ABSTRACT

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, antifreezing agents and medicines. In the present study, 1,3-PDO production was carried out from glycerol using an isolated *Klebsiella pneumoniae* 141B. Initial experiments were carried out for the enrichment, isolation and identification of 1,3-PDO producing microorganism. A total of 40 bacterial isolates were isolated and screened for 1,3-PDO production. Among these strains, the strain 141B-APJ-IICT had shown the maximum 1,3-PDO production at 7.41 and 5.94 g/L under aerobic and anaerobic conditions respectively. Hence, the above potent strain was further identified based on morphological, physiological, biochemical and phylogenetic studies as *Klebsiella pneumoniae*. The comparative 16S rRNA gene sequence evaluation of the isolate 141B-APJ-IICT with literature cited bacterial strains revealed that this isolate showed 98% degree of homology with 16S rRNA gene of *Klebsiella* sp. The EMBL accession number of the present strain was found to be FN820293. It was deposited in IMTECH, Chandigarh with the accession No. MTCC 9751. In the present study, the microbe was referred as *K. pneumoniae* 141B. The identification of the 1,3-PDO produced by *K. pneumoniae* 141B was carried out initially using HPLC and further confirmed by GC-MS (gas chromatography mass spectrometry).

Various fermentation strategies such as optimization studies, cofermentation studies, whole cell immobilization, strain improvement and scale up studies were adopted to maximize the production of 1,3-PDO by the isolated *K. pneumoniae* 141B. A two step methodology was employed for optimization of 1,3-PDO production. In the first stage,

the conventional single variables optimization methodology was employed to select the suitable fermentation parameters. In the second stage, Taguchi methodology was employed to fine tune the levels of the critical parameters those noticed to influence the production of 1,3-PDO drastically during optimization studies carried out via single variables optimization methodology.

For any microbial strain, optimum levels of fermentation process parameters such as pH, temperature, aeration, carbon and nitrogen sources vary for effective and economic productivities. All these parameters have two tire influences at individual and interactive levels elicit a cumulative effect on the growth of microorganism as well as subsequent metabolically related product production. In the present study, the production of 1,3-PDO in shake flask studies was maximized by optimizing the physical parameters such as incubation time, temperature, pH, aeration and inoculum level and nutritional parameters such as carbon, nitrogen and calcium carbonate concentration using single variables optimization methodology. 1,3-PDO production was increased from 7.41 to 11.50 g/L in shake flask using the optimized conditions such as glycerol-2% (w/v), yeast extract-0.75% (w/v), calcium carbonate-0.5% (w/v), pH-6, temperature-37°C, agitation speed-150 rpm, medium volume-75 ml, inoculum level-2% (v/v) and incubation time-8 h. One interesting phenomenon was observed that 1,3-PDO production was not stimulated by vitamin B12 supplementation, which acts as a coenzyme for glycerol dehydratase (GDHt). GDHt is the rate limiting enzyme involved in the synthesis of 1,3-PDO. Along with 1,3-PDO production, GDHt productions was also not increased in presence of vitamin B12. Thus, 1,3-PDO production in vitamin B12-free medium allows the

development of an economical biological fermentation process as vitamin B12 is an expensive compound.

The statistical experimental designs help in obtaining valuable information by running few experimental trials with consistency as these are based on fundamental principles of statistics such as replication and randomization. Hence, Taguchi methodology with L18 orthogonal array was used to improve the production of 1,3-PDO. Six critical fermentation factors such as pH, glycerol, yeast extract, calcium carbonate, inoculum level and temperature were selected for statistical optimization. The design of experimental methodology aided in the identification of most significant parameters that effected 1,3-PDO production at interactive and individual levels. The data suggested that production of 1,3-PDO was mostly influenced by the variation in concentration of glycerol. Approximately a 9.0% improvement was observed in 1,3-PDO yields upon optimization of significant parameters using Taguchi methodology.

The most important step for the development of an economical microbial production process for 1,3-PDO was to access other carbon sources than glycerol. Particularly, glucose is generally a preferred feedstock to build up the fermentation process. An attempt has been made for the production of 1,3-PDO from fermentable sugars such as glucose and fructose using *K. pneumoniae* 141B. Among these two sugars, fructose hasd shown the maximum production of 4.94 g/L followed by glucose at 3.47 g/L with an optimum fermentation time of 9 h. 1,3-PDO yield obtained from fructose and glucose was found to be 2-3 folds lower than the yield obtained from glycerol. Hence, further studies were carried out to evaluate the different sugars such as glucose, fructose and sucrose as cosubstrates in glycerol fermentation at a molar ratio of 0.1 to 0.5 mol/mol

of glycerol. There was a sharp increase in the yield of 1,3-PDO from control (0.70 mol/mol) having glycerol alone to glycerol-sucrose mixture (0.98 mol/mol) followed by glycerol-fructose mixture (0.90 mol/mol) and glycerol-glucose mixture (0.84 mol/mol). Among all the cosubstrates studied, glycerol-sucrose mixture had shown the maximum production of 1,3-PDO at 16.63 g/L with a fermentation time of 10 h at 0.3 mol/mol of sucrose-glycerol ratio.

In general 1,3-PDO production with raw glycerol increases the profitability of overall process as the price of raw glycerol has fallen to about $0.05/lb, primarily because of the increased production of biodiesel. Hence, in the present study, 1,3-PDO production by *K. pneumoniae* 141B using raw glycerol as a substrate has been investigated. The maximum 1,3-PDO production from raw glycerol was observed to be 8.55 g/L with a molar yield of 0.52 mol/mol. To further improve the 1,3-PDO production from raw glycerol, Taguchi L18 orthogonal array was adopted to optimize the nutritional (raw glycerol, yeast extract and calcium carbonate), physical (incubation temperature and medium pH) and microbial (inoculum level) fermentation variables. The data revealed an overall yield of 10.2 g/L of 1,3-PDO, which accounted nearly 19% improvement in production. The molar yield of 1,3-PDO has increased from 0.52 to 0.62 mol/mol.

Immobilization offers several potential advantages to the fermentation systems over free cell systems from the process engineering standpoint, which include ease of handling and cell separation. The rationale for choosing whole cell immobilization is that it provides higher yields after immobilization, higher operational stability, greater resistance to environmental disturbances and lower effective operational costs. Whole cell immobilization of *K. pneumoniae* 141B was attempted using various entrapment and

adsorption matrices. Both the entrapment and adsorption matrices supported 1,3-PDO production. The order of 1,3-PDO production under the influence of various entrapment matrices was polyacrylamide > k-carrageenan > gelatin > alginate > agar. The order of 1,3-PDO production by different adsorption matrices was polypropylene scourer > absorbent cotton > scrubber pad > polyurethane > polystyrene. Comparative evaluation of different supports, suggested that growth of the microorganism should not be restricted and free diffusion of gases and nutrients should be facilitated. Of all the supports that were screened, polypropylene scourer was found to be the ideal matrix for the whole cell immobilization of *K. pneumoniae* 141B cells. The polypropylene scourer adsorbed cells had shown the maximum 1,3-PDO production of 12.3 g/L during the 3^{rd} production cycle and they exhibited a consistent production upto 20^{th} cycle (10.71 g/L). Under immobilized conditions process parameters such as substrate weight and inoculum level were investigated. 1.0 g of polypropylene scourer and 2.0% (v/v) inoculum size were found to support the production of 1,3-PDO. Further reactor studies were carried out in fluidized bed reactor (FBR) and stirred tank reactor (STR) using polypropylene scourer adsorbed *K. pneumoniae* 141B cells. The FBR was made of glass with 38 cm height and 5.3 cm diameter, with total volume of 1.0 L capacity. The working volume of the FBR was 600 ml. The total volume of STR was 1.0 L with working volume of 600 ml. The results from FBR and STR suggested that the polypropylene scourer adsorbed cells of *K. pneumoniae* 141B cells could be effectively used upto > 20 cycles in repeated batch fermentation for 1,3-PDO production. However, the yield of 1,3-PDO observed in FBR was approximately 30% higher than the yield obtained from the STR. Thus, FBR is the most advanced reactor for fermentation products. It has better features when compared to

STR, like good mixing, higher oxygen mass transfer rates, easier CO_2 removal, easier cell regeneration and more uniform cell population. Overall, 1,3-PDO yield exhibited by polypropylene scourer adsorbed *K. pneumoniae* 141B cells was observed to be 7.0% higher than that of free suspended cells.

The industrial potential of 1,3-PDO has stimulated research in the development of methods to improve strains, since the production level of 1,3-PDO in naturally occurring strains is sometimes low for commercial exploitation. For the development of a commercially feasible fermentation process, improvement in yield and overall productivity are essential. Keeping this in view, the isolated *K. pneumoniae* 141B was treated with physical mutagens such as UV rays and with different chemical mutagens such as sodium bromide (NaBr), ethidium bromide (EtBr) and hydroxylamine (NH_2OH) to obtain a mutant with enhanced production of 1,3-PDO. The results revealed that, among all the UV mutants, UV-10 mutant produced the maximum 1,3-PDO production at 12.9 g/L, among NaBr mutants, NaBr-4 had shown the maximum production at 13.4 g/L. Among EtBr mutants, EtBr-4 yielded a maximum titer of 13.6 g/L, while NH_2OH mutant (NH_2OH-3) recorded 13.9 g/L. The production of 1,3-PDO by these mutants followed the order of NH_2OH > EtBr > NaBr > UV. The fermentation time of the NH_2OH-3 mutant had decreased to 7 h, when compared to the 8 h fermentation time of parent strain. *K. pneumoniae* NH_2OH-3 mutant strain exhibited 16% improvement in 1,3-PDO production compared to the parent strain. Hence, it was deposited in IMTECH Chandigarh with the accession No. *Klebsiella pneumoniae* MTCC 10309. A central composite design (CCD) of response surface methodology was applied to determine the optimal conditions for 1,3-PDO production using *K. pneumoniae* NH_2OH-3 mutant. The data revealed that glycerol

was the most important factor affecting the production of 1,3-PDO with a *P* value of 1.55E-09. After optimization, 1,3-PDO production by *K. pneumoniae* NH_2OH-3 mutant had increased to 25%, when compared to the parent strain.

Scale up research reveals key parameters, controlling steps and indicates process routes and constraints in the light of process economics. Hence, scale up studies for the production of 1,3-PDO were carried out using 1.0 L STR by *K. pneumoniae* 141B. the results showed that, 1,3-PDO production was increased in STR, when compared to 1,3-PDO production in shake flask, which corresponds to 5.0%, 10%, 9.0%, 7.0% and 3.0% enhancement at 20, 40, 60, 80 and 100 g/L glycerol concentrations respectively. The reason for increased production in STR might be due to the increase in vessel volume and also supply of external aeration, which helps in uptake of nutrients freely by cells. The optimum process parameters for 1,3-PDO production in STR were observed to be glycerol-40 g/L, inoculum level-10% (v/v), agitation-200 rpm, aeration-1.0 vvm, temperature-37°C and yeast extract-7.5 g/L. Further, 1,3-PDO production was carried out by *K. pneumoniae* NH_2OH-3 mutant strain in STR at the above optimized conditions. The production of 1,3-PDO by mutant strain was improved from 23.8 g/L to 28.2 g/L from shake flask to STR respectively at 40 g/L glycerol content. Overall, the mutant strain had shown approximately 16% improvement in 1,3-PDO production compared to the parent strain in STR.

For economical biological production of 1,3-PDO, an efficient and energy saving strategy for product separation and purification is essential. Since the product shall be used mainly in polymer chemistry, the grade of purification has to be from 95% up to over 99%, depending on the type of impurities and the demanded product properties. In

the present study, the separation of 1,3-PDO from fermentation broth was achieved by four subsequent steps such as removal of biomass, removal of proteins, concentration of broth and fractional distillation of the concentrated broth. Biomass was removed from the fermentation broth by centrifugation. The proteins, colored impurities and residual salts of medium were removed by isopropyl alcohol and charcoal treatment. The charcoal treated broth was further concentrated in rota vapor and the concentrated clarified broth was finally separated using the fractional distillation method. The overall yield of 1,3-PDO recovery was calculated to be 60.65% with 95.21% of purity.

The results of this work indicated that the present isolated strain i.e. *K. pneumoniae* 141B was a potent strain, which posses the potential to produce 1,3-PDO. The investigations suggest that the production of 1,3-PDO by sucrose-glycerol cofermentation, strain improvement and whole cell immobilization strategies were more beneficial as they supported high titers of 1,3-PDO production at 0.98, 0.84 and 0.78 mol/mol of glycerol respectively, from 20 g/L glycerol concentration.