Medium Optimization for Mixed Alcohols Production by Glycerol Utilizing Immobilized *Clostridium pasteurianum* MTCC 116

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Glycerol, a major byproduct of biodiesel production may be utilized by a variety of microorganisms for production of value added products. The production of bioalcohols from anaerobic fermentation of glycerol depends greatly on the composition of the medium. Therefore, optimization of media components holds great significance for achieving higher product yield. The media components for production of mixed alcohols by *Clostridium pasteurianum* immobilized on silica was optimized by Taguchi statistical method in shake flasks. After initial screening using the one-factor-at-a-time approach, a total of 6 media components, KH_2PO_4–K_2HPO_4, yeast extract, $MgSO_4 \cdot 7H_2O$, $CaCl_2 \cdot 2H_2O$, $FeSO_4 \cdot 7H_2O$, $(NH_4)_2SO_4$ each at two different concentrations were employed for optimization experiment (L8 array). The composition of optimized medium was KH_2PO_4–K_2HPO_4 2 g L^{-1}, yeast extract 5 g L^{-1}, $MgSO_4 \cdot 7H_2O$ 0.1 g L^{-1} and $(NH_4)_2SO_4$ 0.1 g L^{-1}. Analysis of Variance (ANOVA) of the optimized factors gave a model F, p and R^2 values of 2003.76, <0.001 and 99.7 respectively. The predicted total product concentration with optimized media was 17.51 g L^{-1}. Fermentation with optimized medium was carried out in a stirred tank bioreactor. The total alcohol production in bioreactor after 10 days of fermentation was 17.67 g L^{-1} at 37 ºC, 25 g L^{-1} carbon source and 3 % w/v support with immobilized cells, which was higher than the value predicted by the model. Hence, Taguchi design of experiment helped in determining the appropriate medium composition for obtaining optimum mixed alcohols yield.

Key words:
Medium optimization, Taguchi method, bioreactor, *Clostridium pasteurianum*, immobilization

Introduction

Glycerol is the principal byproduct of biodiesel manufacture through transesterification of triglycerides. The glycerol so obtained cannot be used in pharmaceutical or cosmetic industry, as it is contaminated with alcohol, alkali and soaps. 130,000 tons of glycerol is estimated to be available from biodiesel industry in Latin American and Asian countries.[1] The need of the hour is effective and economic utilization of this surplus glycerol. A renewable and economically feasible way of utilizing this glycerol is its bioconversion into value added products such as organic acids (citric, succinic, glyceric acid), alcohols (ethanol, butanol, 1,3–propanediol (1,3–PDO), erythritol, 2,3–butanediol), biosurfactants, single cell oils, dihydroxyacetone etc.[1–4, 19, 20]

Clostridium pasteurianum, a micro-aerobe is known to convert glycerol to three bio-alcohols viz: butanol, 1,3–propanediol and ethanol, all of which have economic outlets.[5] Butanol is used as a solvent and its comparable properties to gasoline make it an ideal gasoline substitute. Ethanol is also a biofuel and a good solvent. 1,3–PDO is a monomer for synthesis of various polymers such as polyesters, polyurethanes etc. The use of immobilized cells of *C. pasteurianum* for mixed alcohols production from glycerol based feedstock has already been studied.[6] The high cell density of immobilized cells provides various advantages over free cells, the most important being the ability to reuse immobilized cells for many cycles and ease in downstream processing. The yield and selectivity of products from fermentation of glycerol is influenced greatly by physical

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and chemical process parameters.[7] The optimal production of the three bio-alcohols is dependent on the composition of the medium along with physical parameters of temperature, pH, stirring speed etc. The optimization of media components for enhanced butanol and 1,3–PDO by free cells of *Clostridium pasteurianum* has already been studied[8] but no study has been carried out on optimizing the media components for bioconversion of glycerol by immobilized cells of *Clostridium pasteurianum*. The glycerol metabolism of the cells is likely to change after immobilization, which would also affect the product profile. Hence, *the optimum composition of the media for free and immobilized cells is likely to be different*. This necessitates development of a cheap and appropriate media for enhanced bio-alcohols production by immobilized cells of *Clostridium pasteurianum*. Thus, the present work addresses the issue of optimization of nutrient composition of the media for glycerol fermentation by immobilized cells of *Clostridium pasteurianum*.

We have used the Taguchi statistical method for optimization of the media composition. This method has been widely used for optimization of other fermentation processes.[9–11] The present paper attempts to investigate the effect of media components other than carbon source (glycerol). In preliminary screening experiments employing one component at a time, it was observed that the products concentration was affected to different extents for each media component at fixed glycerol concentration. Taguchi method allows for the analysis of a large number of parameters in minimum number of experiments. This helps in identifying the key parameters that have the largest effect on process output. The initial optimization of media was done in shake flasks, and later, these results were reconfirmed in a bioreactor and a shake flask by carrying out fermentation with the optimized media.

Materials and methods

Microorganism, culture revival and maintenance

Lyophilized cells of *Clostridium pasteurianum* (MTCC 116) were obtained from the Institute of Microbial Technology (Chandigarh, India). The cells were revived on cooked meat media (CMM) agar plates, and in reinforced clostridial media (RCM) broth.[6] The revived cells were maintained on CMM agar slants at 4 °C, and were used as a stock. The cells were sub-cultured every month. The microbiological media and other chemicals used in the study were purchased either from Merck, Germany or Himedia, India.

Immobilization and cross linking of C. *pasteurianum* cells

C. pasteurianum cells were immobilized on column chromatography grade silica (Merck, India) in RCM broth. The silica particles were dried at 120 °C for 24 h in a dry oven prior to use. The support was added to the media at the commencement of log phase of the growth (approx. after 24 h). The support was kept for 48 h with the media at 37 °C with shaking at 200 rpm. The broth was centrifuged to separate silica immobilized cells and non-immobilized cells. The support with immobilized cells was shaken with 50 mM phosphate buffer (pH 7.5) for 10 minutes. It was then washed twice with phosphate buffer (50 mM, pH 7.5) and dried for 24 h at room temperature. The cells immobilized on silica were incubated with 0.1 % glutaraldehyde solution for 1 h, followed by washing with phosphate buffer (50 mM, pH 7.5), and were dried at room temperature for 24 h.

Fermentation conditions

The initial screening of the media constituents was done using the one-factor-at-a-time approach. Total 10 media constituents, NaCl, Biotin, $CoCl_2 \cdot 2H_2O$, KH_2PO_4–K_2HPO_4, PABA (p-aminobenzoic acid), $MgSO_4 \cdot 7H_2O$, $CaCl_2 \cdot 2H_2O$, $FeSO_4 \cdot 7H_2O$, yeast extract, $(NH_4)_2SO_4$ were screened. These media components and their concentrations were selected on the basis of *C. pasteurianum* media used in earlier studies.[5,7,12,13] Out of these 10 components, 6 components (KH_2PO_4–K_2HPO_4, $MgSO_4 \cdot 7H_2O$, $CaCl_2 \cdot 2H_2O$, $FeSO_4 \cdot 7H_2O$, yeast extract, $(NH_4)_2SO_4$) showed significant effect on solvent production. These 6 components were shortlisted for Taguchi analysis, the details of which are described in the next section. The optimization studies were carried out in custom fabricated 250 mL anaerobic Erlenmeyer flasks. Each flask contained solution of media components in desired proportions in 100 mL distilled water. To this we added 3 % w/v immobilized cells with support and 25 % w/v pure glycerol as a sole carbon source. All flasks were sparged with nitrogen at the start and after every 24 h of fermentation to maintain anaerobic conditions. The flasks were kept in an incubator shaker at 200 rpm and 37 °C. The samples of fermentation broth were withdrawn after every 2 days up to a period of 10 days. The fermentation was carried out at uncontrolled pH and the initial pH of each set of medium was adjusted to 6.8 ± 0.2 using 0.1 N HCl and 0.1 N NaOH. All experiments were carried out in triplicate and the results presented are the mean of three trials.

Taguchi's orthogonal array

In order to study the effect of each nutrient component on solvent production by immobilized *C. pasteurianum* and to optimize its concentration for maximum solvent production, a standard L8 Orthogonal array of experiments was used. Six factors (the media components) were examined at 2 levels denoted by 1 and 2 as depicted in Table 1. The value of each nutrient (indicating concentration) was set according to the Taguchi statistical design wherein 8 sets of media composition were identified (Table 2). All experimental runs were carried out in triplicate. The optimal conditions with respect to the factors tested have been determined on the basis of average of signal to noise ratio (S/N) for the factors at each factor level (Table 3). Level 1 value of three factors or nutrients, viz. $MgSO_4 \cdot 7H_2O$, $CaCl_2 \cdot 2H_2O$, and $FeSO_4 \cdot 7H_2O$ was chosen to be zero, on the basis of initial screening experiments, which indicated the influence of zero concentration of certain factors on product pattern. The total solvent production by immobilized *Clostridium pasteurianum* was considered as a desired variable, as a higher concentration of total solvents was required. The statistical significance of each factor was determined using ANOVA. Finally, the optimum conditions for solvent production by immobilized *C. pasteurianum* were determined.

Table 1 – *Factors and their corresponding levels used in Taguchi statistical design for optimal mixed alcohols production by immobilized Clostridium pasteurianum*

Factors	Constituents	Level 1(g L^{-1})	Level 2 (g L^{-1})
A	KH_2PO_4–K_2HPO_4	1	2
B	Yeast Extract	1	5
C	$MgSO_4 \cdot 7H_2O$	0	0.1
D	$FeSO_4 \cdot 7H_2O$	0	0.005
E	$CaCl_2 \cdot 2H_2O$	0	0.01
F	$(NH_4)_2SO_4$	0.1	5

Table 3 – *Taguchi analysis of factors affecting total alcohols production by immobilized C. pasteurianum*

Level	A	B	C	D	E	F
1	3.386	3.954	2.143	12.907	13.137	14.976
2	16.663	16.094	17.905	7.141	6.911	5.072
Delta S/N (main effect mean)	13.277	12.141	15.762	5.767	6.226	9.903
Percentage Contribution	21.05	19.25	24.99	9.14	9.87	15.70

The Taguchi analysis and ANOVA were carried out using Minitab 15 software (trial version).

Confirmatory experiments

Glycerol fermentation with optimized media components were rechecked in 250 mL custom fabricated Erlenmeyer flasks using the protocol, as described previously. Further, the optimized fermentation media was tested in a 2 L microprocessor controlled bioreactor (Zenith, India) 1 L optimized medium, 3 % w/v immobilized cells with support and 25 % w/v glycerol were added to the bioreactor. The temperature was controlled at 37 °C, 99.98 % pure nitrogen was sparged at the start of fermentation and thereafter every 24 h to maintain anaerobic conditions. The initial pH of the media and the agitation rate were set at 6.8 ± 0.2 (by 0.1 M NaOH and 0.1 M HCl) and 200 rpm respectively. The samples of fermentation broth were withdrawn for analysis after every 48 h up to a period of 10 days. The experiments in both shake flask and bioreactor were carried out in duplicate.

Experiments with media optimized for free cells of *Clostridium pasteurianum*

A similar study on media optimization for enhanced butanol and 1,3–PDO production by free cells of *Clostridium pasteurianum* has been recently published by Moon et al (2011).[8] In this study, the optimum media composition for higher butanol

Table 2 – *Taguchi design matrix and corresponding average concentrations (in g L^{-1}) of the products formed and substrate left in shake flask at the end of the fermentation by immobilized Clostridium pasteurianum (MTCC 116)*

Exp No	A	B	C	D	E	F	1,3–PDO	Butanol	Ethanol	Total	Glycerol
1	1	1	1	1	1	1	1.003	0.500	0.028	1.531	9.547
2	1	1	1	2	2	2	0.000	0.000	0.058	0.058	8.462
3	1	2	2	1	1	2	4.220	1.245	0.132	5.597	13.651
4	1	2	2	2	2	1	5.201	4.211	0.132	9.544	5.732
5	2	1	2	1	2	1	4.947	4.844	0.114	9.905	1.185
6	2	1	2	2	1	2	3.351	3.258	0.510	7.119	4.354
7	2	2	1	1	2	2	2.703	1.246	0.502	4.451	13.533
8	2	2	1	2	1	1	3.194	3.108	0.571	6.873	8.410

production was determined to be 0.06 g L^{-1} $FeSO_4 \cdot 7H_2O$, 7.35 g L^{-1} $(NH_4)_2SO_4$ and 5.08 g L^{-1} yeast extract, which happen to be higher values for the first two factors while lower value for the third factor. Similarly, the optimum media components for 1,3–PDO production were 0 g L^{-1} $FeSO_4 \cdot 7H_2O$, 0 g L^{-1} $(NH_4)_2SO_4$ and 8 g L^{-1} yeast extract, which happen to be lower values for the first two factors while higher value for the third factor. To assess whether the same media compositions were also optimum for immobilized cells, experiments were performed with compositions reported by Moon et al (2011) and the results were compared with the present experiments.[8] Except for the media composition, the other protocols were exactly as mentioned in previous sections.

Analysis

The immobilization and cross-linking of *Clostridium pasteurianum* cells on silica support (Fig. 1) was confirmed by scanning electron microscopy (Leo, UK). Solvent production in the fermentation broth was monitored by gas chromatographic analysis using a CP Wax 52CB (250 mm×0.25 mm×0.39 mm) capillary column (Varian) and a Flame Ionization Detector. The injector and detector temperatures were 230 °C and 250 °C, respectively. The oven temperature was programmed from 45 °C to 100 °C with an increment of 3 °C min^{-1} and after 100 °C, an increment of 5 °C min^{-1} up to 200 °C. The utilization of glycerol by immobilized *C. pasteurianum* cells in different media was determined by HPLC analysis using a HiPlex-H column (300 mm × 5 μm × 4.6 mm, Varian). The HPLC apparatus comprised of a pump (Series 200, Perkin Elmer), a refractive index detector (Series 200, Perkin Elmer) and a vacuum degasser (Series 200, Perkin Elmer). HPLC grade water (Milli Q) was used as the mobile phase at a flow rate of 0.5 mL min^{-1}. The analyses were carried out at 37±2 °C. Samples were filtered through a 0.2 μm membrane filter, and diluted appropriately prior to analysis.

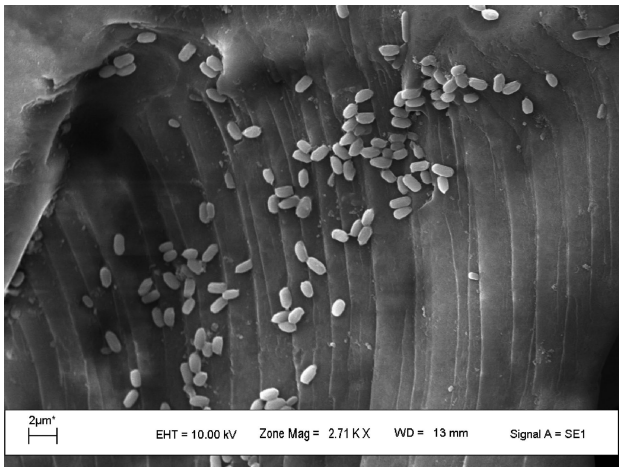

Fig. 1 – *Scanning electron microscopic image of Clostridium pasteurianum cells immobilized on column chromatography grade silica support and cross-linked by glutaraldehyde*

Results and discussion

Initial screening experiments helped in determining the media factors having greater effect on products formation. The results obtained from one component at a time analysis (not included in this manuscript) indicated that components PABA, biotin, $CoCl_2 \cdot 2H_2O$ and NaCl had an insignificant impact on the products yield. The results obtained with PABA and biotin, which are in agreement with earlier study by Sanchez (2009) indicated that *C. pasteurianum is not an auxotroph for both of these nutrients*.[14] Similarly, the study of Sanchez (2009) also showed that the cells could grow even in the absence of micronutrients, viz. Co, Cu, Zn and Ni.[14] This result is in concurrence of our study that the effect of $CoCl_2 \cdot 2H_2O$ on products formation was insignificant.

Taguchi analysis and ANOVA

Table 2 depicts the final concentration of the three bio-alcohols formed with different media compositions and the concentration of substrate left in the fermentation broth. Among all 8 experimental runs, the highest total concentration of alcohols (9.9 g L^{-1}) was obtained in run 5, which also gave the highest concentration of butanol. Also, highest glycerol consumption was observed in medium 5, which also gave highest total solvents concentration. Although, large amount of glycerol was consumed in most of the experiments but there was no concurrent increase in products concentration. This may be attributed to the incomplete conversion of glycerol whereby the glycerol consumed was not completely converted into desired products. In all 8 runs, 1,3–PDO was the major product with butanol formed in second highest concentration. The variation in products concentration with the change in medium composition indicates that the production of alcohols is a major function of media composition. The percentage effect of each factor on alcohols production is depicted in Table 3. Out of the six factors examined, factors A, B, C and F had a major effect on mixed alcohols production, as indicated by their high delta S/N value. In Taguchi statistical design, S/N ratio is an important parameter for identifying optimal conditions for the process. A high S/N ratio indicates higher significance of the factor. Based on this logic, the order of effect of various factors on total alcohol production was $MgSO_4 \cdot 7H_2O$ > KH_2PO_4–K_2HPO_4 > Yeast extract

> $(NH_4)_2SO_4$ > $CaCl_2 \cdot 2H_2O$ > $FeSO_4 \cdot 7H_2O$. The concentration of $MgSO_4 \cdot 7H_2O$ was critical for mixed alcohols production, as it had the highest contribution of 24.99 % to the main effect mean (or delta S/N) while, $FeSO_4 \cdot 7H_2O$ and $CaCl_2 \cdot 2H_2O$ had the least significance. The insignificant effect of calcium on products formation may be explained in terms of immobilization of actively growing *C. pasteurianum* cells. Calcium is a component of endospore and is required for stabilizing endospore wall.[15] In the present study, we have used immobilized actively growing cells of *C. pasteurianum*, and not immobilized spores; and hence, the cells had no very specific requirement for calcium. Among the four significant factors (with 10 % or higher contribution to main effect mean), the total solvent production was higher at level 2 for factors A, B and C, while factor F formed more solvents at lower level (Fig. 2).

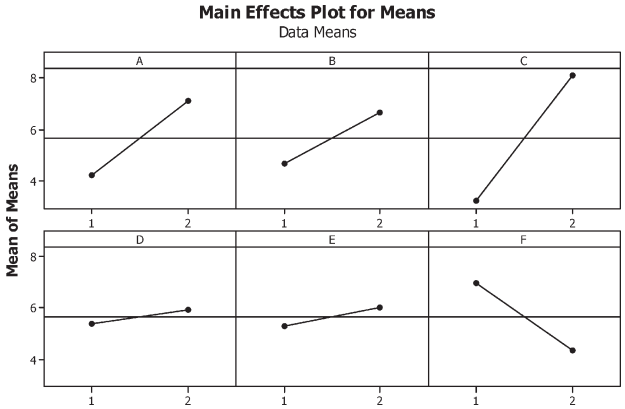

Fig. 2 – *Mean of main effects plot for each medium component at two levels (1 and 2); A) KH_2PO_4–K_2HPO_4 B) Yeast Extract, C) $MgSO_4.7H_2O$, D) $FeSO_4.7H_2O$, E) $CaCl_2.2H_2O$, and F) $(NH_4)_2SO_4$ on mixed alcohols production by immobilized C. pasteurianum. Except $(NH_4)_2SO_4$, all other factors had higher effect at higher factor level.*

ANOVA was done to quantify the variation in products formation caused due to each factor, and also to determine whether the lower or the higher value of a factor is essential for preferred result, i.e. higher products formation. ANOVA of only those factors was done, which had more than 10 % contribution to the main effect.[16, 17] Thus, factors A, B, C and F were considered for further analysis. The ANOVA of total solvent production had a model SS (sum of squares), MS (mean squares), and F value of 63.2970, 15.8243, and 1663.895, respectively (Table 4). The model obtained from ANOVA indicated that R^2 (multiple regression coefficient) is 0.996, which means that the model can explain 99.6 % variation in the response. R^2 value closer to 1.0 indicates the robustness of the model, whereas the value greater than 0.75 implies the fitness of the model. The ANOVA of factors considered clearly demonstrated that all factors had significant effect (as indicated by $p < 0.001$) on total alcohol production. The numerical values of the factors, which provided the highest total concentration of alcohols are KH_2PO_4–K_2HPO_4 2 g L^{-1}, yeast extract 5 g L^{-1}, $MgSO_4 \cdot 2H_2O$ 0.1 g L^{-1}, and $(NH_4)_2SO_4$ 0.1 g L^{-1}. The values of first three factors correspond to higher level of factor, while the value of last factor, i.e. $(NH_4)_2SO_4$ corresponds to the lower level. Higher products concentration at lower concentration of $(NH_4)_2SO_4$ is probably a consequence of the presence of other nitrogen (yeast extract) and sulfur source ($MgSO_4 \cdot 2H_2O$).

Result prediction and confirmatory experiments in shake flasks and bioreactor

The following equation estimates or predicts the total concentration of alcohols formed under optimized conditions:[9, 18]

$$Y_{opt} = T_i + \sum \left(F_{i,avg} - T_{i,avg} \right) \tag{1}$$

Various notations are Y_{opt} = process output, T = average of all trial results/ average of performance, i = significant factors, F_i- average effect of significant factors at each level

For the present study where we have four factors each having two levels, the above equation can be rewritten as:

$$Y_{opt} = T + |A_1 - T| + |A_2 - T| + |B_1 - T| + |B_2 - T| + \\ + |C_1 - T| + |C_2 - T| + |F_1 - T| + |F_2 - T| \tag{2}$$

As per equation 2, the predicted value of total alcohol concentration under optimized conditions

Table 4 – *ANOVA table for the significant factors obtained from Taguchi analysis*

Factors	DF	SS	MS	F-ratio	p value	Confidence percentage
Model	4	63.2970	15.8243	1663.895	p<0.001	99.62
A	1	11.9032	11.9032	714.77	p<0.001	99.44
B	1	5.91332	5.91332	1907.93	p<0.001	99.79
C	1	35.0683	35.0683	3388.59	p<0.001	99.88
F	1	10.4122	10.4122	644.29	p<0.001	99.38

DF – Degree of Freedom, SS – Sum of Squares, MS – Mean of Squares

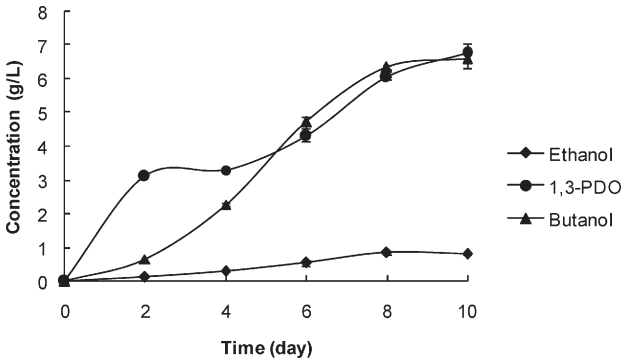

Fig. 3 – *Production of ethanol, butanol and 1,3–PDO in shake flasks containing optimized media. The total product concentration after completion of fermentation was 14.17 g L^{-1}. Fermentation conditions: 200 rpm, 37 °C, pH 6.8 ± 0.2, 3 % w v^{-1} immobilized biocatalyst, and 25 g L^{-1} pure glycerol.*

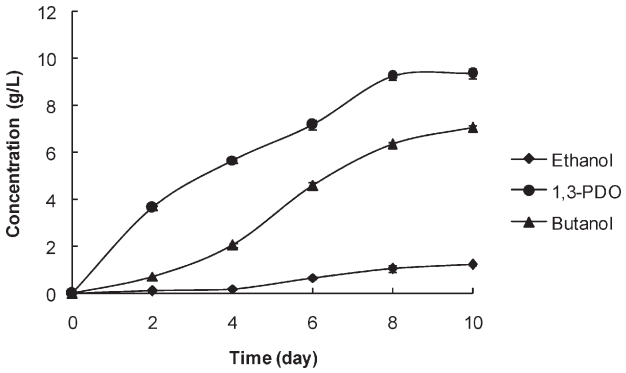

Fig. 4 – *Product formation profiles in stirred tank bioreactor with optimized media components. The final concentration of products achieved was 17.67 g L^{-1} which was higher than the predicted value. Fermentation conditions: 200 rpm, 37 °C, pH 6.8 ± 0.2, 3 % w/v immobilized biocatalyst, and 25 g L^{-1} pure glycerol.*

was 17.51 g L^{-1}. In order to confirm the prediction, fermentation was carried out in shake flasks with media of optimum composition. The trends in the production of 1,3–PDO, ethanol and butanol in shake flask experiments are depicted in Fig. 3. The results presented are the mean of the two experimental sets. The final concentrations (in g L^{-1}) of ethanol, 1,3–PDO and butanol were calculated as 0.81 (0.03 g g^{-1}), 6.77 (0.28 g g^{-1}) and 6.59 (0.26 g g^{-1}), respectively. The total alcohol concentration resulting from shake flask fermentation in optimized medium was 14.17 g L^{-1}, which was lower than the predicted value of 17.51 g L^{-1}. However, the value obtained after optimization of medium components in shake flask was higher than the value obtained in trial 5 (9.90 g L^{-1}). Thus, the effect of medium optimization is evident here. A lower product concentration than estimated value may be attributed to the reduced control of temperature and shaking speed in a shaker incubator. In order to obviate these limitations and to have a better control over fermentation conditions, the experiment was performed in a bioreactor under same conditions. Fig. 4 depicts the trends of production of the three fermentation products, viz. ethanol, butanol and 1,3–PDO in this experiment. The total concentration of products formed after 10 days of fermentation was 17.67 g L^{-1}. The individual concentrations (g L^{-1}) of ethanol, butanol and 1,3–PDO were 1.25 (0.06 g g^{-1}), 7.06 (0.29 g g^{-1}) and 9.36 (0.37 g g^{-1}), respectively. The result obtained is higher than the predicted value of 17.51 g L^{-1}, which not only demonstrates the robustness of the statistical analysis, but also validates the significance of the media components as indicated by the analysis. As can be seen from Figs. 3 and 4, almost similar yields of all three products were obtained after 8 and 10 days of fermentation. Thus, the optimization of medium components also reduced the total glycerol fermentation time.

Fig. 5 depicts the concentration of the glycerol left unutilized in fermentation broth during the course of fermentation in shake flask and bioreactor. The cells in bioreactor study were found to utilize a higher concentration of glycerol (91 %) than the cells in shake flask (86 %) which may be the reason behind higher production of solvents in bioreactor. The trends in ethanol, 1,3–PDO and butanol formation in both shake flask and bioreactor were similar as reported in a paper by Dabrock et al (1992).[7] This paper describes that for glycerol concentration upto 8 % w v^{-1} equal quantities of the three products form; however, for high concentrations of glycerol more 1,3–PDO is produced with a concurrent decrease in ethanol production.[7] In agreement with this result for 25 g L^{-1} glycerol in our study, which is a relatively high concentration, 1,3–PDO was the major product. The yield of the two major products viz. butanol and 1,3–PDO obtained after optimization of medium components was higher than their yields reported by other groups.[5, 7, 12] Only the yields reported by Taconi et al. (2009) are higher than those obtained in the

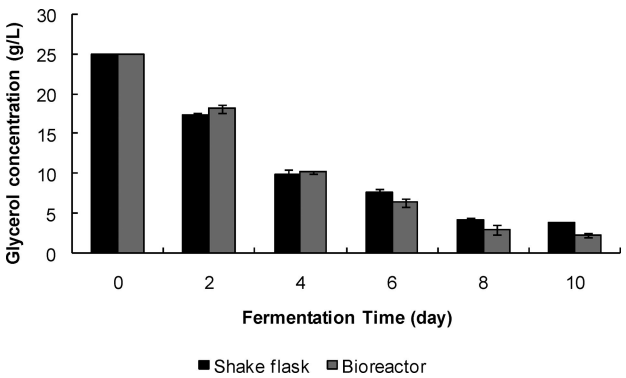

Fig. 5 – *Trends in concentration of glycerol left unutilized in the fermentation broth during the course of fermentation. The final concentration of glycerol utilized by C. pasteurianum was higher in bioreactor than in shake flask.*

present study.[13] An important point to mention here is that the yields reported here are with immobilized *C. pasteurianum* cells while previously reported yields by other researchers were with free *C. pasteurianum* cells. A further increase in products yield is expected with optimization of fermentation parameters such as temperature, pH, agitation rate etc.

Comparative evaluation with optimized media for free cells

The results obtained in the present study match with results of Moon et al (2011) to the extent that we have also found $(NH_4)_2SO_4$ and yeast extract to be the important factors influencing total products concentration.[8] However, in addition to these, our study also establishes $MgSO_4 \cdot 7H_2O$ and K_2HPO_4–KH_2PO_4 as important components for alcohols production by immobilized *C. pasteurianum* cells. But, the total products concentration obtained with immobilized cells with optimized medium for butanol production by free cells was quite low. Ethanol was the major product (0.35 g L^{-1}) with no formation of butanol and 1,3–PDO. Similarly, the medium optimized for 1,3–PDO production by free cells also gave a very low total products concentration with formation of traces of ethanol (0.23 g L^{-1}), 1,3–PDO (0.87 g L^{-1}) and no butanol. This discrepancy in the results indicates that the medium requirement for free and immobilized cells are different.

Conclusion

The results of our study clearly reveal that production of bioalcohols through fermentation of glycerol by immobilized *C. pasteurianum* is a strong function of composition of the medium. Glycerol was metabolized faster by immobilized *C. pasteurianum* cells in an optimized medium. The optimization of medium components by Taguchi design helped in identifying key factors responsible for enhanced production of alcohols. The results of this study could give vital inputs for design of an economic and efficient glycerol fermentation process. Further work must aim at optimizing the fermentation parameters such as temperature, pH, agitation rate etc to obtain higher products yield.

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Abbreviations

SEM – Scanning Electron Microscope
CMM – Cooked Meat Medium
RCM – Reinforced Clostridial Medium
MTCC – Microbial Type Culture Collection
S/N – Signal to Noise ratio
ANOVA – Analysis of Variance
1,3–PDO – 1,3–Propanediol

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