

Full Length Research Paper

Fermentative conversion of raw glycerol into 1,3-propanediol by isolated *Klebsiella pneumoniae* 141B stain: Optimization of culture variables

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Accepted 24 June, 2011

In this study, 1,3-propanediol (1,3-PDO) production by *Klebsiella pneumoniae* 141B strain using raw glycerol as substrate was investigated. Taguchi L18 orthogonal array (OA) was adopted to optimize nutritional (raw glycerol, yeast extract and calcium carbonate), physiological (incubation temperature and medium pH) and microbial (inoculum level) fermentation variables. These controlling fermentation factors were selected based on one variable at a time methodology. Raw glycerol, temperature and calcium carbonate were the most significant factors among the selected factors followed by pH and inoculum level. Yeast extract, however, showed the least significance on 1,3-PDO production at individual level, while in combination, it elucidated a remarkable severity index values of 64.81 and 58.17% with pH and temperature, respectively. At optimized environment, 53% contribution towards 1,3-PDO production was observed from selected fermentation parameters. The model was experimentally validated, yielding a 1,3-PDO production of 0.62 mol mol⁻¹ of raw glycerol, which represented a 19% increase when compared to the non-optimized medium.

Key words: Raw glycerol, 1,3-propanediol, Taguchi methodology, *Klebsiella pneumoniae* and optimization.

INTRODUCTION

Recently, global interest has been directed towards research and commercialization of several microbial fermentation technologies for chemical production. Several studies estimate the share of biotechnological processes in the production of various chemical products to be around 3.0% in 2004, however, they postulated that this figure will soar to about 15% by 2015 (Festel Capital, 2007). Among all these chemical products, 1,3-propanediol (1,3-PDO) is the promising bulk chemical which has attracted worldwide attention, due to its enormous applications in polymers, cosmetics, foods, adhesives, lubricants, laminates, solvents, antifreeze and medicines (Cheng et al., 2007). The development of new polyester, polypropylene terephthalate (PPT or poly-trimethylene terephthalate-PTT) from 1,3-PDO requires a drastic increase in the production of this chemical (Gonzalez-Pajuelo et al., 2006). PPT is biodegradable

polyester that has great potential for use in carpet and textile manufacturing and is related to polyethylene terephthalate (PET) and polybutylene terephthalate (PBT) (Hao et al., 2006). A number of microorganisms are able to grow anaerobically on glycerol as the sole carbon and energy source, such as *Citrobacter freundii*, *Klebsiella pneumoniae*, *Clostridium pasteurianum*, *Clostridium butyricum*, *Enterobacter agglomerans*, *Enterobacter aerogenes* and *Lactobacillus reuteri* for 1,3-PDO production (Da Silva et al., 2009).

The transesterification of renewable biological sources such as vegetable oils and animal fat oils with an alcohol using alkaline or acid catalysts is the most common process for biodiesel production (Ma and Hanna, 1999; Tapanes et al., 2008), yielding 1 mol of glycerol for every three fatty acid methyl esters (FAME). The tremendous growth of the biodiesel industry created a glycerol surplus that has resulted in a dramatic 10-fold decrease in crude glycerol prices to \$0.05/lb (Johnson and Taconi, 2007). For every 100 gal of biodiesel that is produced, 5 to 10 gal of the less glamorous raw glycerol are left behind with a sparking debate about the best uses of this waste (Da

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Silva et al., 2009). Moreover, the excess glycerol generated may become an environmental problem, since it cannot be disposed off in the environment. Hence, the production of value added product like 1,3-PDO from glycerol would not only reduce the production cost of 1,3-PDO, but also improve the economic viability of biodiesel production.

Earlier studies were carried out for the production of 1,3-PDO from pure glycerol by *Klebsiella* species using Taguchi and Box-Behnken experimental designs (Galdeano et al., 2007; Gungormusler et al., 2010). In our previous reports, a new strain of glycerol utilizing bacterium was isolated from the soil samples, which was identified as *K. pneumoniae*, designated as *K. pneumoniae* 141B (Vanajakshi and Annapurna, 2011b) and the effect of process parameters on 1,3-PDO production from pure glycerol were studied by Taguchi methodology (Vanajakshi and Annapurna, 2011a). Owing to its high productivity from pure glycerol, *K. pneumoniae* 141B strain was employed in this study for the production of 1,3-PDO from raw glycerol to make the 1,3-PDO production as an economically feasible process. The specific objective of this investigation was to study the methodological application of Taguchi OA experimental design (DOE) and to optimize the nutritional and cultural parameters involved in the production of 1,3-PDO from raw glycerol.

MATERIALS AND METHODS

In this study, raw glycerol for the production of 1,3-PDO was taken from biodiesel pilot plant, Indian Institute of Chemical Technology, Hyderabad. Biodiesel was synthesized from *Jatropha* (*Jatropha curcas*), which is a non-edible oil having an estimated annual production potential of 200 thousand metric tonnes in India and it can be grown in waste land (Srivastava and Prasad, 2004). Moreover, *Jatropha* oil contains about 14% free fatty acid (FFA), which is far beyond the limit of 1% FFA level that can be converted into biodiesel by transesterification using an alkaline catalyst (Tiwari et al., 2007). The transesterification reaction was carried out with methanol-to-*Jatropha* oil in 5:1 molar ratio using 0.5% (w/v) KOH as an alkaline catalyst at 60°C for half an hour and the products were allowed to settle for overnight. The bottom glycerol layer was separated from top ester layer through separating funnel and the raw glycerol layer was further processed for the methanol recovery by distillation. Finally, the raw glycerol obtained with a purity 74% (w/w), was utilized as a cheap substrate for 1,3-PDO production. All other chemicals used were commercially obtained and were of analytical grade.

Microorganism

In this study, the organism used for the production of 1,3-PDO was isolated from soil samples collected from Indian Institute of Chemical Technology, Hyderabad. The isolated organism was identified as *K. pneumoniae* based on morphological, physiological and biochemical characteristics and it was deposited in IMTECH Chandigarh with the accession number *K. pneumoniae* MTCC 9751. The 16S rDNA sequence of this organism displayed the highest degree of homology (98%) with those of *Klebsiella* sp. and its EMBL accession number was FN820293.

Medium and culture conditions

The strain was maintained on growth medium (pH 7) containing (g l⁻¹) yeast extract, 5; peptone, 10; sodium chloride, 9; glycerol, 20 and agar 20 and stored at 4°C. For seed culture development, the strain was grown on the medium consisting of all earlier mentioned components except agar. The production medium used for 1,3-PDO production contained (g l⁻¹) raw glycerol, 20; K₂HPO₄, 0.69; KH₂PO₄, 0.25; (NH₄)₂SO₄, 6; MgSO₄·7H₂O, 0.2; yeast extract, 1.5; and 0.1% (v/v) of trace element solution. The composition of the trace element solution was (mg l⁻¹): MnSO₄·4H₂O, 100; ZnCl₂, 70; Na₂MoO₄·2H₂O, 35; H₃BO₃, 60; CoCl₂·6H₂O, 200; CuSO₄·5H₂O, 29.28; NiCl₂·6H₂O, 25; FeSO₄ solution, 0.2% (v/v) and 37% HCl, 0.9 ml. The composition of the FeSO₄ solution was 5 g l⁻¹. The experiments were carried out by batch fermentation in 250 ml Erlenmeyer flasks containing 100 ml of the production medium with 2% (v/v) seed culture. After inoculation, the flasks were incubated in an orbital shaker (Labtech, Germany) at 37°C, 150 rpm for 8 h and analyzed for 1,3-PDO production. All experimental trials were performed in triplicate.

Analytical methods

The concentration of 1,3-PDO, glycerol, ethanol, 2,3-butanediol and succinic acid were determined by a high-performance liquid chromatography system (SHIMADZU 10A) equipped with an Aminex HPX-87H column (Bio-Rad) with a refractive index detector. The working conditions were: 0.005M H₂SO₄ as a mobile phase with a flow rate of 0.5 ml min⁻¹ and 65°C as the working temperature. Biomass was determined by dry cell weight (DCW) method (Herbert et al., 1971).

Statistical optimization and analysis of the results

Six different fermentation parameters such as pH, yeast extract, raw glycerol, calcium carbonate, inoculum and temperature were selected at three different levels and their ranges were further assigned based on the preliminary investigation data. L-18 orthogonal array of Taguchi experimental design was used for optimization of 1,3-PDO production. The experimental data obtained was processed using Qualitek-4 (Nutek Inc., Bloomfield Hills, CA) software. It mainly evaluates the influence of individual factors, multiple interactions of the selected factors on the process performance and determination of optimum conditions for effective production. Software operation for optimization was performed at 'bigger is better' performance characteristics for all the runs. To validate the method, 1,3-PDO production experiments were further studied by employing the established optimized process conditions from the proposed methodology and the production efficiency was evaluated.

RESULTS

Effect of raw glycerol on 1,3-PDO production

To study the effect of raw glycerol on the production of 1,3-PDO, shake flask studies were performed by the isolated *K. pneumoniae* 141B strain with raw glycerol and pure glycerol as substrates at 37°C in pH 7 medium containing (w/v, g l⁻¹) glycerol, 20; yeast extract, 7.5; calcium carbonate, 5; trace elemental solution, 0.1% (v/v) and with the inoculum level of 2% (v/v), incubated at 150 rpm for 8h. The fermentation profile of 1,3-PDO

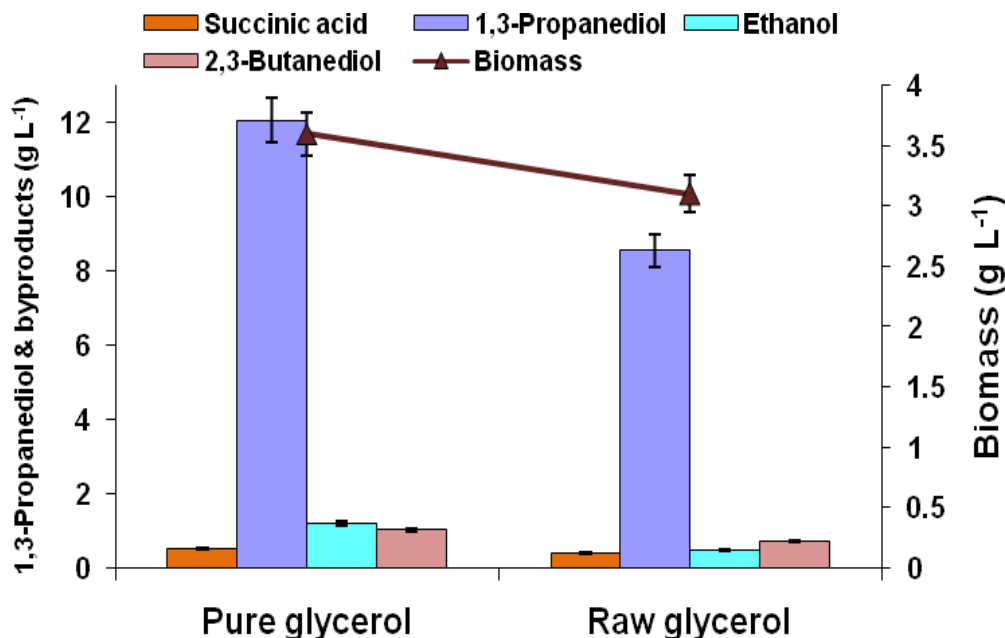


Figure 1. Production of 1,3-PDO from pure and raw glycerol by isolated *K. pneumoniae*.

production from pure and raw glycerol is shown in the Figure 1. The 1,3-PDO concentration obtained from pure glycerol (12.06 g l^{-1}) was more than that of raw glycerol (8.55 g l^{-1}) and the molar yields of 1,3-PDO on pure and raw glycerol were 0.73 and 0.52, respectively. The byproducts formed by this strain during 1,3-PDO production were ethanol, 2,3-butanediol and succinic acid. Ethanol was obtained as a principle byproduct at a concentration of 1.21 g l^{-1} with pure glycerol and 2,3-butanediol was produced as a major byproduct from raw glycerol at a concentration of 0.72 g l^{-1} . The highest biomass of 3.6 g l^{-1} was reached in the culture with pure glycerol, while it reached 3.1 g l^{-1} in the cultures with raw glycerol. Owing to its vast availability, renewable nature and low cost, raw glycerol was chosen as a substrate for further optimization studies by *K. pneumoniae* strain.

Preliminary fermentation studies revealed that apart from raw glycerol, yeast extract, calcium carbonate, pH, temperature and inoculum level were the critical medium components for 1,3-PDO production by this isolated strain (results not shown). These six factors were taken at different concentrations in three levels for further evaluation (Table 1) with Taguchi experimental design. The Qualitek-4 software was equipped to use L-4 to L-64 arrays along with a selection of 2–63 factors with two, three and four levels to each factor. The automatic design option allowed Qualitek-4 to select the array used and assign factors to the appropriate columns. L-18 orthogonal experimental plan layout, along with 1,3-PDO production values is depicted in Table 2. Evaluation of data based on L-18 orthogonal array (OA) experimental design indicated a variation of 1,3-PDO production values

from 4.2 to 23.4 g l^{-1} , suggesting the imperative role of selected factors and their concentration on product yield.

Trend of influence of factors on 1,3-PDO production

By studying the main effects of each of the factors, the general trends of the influence of the factors toward the process can be characterized. The process efficiency has been found to be very much dependent on the selected process conditions. The average effect of the factors along with the interaction at the assigned levels on the performance of 1,3-PDO production is depicted in Table 4 and Figure 2. Factor analysis at individual level revealed that pH, yeast extract, inoculum and temperature at level 2, raw glycerol and calcium carbonate at level 3 were optimum for 1,3-PDO production. Among all selected factors, raw glycerol was the most influential factor for 1,3-PDO production as it contributed maximum variability of 16.31 g l^{-1} . Temperature was the next factor that exhibited significant contribution to variability at 14.33 g l^{-1} , followed by calcium carbonate at 13.63 g l^{-1} , pH at 13.58 g l^{-1} , inoculum level at 13.43 g l^{-1} and least influence was exhibited by yeast extract concentration at 12.61 g l^{-1} .

Factors interactions

Understanding the impact of each individual factor is the key for a successful fermentation process. The interaction effect between two factors was measured in terms

Table 1. Selected fermentation factors for the production of 1,3-PDO from raw glycerol by *K. pneumoniae*.

S/N	Factor	Level 1	Level 2	Level 3
1	Unused	-	-	-
2	pH	6	7	8
3	Yeast extract (g l ⁻¹)	5.0	7.5	10
4	Raw glycerol (g l ⁻¹)	20	40	60
5	CaCO ₃ (g l ⁻¹)	2.5	5.0	7.5
6	Inoculum (% v/v)	1	2	3
7	Temperature (°C)	34	37	40
8	Unused	-	-	-

Table 2. L18 orthogonal experimental array for the production of 1,3-PDO by *K. pneumoniae*.

S/N	Factor								1,3-PDO (g l ⁻¹)
	1	2	3	4	5	6	7	8	
1	0	1	1	1	1	1	1	0	6.40
2	0	1	2	2	2	2	2	0	17.5
3	0	1	3	3	3	3	3	0	16.1
4	0	2	1	1	2	2	3	0	4.20
5	0	2	2	2	3	3	1	0	17.3
6	0	2	3	3	1	1	2	0	19.2
7	0	3	1	2	1	3	2	0	10.6
8	0	3	2	3	2	1	3	0	7.40
9	0	3	3	1	3	2	1	0	7.80
10	0	1	1	3	3	2	2	0	23.4
11	0	1	2	1	1	3	3	0	5.10
12	0	1	3	2	2	1	1	0	11.7
13	0	2	1	2	3	1	3	0	9.70
14	0	2	2	3	1	2	1	0	20.9
15	0	2	3	1	2	3	2	0	10.2
16	0	3	1	3	2	3	1	0	10.9
17	0	3	2	1	3	1	2	0	7.50
18	0	3	3	2	1	2	3	0	6.80

of a numerical quantity known as interaction severity index (SI) values, calculated by Qualitek-4 software program and expressed in terms of %. The SI interactions of the factors on the process performance are shown in Table 3. The SI interaction presents 100% SI for a 90° angle between the lines and it shows 0% SI for the parallel lines. It is evident from Table 3 that interactions exist between all the factors. Further analysis indicated that the highest strength of interaction (75.87%) was existed between pH and calcium carbonate. Interaction between calcium carbonate and inoculum level elicited least SI value of 1.41%. It was interesting to observe that calcium carbonate was the same factor involved in the highest and least SI values indicating that

fermentation factors were interacted differentially in the fermentation process. Effect of calcium carbonate on the production of 1,3-PDO is shown in the Figure 3. It was evident from the figure that the 1,3-PDO production was increased from 1.8 to 8.51 g l⁻¹ from the control to calcium carbonate supplemented medium, respectively. Though raw glycerol influenced 1,3-PDO production greatly at individual level (Figure 2), its interaction with other factors did not show significant effect on the 1,3-PDO yield (Table 3). Yeast extract at individual level (Figure 2) showed least influence on 1,3-PDO production, but its interaction with other factors such as pH and temperature elucidated a remarkable SI values of 64.81 and 58.17%, respectively and moreover, these two factors were

Table 3. Estimated interaction (as severity index) for the selected factors during 1,3-PDO production by *K. pneumoniae*.

S/N	Interacting factor pair	Column	SI (%)	Reserved column	Level
1	pH × CaCO ₃	2 × 5	75.87	7	[2,1]
2	pH × yeast extract	2 × 3	64.81	1	[2,2]
3	pH × temperature	2 × 7	58.51	5	[1,2]
4	Yeast extract × temperature	3 × 7	58.17	4	[2,1]
5	pH × inoculum	2 × 6	50.57	4	[1,2]
6	Yeast extract × raw glycerol	3 × 4	25.3	7	[3,3]
7	Yeast extract × inoculum	3 × 6	25.21	5	[2,2]
8	Raw glycerol × CaCO ₃	4 × 5	15.55	1	[3,1]
9	pH × raw glycerol	2 × 4	8.91	6	[2,3]
10	Raw glycerol × inoculum	4 × 6	7.43	2	[3,2]
11	CaCO ₃ × temperature	5 × 7	6.73	2	[3,2]
12	Raw glycerol × temperature	4 × 7	6.6	3	[3,2]
13	Inoculum × temperature	6 × 7	6.02	1	[2,2]
14	Yeast extract × CaCO ₃	3 × 5	2.22	6	[1,3]
15	CaCO ₃ × inoculum	5 × 6	1.41	3	[3,3]

Table 4. Main effects, ANOVA and optimum performance conditions for 1,3-PDO production by *K. pneumoniae* with raw glycerol as a substrate.

Factor	pH	Yeast extract	Raw glycerol	CaCO ₃	Inoculum	Temp	Other/error	Total
Main effects								
L1	13.366	10.866	6.866	11.5	10.316	12.5	-	-
L2	13.583	12.616	12.266	10.316	13.433	14.733	-	-
L3	8.499	11.966	16.316	13.633	11.699	8.216	-	-
L2-L1	0.217	1.75	5.4	-1.184	3.116	2.233	-	-
ANOVA								
DOF	2	2	2	2	2	2	5	17
Sum of spurs	99.143	9.389	269.73	33.903	29.263	131.60	0.21	573.2
Variance	49.571	4.694	134.865	16.951	14.631	65.801	0.04	-
F-ratio	1170.4	110.84	3184.22	400.23	345.461	1553.6	-	-
Pure sum	99.058	9.305	269.645	33.818	29.178	131.51	-	-
Percentage	17.28	1.623	47.038	5.899	5.09	22.942	0.128	100%
Optimum conditions and performance								
Level desc	7	7.5	60	7.5	2	37	-	-
Level	2	2	3	3	2	2	-	-
Contribution	1.766	0.799	4.5	1.816	1.616	2.916	-	-

Total contribution from all factors was 13.413 g l⁻¹; current grand average of performance was 11.816 g l⁻¹; expected result at optimum condition was 25.229 g l⁻¹.

physical factors. pH interaction was noticed to be highest among all the studied factors (Table 3) suggesting its importance in 1,3-PDO production by this strain, even though it was the fourth important factor at individual level. This could be evidenced based on the observation

that it showed maximum interaction with yeast extract (64.81%), temperature (58.51%) and inoculum (50.57%) in addition to calcium carbonate (75.87%). However, in the experiment, a very less interaction of 8.91% was noticed between pH and glycerol, a high influencing

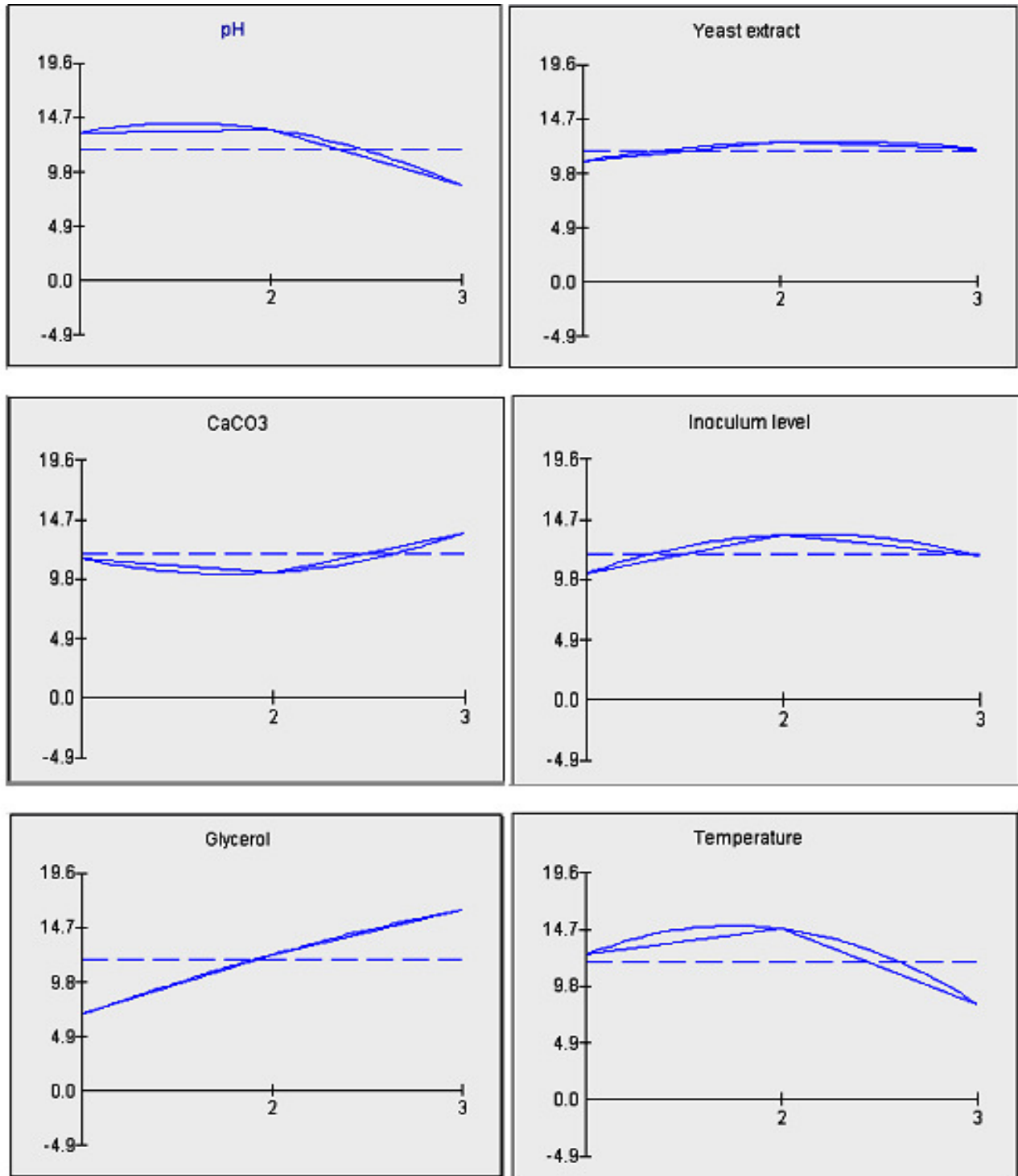


Figure 2. 1,3-PDO production at selected fermentation parameter levels by *K. pneumoniae*.

factor at individual level in 1,3-PDO fermentation process.

ANOVA (analysis of variance)

The contribution of individual factors is the key for the

control to be enforced on the 1,3-PDO production processes. ANOVA was used to analyze the results of the OA experiment and to determine how much variation each factor had contributed. ANOVA with the percentage of contribution of each factor with interactions is shown in Table 4. F-ratios (in ANOVA), reveals the significance of

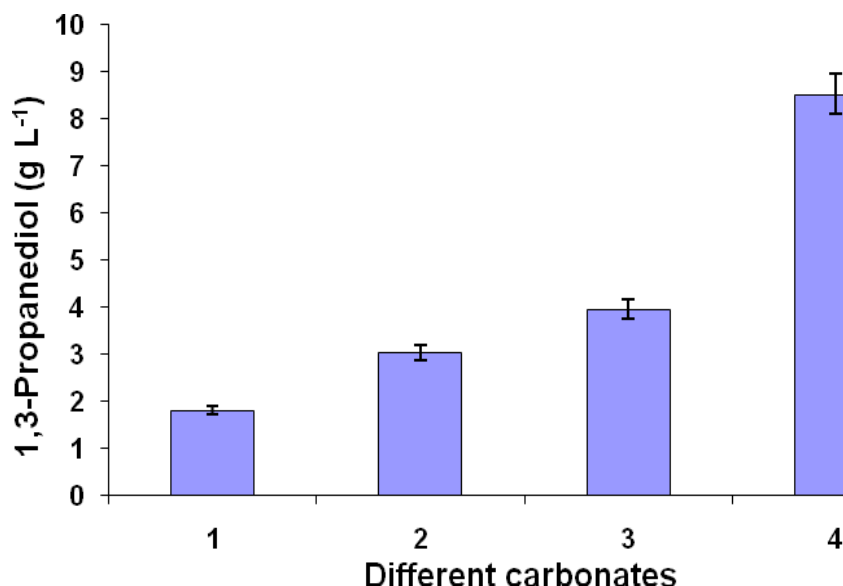


Figure 3. Effect of different carbonates on the production of 1,3-PDO. 1-control; 2- Na_2CO_3 ; 3- KCO_3 ; 4- CaCO_3 .

the controlling factors for the fermentation process. It is evident from F-ratios, that all the factors and interactions considered in the experimental design had statistically significant effects at 95% confidence limit. The variability of the experimental data was explained in terms of significant effects. It can be observed from Table 4 that experimental DOF was 17, while factors-DOF was 2. The percentage contribution was calculated for each individual factor by the ratio of pure sum to the total sum of the squares. The most influential factor was the raw glycerol for 47.03% of the overall variance of the experimental data followed by temperature (22.94%), pH (17.28), calcium carbonate (5.89), inoculum level (5.09) and yeast extract (1.62%). Nearly 50% contribution on 1,3-PDO production was observed with raw glycerol alone and 40% contribution noticed was with the physical parameters such as temperature and pH. Of all the selected factors, yeast extract was found to be the least significant at individual level.

Optimum conditions and validation

Optimum conditions and individual parameter performance in terms of contribution for achieving higher 1,3-PDO yield are shown in Table 4. Based on this information, optimum 1,3-PDO yield was achieved at pH 7 with 7.5 g l⁻¹ of yeast extract, 60 g l⁻¹ glycerol, 7.5 g l⁻¹ calcium carbonate using 2% (v/v) inoculum at a fermentation temperature of 37°C. These optimum conditions revealed the importance of physical factors and media components along with their concentrations in 1,3-PDO

production with this microbial strain. The average performance of this strain in 1,3-PDO production was observed to be 11.81 g l⁻¹ which represented > 46% in the overall production (Table 4). However, all fermentation factors contribution was noticed to be 13.41 g l⁻¹, which represented > 53% in the overall production. The model predicted that a maximum of 25.22 g l⁻¹ of 1,3-PDO could be obtained using the mentioned optimal conditions. The experimental production of 1,3-PDO yielded 24.82 g l⁻¹, which was in close agreement with the model predicted value. At optimized environment, 1,3-PDO production was increased from 8.55 to 10.2 g l⁻¹ at initial 20 g l⁻¹ raw glycerol concentration, representing an overall improvement of 19% than that of the conventional approach. The process variation at current and improved conditions with the function of frequency distribution is shown in Figure 4. It can be observed from the figure, that a substantial increase in frequency distribution was observed with the optimal factors.

DISCUSSION

Commercial success of any biotechnological product depends on several factors such as microbial strain potential, substrate cost, fermentation economics and product yield. In this study, the isolated strain, *K. pneumoniae* 141B is a potent strain as it has higher 1,3-PDO production potential at a molar yield of 0.73 mol mol⁻¹ from glycerol. In most of the cases, the observed molar yield of 1,3-PDO to glycerol was between 0.35 to 0.65 (Hao et al., 2008; Barbirato et al., 1998). When

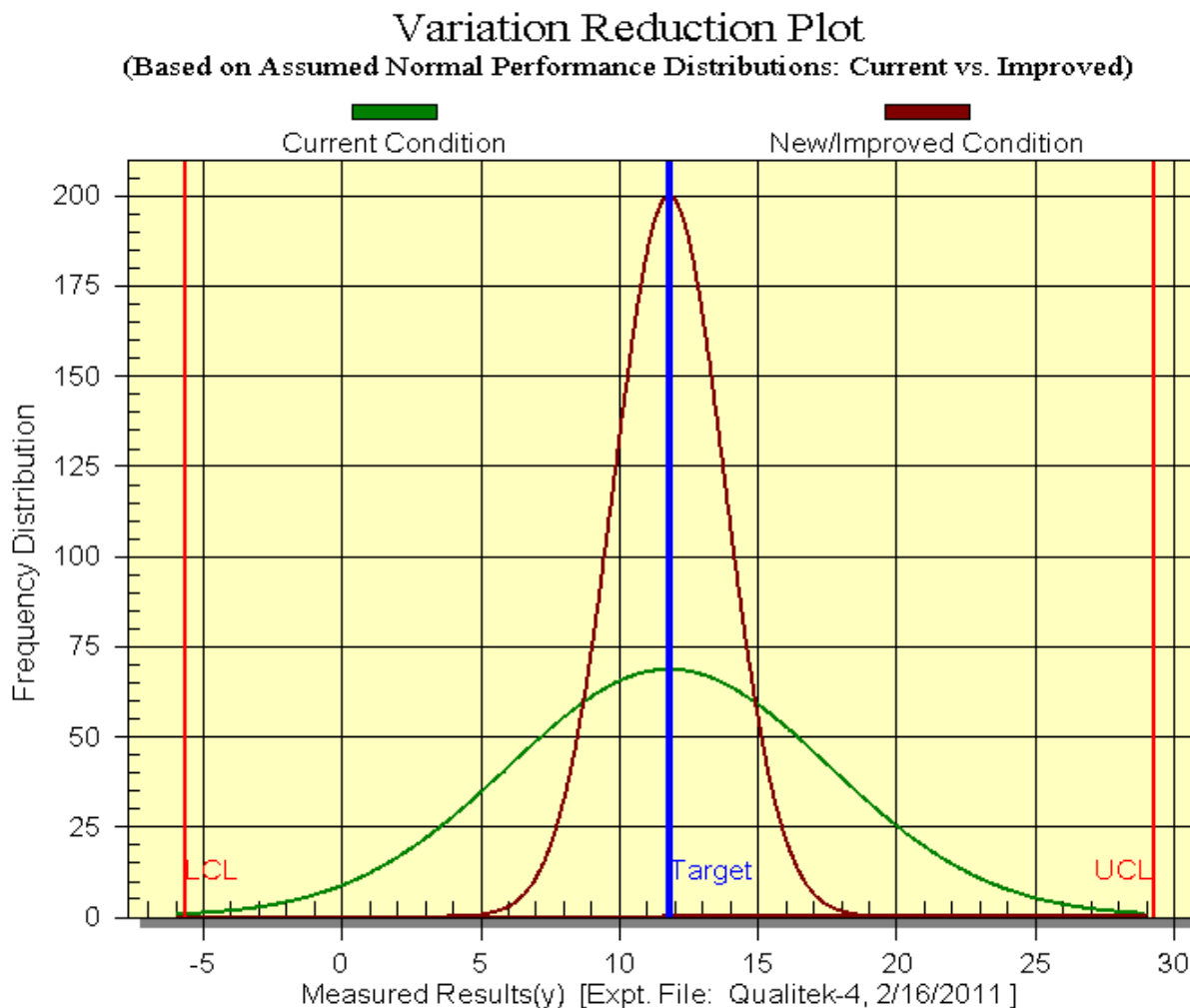


Figure 4. Performance distribution of current and improved condition of 1,3-PDO production.

compared with pure glycerol, the organism reported low molar yield with raw glycerol at $0.52 \text{ mol mol}^{-1}$. The reason for this could be attributed to the dilution of raw glycerol concentration to 74% in comparison to 99% pure glycerol and impurities present in the raw glycerol derived from biodiesel preparation also reduced the 1,3-PDO yield. In general, 1,3-PDO production from pure glycerol is not an economically feasible process since it has fairly high value commercial chemical, historically valued at \$ 0.60 to \$0.90/lb. 1,3-PDO production with raw glycerol increases the profitability of the overall process as the price of raw glycerol has fallen to about \$0.05/lb, primarily because of the increased production of biodiesel (Johnson and Taconi, 2007). Owing to higher 1,3-PDO production potential of this isolate, further improved studies were undertaken by exploiting Taguchi methodology with raw glycerol as a cheaper substrate because the statistical optimization methods improve the productivity with minimum number of experiments.

All experimental trials in the selected design supported the production of 1,3-PDO, although its concentration

varied from 4.2 to 23.4 g l^{-1} (Table 2). From this, it is evident that not only the presence of key components but also appropriate proportion of each of these factors were essential for maximum production of 1,3-PDO. Among all selected factors, raw glycerol concentration had a detrimental role on 1,3-PDO production (Figure 2), such carbon source concentration dependent on 1,3-PDO production regulation has been noticed in different microbial strains (Barbirato et al., 1998). With increase in raw glycerol concentration from level 1 (20 g l^{-1}) to level 3 (60 g l^{-1}), the yield of 1,3-PDO was also increased from 10.2 to 23.4 g l^{-1} . The volumetric productivity of the strain was improved from 1.27 to $1.44 \text{ g l}^{-1} \text{ h}^{-1}$, with increasing initial raw glycerol concentration from 20 to 40 g l^{-1} , respectively and it was decreased to $1.23 \text{ g l}^{-1} \text{ h}^{-1}$ at higher glycerol concentration of 60 g l^{-1} . The productivity usually decreases at higher glycerol concentration, due to the increased fermentation time and feedback inhibition exerted by high substrate concentration. This observation correlated well with the reported data on 1,3-PDO production with earlier reports (Zhang et al., 2007; Zeng

et al., 1994).

In general, calcium carbonate role was considered as maintenance of the medium pH (buffer) during fermentation. Figure 3 reveals that absence of calcium carbonate in the medium (control flask) resulted in the drastic decrease in the production of 1,3-PDO to 1.8 g l^{-1} and moreover, supplementation of carbonates in the form of sodium and potassium carbonates was also not aided much in the production. Presence of calcium carbonate in the medium enhanced the production of 1,3-PDO to 8.51 g l^{-1} , which indicates that the organism utilized carbonates for buffering action and calcium ion as cofactor for enzymes involved in the 1,3-PDO synthesis. Toraya et al. (2010) reported that the calcium ion was required for the catalysis of diol dehydratase which catalyzes the conversion of glycerol to 3-hydroxypropionaldehyde, an intermediate compound in the metabolic pathway of 1,3-PDO. This could explain the dual role of calcium carbonate as an activator for proteins as well as a buffering agent in the medium. It was further confirmed based on the observation that calcium carbonate had the highest strength of interaction of 75.87% with pH as well as it was the third most significant factor that affects the production of 1,3-PDO.

Rosso et al. (1995) described the maximum microbial specific growth rate as a function of pH and temperature in his new model for microbial growth. In this study, the physical parameters such as temperature and pH had a significant role in the microorganism growth and fermentation as they contributed 40% for the production of 1,3-PDO. The formation of 1,3-PDO increased, when the pH was increased from level 1 to level 2 (near neutral) and subsequent increase to level 3 resulted in declined production. Highly acidic or alkaline conditions generally inhibit microbial activity and most bacteria favor neutral conditions. Bacterial species generally grow well in the temperature range of 25 to 45°C and with increase in operation temperature from level 1 (34°C) to level 2 (37°C), higher production was documented and further increase to level 3 (40°C) resulted in the lower yield. These results were consistent with the previous reports (He et al., 2002); the optimum pH value and temperature for the growth of *K. pneumoniae* for 1,3-PDO formation were 7.0 and 30 to 37°C, respectively. The fermentation of glycerol by *C. pasteurianum* was regulated under a pH of 6.5 at 30°C (Biebl, 2001).

The inoculum level was also an important factor for the production of 1,3-PDO. The production was found higher up to 2% (v/v) inoculum (level 2), and subsequent increase in the inoculum concentration (level 3, 3%) reduced the production efficiency. The reason could be due to exhaustion of nutrients in the fermentation broth. Yeast extract was the least significant factor of 1,3-PDO production among all selected factors as it contributed 1.62%, although its absence in the fermentation medium effected the production drastically with this strain (results not shown). Among various organic nitrogen sources,

yeast extract was considered as the best source for the growth of *K. pneumoniae* during 1,3-PDO production (Gunzel et al., 1991; Chen et al., 2005). It is obvious that high level of glycerol and calcium carbonate, medium level of pH, temperature, inoculum level and yeast extract favoured high titers of 1,3-PDO production.

The molar yield of 1,3-PDO was increased from 0.52 to $0.62 \text{ mol mol}^{-1}$ of glycerol from conventional method to statistical approach, representing an overall improvement of 19% in the production. The 1,3-PDO yield obtained by the present stain (*K. pneumoniae* 141B strain) from raw glycerol derived from *Jatropha* oil transesterification was higher than that of the earlier reports, who reported 0.54 and 0.47 for crude glycerol derived from lipase and alkali catalyzed transesterifications (Mu et al., 2006). The productivity of the stain was also increased from 1.06 to $1.27 \text{ g l}^{-1} \text{ h}^{-1}$ by this Taguchi methodology at initial glycerol concentration of 20 g l^{-1} . This study using L-18 orthogonal array confirmed Taguchi methodology as a qualified method to optimize the 1,3-PDO culture conditions by *K. pneumoniae* 141B strain.

Conclusion

The impact of different fermentation variables on 1,3-PDO production by the isolated *K. pneumoniae* 141B was investigated using Taguchi methodology. On the whole, all selected factors showed impact on 1,3-PDO production by this isolated microbial strain, either at individual or interactive level. The raw glycerol concentration individually showed significant influence on the process performance, followed by temperature, calcium carbonate, pH, inoculum level, and yeast extract. While in combination, pH with calcium carbonate and pH with yeast extract showed positive influence over the process performance. A more than 19% overall increase in the production of 1,3-PDO was observed with the statistical design. Hence, this design proved to be crucial in achieving exponential increases in the production of 1,3-PDO.

ACKNOWLEDGEMENTS

The authors are thankful to Dr. J. S. Yadav director, IICT for his co-operation and one of the authors, Jalasutram Vanajakshi is thankful to the Council of Scientific and Industrial Research, New Delhi, for the award of senior research fellowship.

REFERENCES

- Barbirato F, Himmi EH, Conte T, Bories A (1998). 1,3-propanediol production by fermentation: An interesting way to valorize glycerin from the ester and ethanol industries. *Ind. Crops Prod.*, 7: 281-289.
- Biebl H (2001). Fermentation of glycerol by *Clostridium pasteurianum*-batch and continuous culture studies. *J. Ind. Microbiol. Biotechnol.*

- 27: 18-26.
- Chen HW, Fang BS, Hu ZD (2005). Optimization of process parameters for key enzymes accumulation of 1,3-propanediol production from *Klebsiella pneumoniae*. *Biochem. Eng. J.* 25: 47-53.
- Cheng KK, Zhang JA, Liu DH, Sun Y, Liu HJ, Yang MD (2007). Pilot-scale production of 1,3-propanediol using *Klebsiella pneumoniae*. *Process Biochem.* 42: 740-744.
- Da Silva GP, Mack M, Contiero J (2009). Glycerol: a promising and abundant carbon source for industrial microbiology. *Biotechnol. Adv.* 27: 30-39.
- Festel Capital (2007). (available on <http://www.Festel.com>).
- Galdeano C, Zazo M, Santos VE, Garcia JL, Garcia-Ochoa F (2007). Production of 1,3-Propanediol using *Klebsiella oxytoca* NRTL B-199 growing cells: medium composition optimization using Taguchi method. *European Congress of Chemical Engineering-6*, Copenhagen, pp. 16-20.
- Gonzalez-Pajuelo M, Meynial-Salles I, Mendes F, Soucaille P, Vasconcelos I (2006). Microbial conversion of glycerol to 1,3-propanediol: physiological comparison of a natural producer, *Clostridium butyricum* VPI 3266, and an engineered strain, *Clostridium acetobutylicum* DGI(pSPD5). *Appl. Environ. Microbiol.* 72(1): 96-101.
- Gungormuşler M, Gonen C, Ozdemir G, Azbar N (2010). Fermentation medium optimization for 1,3 propanediol production using Taguchi and Box-Behnken experimental designs. *The 5th Int. Bioeng. Congress*, Izmir, Turkey, pp. 16-19.
- Gunzel B, Yonsel S, Deckwer WD (1991). Fermentative production of 1,3-propanediol from glycerol by *Clostridium butyricum* up to a scale of 2m². *Appl. Microbiol. Biotechnol.* 36: 289-294.
- Hao J, Lin R, Zheng Z, Liu H, Liu D (2008). Isolation and characterization of microorganisms able to produce 1,3-propanediol under aerobic conditions. *World J. Microbiol. Biotechnol.* 24: 731-740.
- Hao J, Xu F, Liu HJ, Liu DH (2006). Downstream processing of 1,3-propanediol fermentation broth. *J. Chem. Technol. Biotechnol.* 81: 102-108.
- He H, Gong CS, Tsao GT (2002). Production of 1, 3-propanediol by *Klebsiella pneumoniae*. *Appl. Biochem. Biotechnol.* 98: 687-698.
- Herbert D, Phipps PJ, Strange RE (1971). Chemical analysis of microbial cells. In: Norris JR, Ribbons DW, editors. *Methods in Microbiology*, Academic Press, London, pp. 209-344.
- Johnson DT, Taconi KA (2007). The Glycerin Glut: Options for the Value-Added Conversion of Crude Glycerol Resulting from Biodiesel Production. *Environ. Prog.* 26: 338-348.
- Ma F, Hanna MA (1999). Biodiesel production: a review. *Bioresour. Technol.* 70: 1-15.
- Mu Y, Teng H, Zhang DJ, Wang, Xiu ZL (2006). Microbial production of 1,3-propanediol by *Klebsiella pneumoniae* using crude glycerol from biodiesel preparations. *Biotechnol. Lett.* 28: 1755-1759.
- Rosso L, Lobry JR, Bajard S, Flandrois JP (1995). Convenient model to describe the combined effects of temperature and pH on microbial growth. *Appl. Environ. Microbiol.* 61: 610-616.
- Srivastava A, Prasad R (2004). Triglycerides-based diesel fuels. *Renew. Sust. Energ. Rev.* 4: 111-133.
- Tapanes NCO, Aranda DAG, Mesquita Carneiro JWD, Antunes OAC (2008). Transesterification of *Jatropha curcas* oil glycerides: theoretical and experimental studies of biodiesel reaction. *Fuel*, 87: 2286-2295.
- Tiwari AK, Kumar A, Raheman H (2007). Biodiesel production from jatropha oil (*Jatropha curcas*) with high free fatty acids: An optimized process. *Biomass Bioenerg.* 31: 569-575.
- Toraya T, Honda S, Mori K (2010). Coenzyme B₁₂-dependent diol dehydratase is a potassium ion-requiring calcium metalloenzyme: evidence that the substrate-coordinated metal ion is calcium. *Biochemistry*, 49: 7210-7217.
- Vanajakshi J, Annapurna J (2011a). Optimization of 1, 3-Propanediol production by *Klebsiella pneumoniae* 141B using Taguchi methodology: Improvement in production by cofermentation studies. *Res. Biotechnol.* 2(2): 90-104.
- Vanajakshi J, Annapurna J (2011b). Isolation and Identification of 1, 3-Propanediol producing Strain of *K. Pneumoniae* 141B from Soil and Optimization of process Parameters. *Res. J. Biotechnol.* 6(2): 29-36.
- Zeng AP, Ross A, Biehl H, Tag C, Gunzel B, Deckwer WD (1994). Multiple product inhibition and growth modeling of *Clostridium butyricum* and *Klebsiella pneumoniae* in glycerol fermentation. *Biotechnol. Bioeng.* 44: 902-911.
- Zhang GL, Ma BB, Xu XL, Li C, Wang L (2007). Fast conversion of glycerol to 1,3-propanediol by a new strain of *Klebsiella pneumoniae*. *Biochem. Eng. J.* 37: 256-260.