 0.45 µm gas inlet-outlet filter attached on its base. This prevented air from entering, but allowed gaseous products to escape, and preventing pressure build-up of bioreactor system. The fermentation pH was adjusted using 0.1M HCl and 0.1M NaOH. All experiments trials were performed in triplicate.

Table 1: Selected fermentation parameters for the production of bioethanol from glycerol by *E. coli*

Parameters	Level 1	Level 2	Level 3	Level 4
A: Temperature C°	30	32	34	36
B: Medium pH	6.0	6.5	7.0	7.5
C: Glycerol concentration (g/l)	75	100	125	250
D: Tryptone concentration (g/l)	2	5	10	15
E: Agitation speed (rpm)	100	120	150	200

ISSN: 0975-8585

Optimization of Fermentation Parameters of Bioethanol Production Using Taguchi Method Orthogonal Array Design

Optimization of bioprocesses with multiple parameters requires a robust experimental design. Taguchi experiment design matrix with a standard $L_{16}(4^5)$ orthogonal array (16 experiment runs, 5 variables, 4 levels) with 15 degree of freedom was employed to examine five parameters, namely, temperature, pH, glycerol concentration, tryptone concentration, and agitation speed in four levels, namely 1 to level 4 (table 1). The lower and upper levels of optimized parameters were selected on the basis of the suitable condition for the desired growth of the microbes for efficient bioethanol production. This orthogonal array is a fractional factorial design and was chosen because of its minimum number of required experimental trials. Each row of the matrix represented three trials. The levels of the parameters studied and the layout of the L_{16} Taguchi orthogonal array are represented in tables 1 and 2. Each of the sixteen runs represents the combinations of experimental conditions adopted as per the design, along with bioethanol concentration as the observed response in each run.

Taguchi Analysis of the Orthogonal Array Experiments

The MINITAB statistical software (version 15.1, PA, USA), and design of experiment (DOE) software Design-Expert® (version 7.0.0, Stat-Ease Inc., USA) were used to determine the outcomes of the fermentation experiments. Taguchi orthogonal array design utilizes a distinctive design of orthogonal arrays and signal-to-noise ratio (S/N ratio) to find the optimum experimental conditions. The signal-to-noise ratio (S/N), which is the logarithmic function of desired output, served as objective function for optimization. The optimal conditions with respect to the experimental parameters have been determined on the basis of average for mean of the response (bioethanol) for the parameter at each parameter level (table 3). Bioethanol production by immobilized mutant *E.coli* was considered as a desired output. The mean denotes "signal" and the standard deviation from the mean are signified by "noise". In each experimental run, the response was recorded as the bioethanol production and corresponding S/N ratio was calculated. Larger-the–better S/N ratio characteristics were employed to accomplish a high production of bioethanol. Larger-the-better objective function was calculated using equation in 1.

$$\frac{S}{N} = -10\log\frac{1}{n}\sum_{i=1}^{n}\frac{1}{y_i^2} \qquad (1)$$

Where 'i' is the number of a trial; 'Y^{ij}' is the measured value of quality characteristic for the i^{th} trial and j^{th} experiment; 'n' is the number of repetitions for the experimental combination. The mean response values in terms of bioethanol concentration (g/l) and S/N ratios of Taguchi experimental design in 16 runs were analysed to extract independently the main effects of the parameters. The analysis of variance technique (ANOVA) was then applied to determine which parameters were statistically significant (delta values). The controlling parameters were identified and rank obtained from the delta values was used to assist in evaluating the significance of the parameters from greatest to lowest impact on the bioethanol production. Accordingly, the optimal conditions were determined by combining the levels of parameters that had the highest main effect value (Table 3).

Optimum Conditions and Validation of the Experiment Design

The experiment was validated by performing the bioethanol production with another three replications trial at the optimal settings of the process parameters determined from the analysis of mean characteristics in 3.5 L flask. The optimum fermentation parameters were; temperature 36°C, medium pH 7.0, glycerol concentration 250 (g/l), and organic source concentration 5 (g/l).

RESULTS AND DISCUSSION

Statistical Evaluation and Taguchi Analysis of Parameters for Bioethanol Production

The effects of some process parameters on the production of bioethanol from glycerol in batch fermentation are not fully elucidated. Conventional optimization procedures which involves altering of one

ISSN: 0975-8585

parameter at a time keeping all other parameters constant, are time consuming, cumbersome, require more experimental data sets and resources (Beg, et al., 2003). Taguchi method possesses the advantage that many factors can be examined simultaneously and much quantitative information can be extracted by only a few experimental trials (Stone and Veevers, 1994; Houng, et al., 2006). The basic principle of this method examine the effects of many process variables and identify those factors which have major effects on process using a few experiments (Dasu, et al., 2003). The experimental design proposed by Taguchi involves using orthogonal arrays to organize the parameters affecting the process and the levels at which they should be varies. Instead of having to test all possible combinations like the factorial design, the Taguchi method tests pairs of combinations. This allows for the collection of the necessary data to determine which factors most affect product quality with a minimum amount of experimentation. In the design of orthogonal arrays (OA), the selection of is based on the number of parameters and the levels of variation for each factor (parameter) (Krishna Prasad, et al., 2005). Three observations (corresponding to the three replications) are recorded for each experimental condition as shown in Table 2. The bioethanol production (as in Table 2) ranging from (31.45 – 115.3) g/L corresponding to the combined effect of the five factors in their specific ranges. The experimental results suggest that these parameters at optimum level strongly support the production of bioethanol. Conventionally, data from a designed experiment are used to analyse the mean response (product). In the Taguchi technique, it is shown that the variation of the response values at each inner array design point can be summarized by a performance criterion called the signal to noise ratio (S/N). Broadly speaking, the S/N ratio is the ratio of the mean (signal) to the standard deviation (noise). Generally, three standard S/N equations are widely used to classify the objective function as: 'larger the better', 'smaller the better', or 'nominal the best'. In the present study, the objective is to have large bioethanol production and hence a 'larger the better' type of S/N equations is required. The formula for calculating the S/N ratio for this type of response is as in equation (1).

$$\frac{S}{N} = -10\log\frac{1}{n}\sum_{i=1}^{n}\frac{1}{Y_i^2} \qquad (1)$$

Where 'i' is the number of a trial; 'Y^{ij}' is the measured value of quality characteristic for the i^{th} trial and j^{th} experiment; 'n' is the number of repetitions for the experimental combination.

Table 2: L_{16} (4^5) orthogonal array of Taguchi design of experiments and corresponding Bioethanol production by immobilized microwave-irradiated mutant *E.coli*

Experiment No.	A	B	C	D	E	Observation (1)	Observation (2)	Observation (3)	Mean-bioethanol production (g/l)	(S/N) ratio
1	1	1	1	1	1	34.5	35.2	34.6	34.76	30.82
2	1	2	2	2	2	46.0	48.22	49.35	47.85	33.59
3	1	3	3	3	3	57.5	55.14	57.78	56.80	35.08
4	1	4	4	4	4	115.0	115.5	114.30	114.93	41.20
5	2	1	2	3	4	44.0	43.71	44.5	44.07	32.88
6	2	2	1	4	3	33.0	31.91	35.37	33.42	30.45
7	2	3	4	1	2	110.0	108.74	115.5	111.41	40.92
8	2	4	3	2	1	55.0	59.20	56.40	56.86	35.08
9	3	1	3	4	2	48.75	45.60	47.01	47.12	33.45
10	3	2	4	3	1	97.5	96.70	98.48	97.56	39.78
11	3	3	1	2	4	29.25	31.34	33.76	31.45	29.90
12	3	4	2	1	3	39.0	39.5	41.61	40.03	32.03
13	4	1	4	2	3	113.75	116.65	115.5	115.3	41.23
14	4	2	3	1	4	56.87	57.70	57.15	57.24	35.15
15	4	3	2	4	1	54.5	53.49	52.66	53.55	34.57
16	4	4	1	3	2	34.12	39.55	37.69	37.12	31.34

Signal-to-noise ratios are computed using equation (1) for each of the sixteen experimental conditions and are reported in Table 2. Since the experimental design is orthogonal, the parameter effects can be separated out in terms of the S/N ratio and in terms of the mean response. The average values of S/N ratios of

ISSN: 0975-8585

the five control parameters at each of the levels are shown in Figure 2 (a), and from which the levels corresponding to the highest S/N ratio values are chosen for each parameter representing the optimum condition. It is clear from Figure 2(a) that the optimum levels for maximum production of bioethanol are: A_4 (temperature: 35.59), B_3 (pH: 35.14), C_4 (glycerol concentration: 40.79) D_2 (tryptone concentration: 34.97), and E_1 (agitation speed: 35.07) respectively. In addition to S/N analysis, main effects of the process parameters on the mean response are also analysed. The mean response refers to the average value of the quality characteristic for each factor at different levels. Thus the average values of the bioethanol production for each parameter at the four levels have been calculated and are plotted as depicted in 2(b). The mean response analysis (Figure 2b) also indicates the same optimum level of the parameters (A_4, B_3, C_4, D_2 and E_4,) that is obtained in S/N ratio analysis, except the agitation speed, which showed contrast value in terms of the level in both cases. Chaulia and Das, (2008) stated in their work that the effect of the average values of S/N ratio of control factors is compatible with performance characteristic for the mean response data. According to their results, the estimated optimum values of the process parameters were confirmed with corresponding to the performance characteristic of the mean response value.

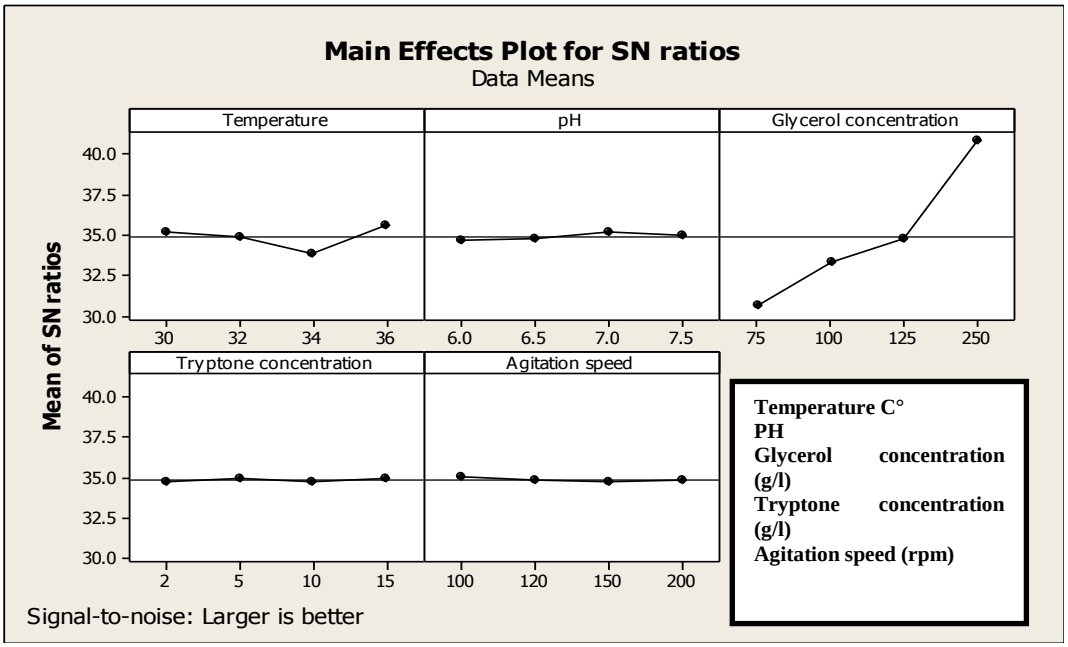


Figure.2 (a) Main effect plot on average S/N ratio of the process parameters

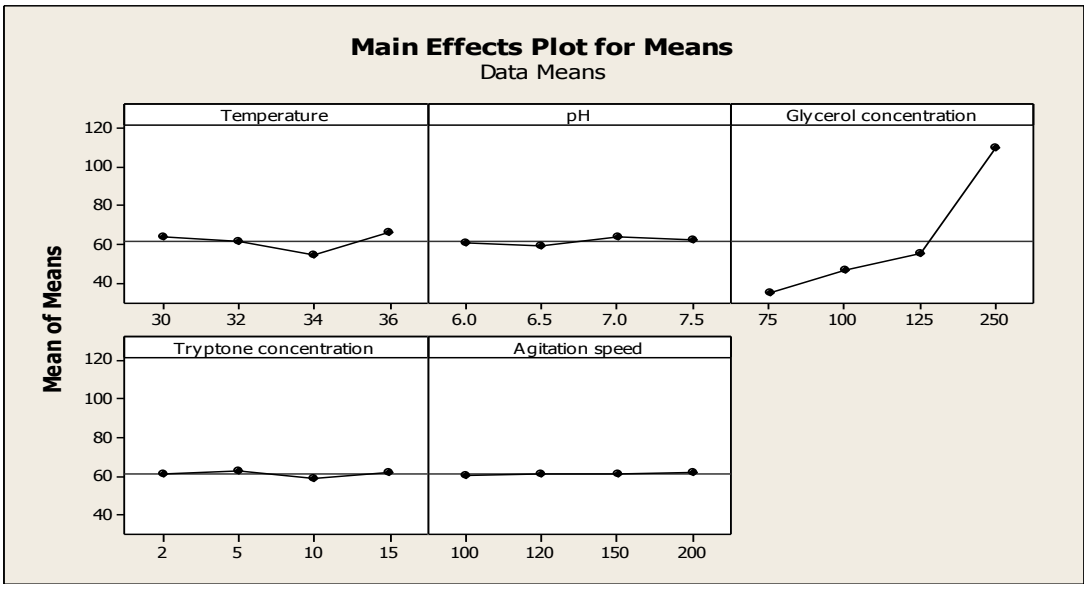


Figure.2 (b) Mean of mean plot on characteristics (Bioethanol) of the process parameters

ISSN: 0975-8585

Out of the five parameters examined, parameters A, B, C and D had a major effect on bioethanol production, as indicated by their high delta (main effects mean value). However, agitation speed (E) showed insignificant variation on the process parameter in different magnitudes regarding with S/N ratio and with mean response. The percentage contribution of each parameter on bioethanol production from partial pretreated crude glycerol by immobilized *E.coli* is shown in table 3. It is apparent that glycerol concentration, temperature, pH and tryptone concentration respectively contribute 75.61, 11.76, 4.28 and 3.98 % impact on the bioethanol production. Agitation speed showed insignificant parameters with lower percent contribution for bioethanol production among the parameters studied.

Table 3: The response values for mean (larger is better) through analysis of parameters affecting bioethanol production by immobilized *E.coli*

level	A	B	C	D	E
1	63.59	60.31	34.19	60.86	60.68*
2	61.44	59.02	46.38	62.86*	60.88
3	54.04	63.30*	54.51	58.89	61.39
4	65.80*	62.24	109.80*	62.26	61.92
Delta (main effect mean)	11.76	4.28	75.61	3.98	1.24
Rank	2	3	1	4	5

**** Optimum parameters***

The analysis of variance (ANOVA) is also performed to study the relative significance of the process parameters on the OA experiments and determine how much variation of each parameter has contributed. By studying the main effects of each of the parameters, the general trends of the influence of the parameters towards the process can be characterized. The characteristics can be controlled such that a lower or a higher value in a particular influencing parameter produces the preferred result. Hence, the parameters A, B, C, and D were considered for further analysis. Accordingly, the ANOVA of bioethanol production had a model SS (sum of squares), MS (mean squares), and F value of 13819.28, 1151.61, and 929.26, respectively (Table 4).

Table 4: ANOVA table for the significant parameters obtained from mean response values Analysis

Parameters	Sum of squares	DF	Mean square	F value	p-value Prob >F	Percentage contribution
Model	13819.28	12	1151.61	929.26	<0.0001	**Significant**
A-Temperature	312.77	3	104.26	84.13	0.0022	2.26
B-pH	44.17	3	14.72	11.88	0.0359	0.32
C-Glycerol Concentration	13424.95	3	4474.98	3610.98	<0.0001	97.12
D-Tryptone Concentration	37.39	3	12.46	10.06	0.0449	0.27
Residual	3.72	3	1.24			0.03
Corrected Totals	13823.00	15				100

F-ratios (in ANOVA), reveals the significance of the controlling parameters for the fermentation process. It is apparent from F-ratios, that all the parameters considered in the experimental design by Taguchi had statistically significant effects at 95% confidence limit. The ANOVA of bioethanol production has the model F value of 0.0500 that implies the model is significant. The model obtained from ANOVA indicated that the multiple correlation coefficient of R^2 is 0.9923 i.e. the model can explain 99.87% variation in the response. Also, the model has an adequate precision value of 84.095; this suggests that the model can be used to navigate the design space. The “adequate precision value” is an index of the signal to noise ratio and a value > 4 is an essential prerequisite for a model to be a good fit. The model shows standard deviation, mean, C.V. and predicted residual sum of square (PRESS) values of 1.11, 61.22, 1.82 and 105.75 respectively. The variability of the experimental data was explained in terms of significant effects. It can be observed from Table 4 that experimental degree of freedom (DOF) was 15, while parameters-DOF was 3. The percentage contribution was calculated for each individual parameter by the ratio of pure sum to the total sum of the squares. Since the aim of this research is to obtain a high percentage value i.e. parameter with highest percentage (p)

ISSN: 0975-8585

contribution value may rank first and parameter with lowest (p) value will placed in last rank. Hence, it is obvious that the most influential parameter was the raw glycerol for 97.12% of the overall variance of the experimental data followed by temperature (2.26%). The contribution on bioethanol production was observed with raw glycerol alone and contribution noticed was with the physical parameters such as temperature and pH. Of all the selected parameters, tryptone organic source was found to be the least significant at individual level. The ANOVA break down with the percentage of contribution of each parameter is shown in Table 4.

Optimization Bioethanol Production under Optimal Key Parameters from glycerol by immobilized E.coli

Since the interest was based on a larger-the-better characteristic, the focus was to determine the levels of each significant parameter that resulted in the highest ratio response. From the analyses of (S/N) ratio and the mean response characteristic for bioethanol production, the optimum settings levels of the control parameters are verified as: A_4, B_3, C_4, and D_2. Thus, the predicted mean of the quality characteristic (maximize bioethanol) production has been estimated using the following formula (2) with some modification (Chaulia and Das, 2008).

$$Y_{OPT} = \bar{T} + (\overline{A_4} - \bar{T}) + (\overline{B_3} - \bar{T}) + (\overline{C_4} - \bar{T}) + (\overline{D_2} - \bar{T}) \quad (2)$$

Where, $\bar{T}$ is the average mean of bioethanol production in all trial results observation in table 2. $\overline{A_4}$, $\overline{B_3}$, $\overline{C_4}$, and $\overline{D_2}$ are the average values of the bioethanol production with process parameters at their optimal levels and Y_{opt} denotes the predicted mean of the bioethanol production at optimum condition.

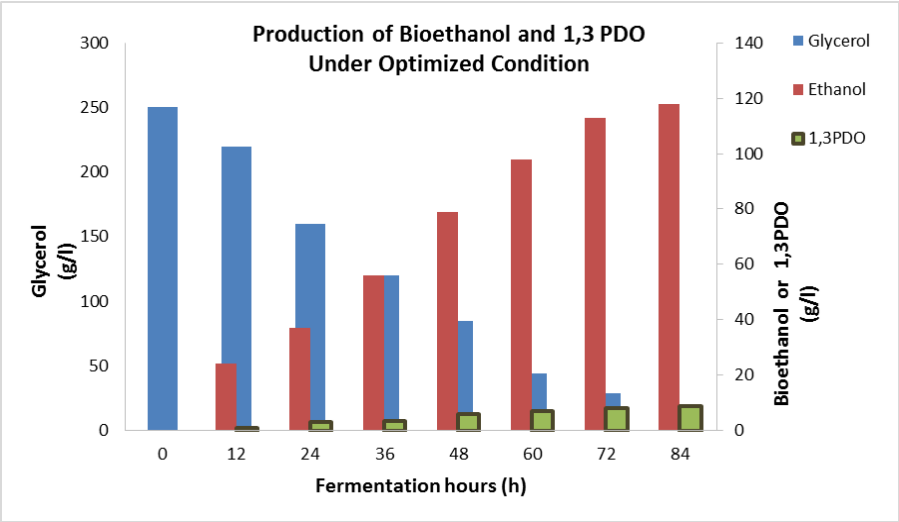


Figure 4: Profile of substrate and products formation during the fermentation experiment using optimized values of the four parameters (T= 36°C, pH = 7.0, glycerol = 250 g/l and organic source = 5 g/l).

As per equation 2, the predicted value for bioethanol concentration from crude glycerol under optimized conditions was 117.6 g/L. In order to validate the prediction, another three confirmation experiments were conducted in 3.5-L flask fermenter at the optimal settings. The figure 4 shows the trends in production of bioethanol, and 1, 3 PDO in fermentation flask experiments with almost complete utilization of crude glycerol within four days under optimized fermentation conditions. The reducing glycerol profile was inversely proportional to the rate of bioethanol formation. In a report by Dharmadi, et al., (2006), showed that 80% of glycerol was consumed at 84 h, however, this trend was different from the trend observed in this study, which may be due to different microbial strains used as well as varied experimental conditions. It is worth noting that the optimized conditions of the process led to the most efficient substrate utilization for the bacteria. As shown in figure 4, the bioethanol production from the fermentation experiment using optimized conditions was 118.75 g/l, while the by-product, 1,3 PDO, formed by this strain was 8.2 g/L. The value bioethanol production is in close agreement with the predicted value. Hence, the experiment confirmed that the model developed by application of Taguchi analysis could reliably predict the concentration of bioethanol produced. It can be deduced from these results that reduction in fermentation time, elevated bioethanol production and higher substrate utilization profile could be attributed to control of physical parameters with

great precision. Furthermore, this result not only demonstrates the robustness of the statistical analysis, but also validates the significance of the process parameters indicated by the analysis. In addition, it can be seen that the techniques of using permeabilized and immobilized mutant *E.coli* cells on glutaraldehyde cross-linked chitosan under high glycerol concentration successfully increased the yield of bioethanol. In the current study, under optimized conditions, up to 250 g/L of crude glycerol, derived from the biodiesel industry, were completely degraded, with a bioethanol yield of 0.96 mol bioethanol per mol glycerol. In a study by Varrone, et al., (2012), it was reported that under the optimized conditions, it was possible to convert more than 50% of glycerol into bioethanol, which represented the main fermentation product. The result in this study indicates that substrate inhibition did not occur and that the *E.coli* (MW-EC) strain is able to achieve very good yields. To the best of our knowledge, the bioethanol yield obtained in this study is among the best in terms of the yield obtained using high glycerol concentration as substrate.

It is well known that determining the optimum value of the main parameter during fermentation is crucial as they directly affect the metabolic process of the microbes during fermentation process. In this study four parameter were studied (temperature, pH, glycerol concentration and tryptone amount). Khanna, et al., (2013) stated that the temperature is one of the most important environmental factors affecting microbial activity. The temperature of fermentation influences the growth and glycerol metabolism as the enzymes involved in metabolic cycle are known to have an optimum temperature for maximum activity. The pH requirements vary from species to species, and even between different strains of the same species isolated from different habitats. Thapa, et al., (2013) reported in their work that the optimal condition for the conversion of glycerol into bioethanol was at pH 7.6. In addition, Barbirato, et al., (1998) observed that the amount of glycerol consumed increased with increasing pH. Meanwhile, when pH levels are low and slightly acidic, the co-production of hydrogen is likely to occur because metabolic pathways are altered by changing NADH to NAD^+ ratios (Zhang, et al., 2009). Nakashimada, et al., (2002) demonstrated that at a pH range of 6.0-6.7, the NADH to NAD^+ ratios were higher than at other pH values, and this increased the hydrogen production. Several authors had also reported that the transport of chemical products and enzymes across the cell membrane is affected by the pH of the fermentation medium, influencing many enzymatic reactions (Liang, et al., 2010). The concentration of glycerol is an important factor, as our earlier studies (Saifuddin and Refal, 2015) have revealed that with high concentrations of glycerol (typically above 250 g/l) there was increase in bioethanol production. The observed increase in bioethanol production with increasing substrate concentration is likewise in agreement with other research findings (Reungsang, et al., 2013; Jitrwung and Yargeau, 2011; Fabiano and Perego, 2002). The use of organic nitrogen sources in fermentation can enhance microbial growth (Seifert, et al., 2009). Suitable nitrogen sources can supress the formation of by-products and increase bioethanol yields (Yue, et al., 2012). Tryptone is an organic nitrogen source, which exhibited a significant positive effect on bioethanol production. This result confirms the specific requirement of tryptone as a nitrogen source for *E. coli* and is consistent with the result of many previous studies that showed that nitrogen sources affect microbial biosynthesis of bioethanol, and different types of nitrogen sources are generally effective in different microbial strains (Shaikh, et al., 1997; Choi, et al., 2011). Moreover, Thompson and He, (2006) reported that carbon, nitrogen, and some metals are present in the crude glycerol derived from biodiesel production and that the bioethanol yield from crude glycerol had exceeded the theoretical yield. It is evident that not only the presences of key parameters but also appropriate proportion of each of these parameters were essential for maximum production of bioethanol.

Table 5: Comparison of the maximum yields of bioethanol from glycerol by various researches

Strain	Crude glycerol concentration (g/L)	Ethanol yield (mol/mol)	References
E. aerogenes HU-101	10	0.86	(Ito, et al., 2005)
Mixed culture	15	1.00	(Hafizi,, et al., 2013)
Klebsiella sp. HE1	10-70	0.26 (70) 0.80 (10)	(Wu, et al., 2011)
E. aerogenes KKU-S1	31.2	0.83	(Tiwari, et al., 2008)
E. coli SS1	34.5	1.00	(Adnan, et al., 2014)
Clostridium pasteurianum ATCC	25	0.13	(Taconi, et al., 2009)
Immobilized Clostridium pasteurianum	25	0.15	(Khanna, et al.,2013)
E.coli MW-EC	250	0.96	This study

Finally to highlight the successful implementation of the optimized fermentation using the mutant *E.coli MW-EC,* a comparison is presented in Table 5. It shows the bioethanol yield obtained by other researchers using different concentrations of crude glycerol as the substrate and various types of microorganisms.

CONCLUSIONS

Partial purified crude glycerol is beneficial raw material as it is available abundantly and more importantly it is renewable. Taguchi design was achieved to identify the optimal key parameters conditions for bioethanol production. Four parameters out of five were most significant factors affecting bioethanol production by immobilized mutant *Escherichia coli*. Our experimental results indicated that the temperature 36°C, medium pH 7.0, initial glycerol concentration (250 g/l), and organic source concentration (5 g/l) exert significant effects on bioethanol production of (118.75 g/l). The results obtained in this study are among the best reported to date for glycerol fermentation in terms of both substrate concentration and yield. This study has successfully demonstrated the feasibility of bioethanol production by *E. coli* EC-MW using high glycerol concentrations of up to (250 g/L). The ethanol production was found to be reasonably higher in comparison to other processes utilizing different crude glycerol waste substrate for bioethanol production. Moreover, the ability of the mutant *E. coli* to convert glycerol to bioethanol at effectiveness of close to the theoretical yield in high glycerol concentration medium is potentially of great importance to biofuel industry. Furthermore, ANOVA was applied to validate the consistency that is proposed by Taguchi methodology and to estimate the relative contribution of each factor. Future study could be done for optimizing the fermentation composition in terms of other supplement to obtain higher biofuel yield.

ACKNOWLEDGMENT

The authors would like to thank the generosity of the invaluable input provided by the Malaysian Ministry of Education through the Exploratory Research Grant Scheme (ERGS) (12012013ERGS Project) to carry out this research. We also would like to thank Sime Darby Malaysia, for supplying the crude glycerol and Universiti Tenaga Nasional for the reinforcement research facilities.

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