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# Efficient production of (R,R)-2,3-butanediol from cellulosic hydrolysate using *Paenibacillus polymyxa* ICGEB2008

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**Abstract** We report here the production of pure (R,R)-2,3-butanediol (2,3-BDO) isomer by the non-pathogenic *Paenibacillus polymyxa* ICGEB2008 using lignocellulosic hydrolysate as substrate. Experimental design based on Plackett-Burman resulted in identification of Mn and K as most crucial salt elements along with the yeast extract for 2,3-BDO production. Further experiments using Box-Behnken design indicated that both KCl and yeast extract together had major impact on 2,3-BDO production. Optimized medium resulted in 2,3-BDO production with 2.3-fold higher maximum volumetric productivity (2.01 g/L/h) and similar yield (0.33 g/g sugar) as compared to rich yeast extract-peptone-dextrose medium in the bioreactor studies. Considering that the balance substrate was channeled towards ethanol, carbon recovery was close to theoretical yield between the two solvents, i.e., 2,3-BDO and ethanol. Biomass hydrolysate and corn-steep liquor was used further to produce 2,3-BDO without impacting its yield. In addition, 2,3-BDO was also produced via simultaneous saccharification and fermentation, signifying robustness of the strain.

**Keywords** 2,3-butanediol · *Paenibacillus* · Corn steep liquor · Biomass hydrolysate · Media optimization

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## Introduction

2,3-Butanediol is a versatile bulk chemical with huge market of ~320 billion USD, owing to its wide application in the manufacture of printing inks, perfumes, fumigants, moistening and softening agents, explosives, plasticizers, foods, pharmaceuticals, etc. [16]. Currently, it is derived from crude oil fractionation, but due to limited reserves and increasing prices of crude oil, the price hike for 2,3-butanediol (2,3-BDO) is indispensable. Thus, there is an emergent need to search for an alternate source for making 2,3-BDO. Lignocellulosic biomass is an attractive feedstock for 2,3-BDO industries since it is available at low cost and is abundant and renewable [8]. Lignocellulose comprises mainly of cellulose (40–50 %) and hemicellulose (20–30 %), and its efficient hydrolysis results in production of mixtures of hexoses, pentoses and oligosaccharides [15]. Therefore, the major challenge in cellulosic 2,3-BDO production lies in the development of strain that can ferment mixture of sugars efficiently to produce 2,3-BDO.

A variety of obligate anaerobes (e.g., Clostridia) and facultative anaerobes (e.g., *Klebsiella*, *Bacillus polymyxa,* etc.) can convert mixed reducing sugars to 2,3-butanediol under aerobic and anaerobic fermentation conditions [13, 28]. Amongst the reported microbes, *Paenibacillus polymyxa* is preferred host for 2,3-BDO production since it is a nonpathogenic microorganism (risk group 1 according to German legislation) while commonly reported *Enterobacter*, *Klebsiella* and *Serratia* sp. are pathogenic (genera of risk group 2) [6, 16]. Moreover, *P. polymyxa* is also reported to preferentially produce optically active (R,R)-2,3-BDO or D-(—)-2,3-BDO resulting in easy purification of dominant single isoform produced unlike other strains, which produce mixture of stereoisomers. Pure R,R-BDO is majorly used in rubber industry, plasticizer and also as anti-freeze agent [6].

Recent reports have shown the potential of bacteria related to *P. polymyxa* to produce high titers of 2,3-BDO using glucose as the carbon source in the media containing 60 g/L yeast extract [17]. But the use of expensive yeast extract and pure glucose as substrate has made the process cost inefficient. In our study, we have focused on three strategies to make the process economical. First strategy involves development of low-cost industrial media. In the last decade, major efforts were being diverted towards optimization of media components by factorial design and response surface methodology, which have particularly led to design of cost-effective production media for optimal cell growth. Second, emphasis is given to replace yeast extract with low-cost nitrogen source. Various studies have reported alternative nitrogen sources, but in-depth analysis revealed use of corn steep liquor (CSL) as the best and low cost alternative for microbial growth [4]. Third strategy focuses on use of low cost substrate for 2,3-BDO production, i.e., lignocellulosic biomass. Moreover, we also produced 2,3-BDO via simultaneous saccharification and fermentation (SSF). SSF is superior compared to separate hydrolysis and fermentation since combining the two process steps results in shorter process time and lower capital cost [18].

The cumulative effect of the above-mentioned strategies have not been studied for 2,3-BDO production. We have earlier reported isolation of a cellulolytic microbe, *Paenibacillus* sp. ICGEB2008 [3], whose genome sequence showed close relatedness with *P. polymyxa* species [1]. Therefore, we used *P. polymyxa* ICGEB2008 as a non-pathogenic host organism to develop cost-effective platform for (R,R)-2,3-BDO production through use of low-cost substrates, such as biomass hydrolysate and CSL.

## Materials and methods

### Bacterial strain and culture condition

*Paenibacillus polymyxa* ICGEB2008 (MTCC culture collection number: MTCC5684) used in this study was isolated from the gut of *Helicoverpa armigera* [3]. The culture was maintained in complex TSB medium (17 g/L Tryptone, 5 g/L NaCl, 2.5 g/L $K_2HPO_4$, 3 g/L peptone). The anaerobic growth of the culture was achieved by inoculating 0.2 mL of glycerol stock into 125-mL serum bottle containing 50 ml of YEPD (10 g/L yeast extract, 20 g/L soya peptone and 10 g/L glucose) medium purged with ultra-pure argon. The bottles were incubated in a rotary shaker for 16 h at 37 °C, 200 rpm and used for monitoring growth and metabolite profile. Effect of culture pH in the range of 5.0–7.0 was investigated on 2,3-BDO production at 37 °C in YEPD medium. All experiments were performed in duplicate and the average of the observations was used for final result analysis.

### Analytical methods

Extracellular metabolites of the grown culture were analyzed as follows. Culture of the grown cells was centrifuged at 10,000*g* for 5 min and the supernatant obtained was filtered and analyzed using HPLC (Agilent technologies) attached with Aminex HPX-87H anion exchange column (Bio-Rad). The filtered and degassed mobile phase (4 mM $H_2SO_4$) was used at a constant rate of 0.3 mL/min with column temperatures maintained at 40 °C. Standards of all the major metabolites at 1 g/L (Absolute Standards) were separated on HPLC column and used to estimate the concentration of metabolites present in test samples [25]. Cell density was measured at an optical density 600 nm ($OD_{600}$) in a spectrophotometer (BioRad).

### Media optimization through experimental design

#### *Plackett–Burman design*

The Plackett–Burman design was used to determine the role of media components in 2,3-BDO production [27]. All the experiments were carried out according to matrix designed using JMP10 software (http://www.jmp.com/) in serum bottles. Total nine variables were screened that included $K_2HPO_4$, $MgSO_4{\cdot}7H_2O$, KCl, sodium acetate, $KH_2PO_4$, $MnSO_4$, $FeSO_4$, $NH_4Cl$ and yeast extract (Table 1). Each independent variable was investigated at a high (+1) and a low (−1) level, which represents two different nutrient concentrations (Supplementary Table 1). In this design, it is assumed that the main factors have no interaction and a first order multiple regression model is appropriate as follows:

$$Y = \beta_0 + \sum \beta_i x_i (i = 1, \ldots, k)$$

where $Y$ is the response function (2,3-BDO production) and $\beta_i$ is the regression coefficient. A number of associated control (or input) variables are denoted by $x_1$, $x_2$, …, $x_k$. The most important factor was determined by the $p$ value ($p < 0.05$) and $t$ value ($t > 0$) evaluation of each individual effect. The $p$ value is the probability of magnitude of contrast coefficient due to random process variability and serves as a tool for checking significance of each coefficient. The components were screened at the confidence level of 95 % on the basis of their effects [27].

#### *Box-Behnken design*

The three significant variables (KCl, $MnSO_4$ and yeast extract) were selected from Plackett-Burman to optimize its concentrations using Box-Behnken design [24]. $KH_2PO_4$ (0.2 g/L) and $K_2HPO_4$ (2 g/L) were kept constant as buffering reagents. The experimental design consists of a set of

**Table 1** Statistical analysis of Plackett-Burman design results showing effect of each variable on 2,3-butanediol yield

| Code | Variable | Low level (g/L) (−1) | High level (g/L) (+1) | Effects | Standard error | *t* value | Prob > \|*t*\| |
|---|---|---|---|---|---|---|---|
| X1 | Yeast extract | 5 | 15 | 0.311667 | 0.023784 | 13.1 | <0.0001 |
| X2 | $KH_2PO_4$ | 0.2 | 0.5 | 0.135 | 0.023784 | 5.68 | <0.0001 |
| X3 | $K_2HPO_4$ | 0.2 | 2 | 0.110833 | 0.023784 | 4.66 | 0.0002 |
| X4 | Sodium acetate | 0 | 2 | 0.024583 | 0.022771 | 1.08 | 0.2946 |
| X5 | $FeSO_4$ | 0 | 0.015 | −0.03292 | 0.022771 | −1.45 | 0.1655 |
| X6 | $MnSO_4$ | 0 | 0.015 | 0.057917 | 0.022771 | 2.54 | 0.0204 |
| X7 | $MgSO_4$ | 0 | 0.5 | −0.09375 | 0.022771 | −4.12 | 0.0006 |
| X8 | $NH_4Cl$ | 0 | 2 | −0.03875 | 0.022771 | −1.7 | 0.106 |
| X9 | KCl | 0 | 0.2 | 0.052143 | 0.02202 | 2.37 | 0.0293 |

The value of the determination coefficient ($R^2 = 0.9352$) being a measure of goodness of fit to the model indicated that only about 6.5 % of the total variations were not explained by the model

The adjusted $R^2$ value (0.9029) was also very high, making the model very significant

points lying at the midpoint of each edge and the replicated center point of the multidimensional cube. The equation generated by this model is as follows:

$$Yi = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2$$

where *Yi* is the dependent variable; $b_0$ is the intercept; $b_1$–$b_{33}$ are the regression coefficients; and $X_1$, $X_2$ and $X_3$ are the independent variable that was selected from the preliminary experiments. The ANOVA was used to evaluate the effect of independent variables on the response and significant results were identified by a $p$ value of $< 0.05$ [12]. The results were further analyzed using Fisher's statistical analysis in which the $F$ value is the ratio of the mean square due to regression to the mean square due to error and indicates the influence (significance) of each controlled factor on the tested model [10]. Multiple correlation coefficient ($R^2$) and adjusted $R^2$ were used as quality indicators to evaluate the fitness of the second order polynomial equation. Contour plots were employed to demonstrate the relationship and interaction between the coded variables and the response.

*Bioreactor cultivation conditions*

Cultivations in the bioreactor were performed in a multi-vessel fermentor system (Biostat Qplus, Sartorius) with 350 mL working volume. Anaerobic condition was maintained in the bioreactor by passing filtered argon gas through a sparger at the bottom of the vessel. The bioreactors were equipped with sensors for pH, temperature, and dissolved oxygen. The inoculum was prepared by growing ICGEB2008 strain in 50 mL fermentation medium at 37 °C for 16 h under anaerobic condition. The grown inoculum was centrifuged and washed with fermentation medium and was used to inoculate the bioreactor containing different medium. During cultivation the pH and temperature were automatically maintained at pH 6.3 and 37 °C, respectively. No gas was passed through the bioreactor initially to allow exchange of air through the headspace for faster cell growth. Argon gas was sparged at the rate of 0.2 mL/min after 12 h of cultivation to maintain strict anaerobic condition. Strict anaerobic condition allowed higher product yield. Samples were withdrawn at various time points to analyze the biomass and various metabolites.

*Biomass hydrolysate preparation*

The wheat straw-based, ammonia-treated biomass [22] was used as substrate. The enzymatic hydrolysis of biomass was done as follows. Pretreated biomass (25 g dry weight) was hydrolyzed in 200 mL citrate–phosphate buffer (50 mM, pH 4.8) with enzyme cocktail (100 FPU/g, Advanced Enzyme) by incubating in rotary shaker at 50 °C for 20 h. pH of hydrolysate was then adjusted to 6.3 using NaOH and was filter sterilized before use.

*Simultaneous saccharification and fermentation (SSF)*

SSF was achieved by growing ICGEB2008 strain in the bioreactor under similar conditions as mentioned earlier [7]. Briefly, seed culture was prepared by inoculating ICGEB2008 strain in the optimized medium containing glucose (10 g/L) under anaerobic condition. After 24 h of growth at 37 °C, the culture was centrifuged at 4,000*g* for 10 min and washed before inoculating into fermentor vessel containing optimized medium components (pH 6.3), 15 g/L CSL, 57 g/L cellulosic biomass on dry basis, and cellulolytic enzymes at 100 FPU/g of biomass. The cultivation was performed without sparging the inert gas to allow air to diffuse through the headspace to facilitate the enzymatic hydrolysis of the biomass. ICGEB2008 strain was allowed to grow for 48 h at 37 °C and the metabolites produced were analyzed using HPLC.

## Results and discussion

### (R,R)-2, 3-butanediol production by *Paenibacillus polymyxa* ICGEB2008

*Paenibacillus* sp. ICGEB2008, isolated from the gut of cotton bollworm, showed genomic properties close to *Paenibacillus polymyxa* [1]. *P. polymyxa* has been reported earlier as GRAS microbe for the production of 2,3-BDO [26]. Upon analysis, we indeed found production of 2,3-BDO by the ICGEB2008 strain as major metabolite along with ethanol and small quantity of organic acids (Fig. 1a). Gas chromatography-based mass spectroscopic analysis revealed production of single (R,R) form of 2,3-BDO by the ICGEB2008 strain (Supplementary Fig. 1). We further tested impact of culture pH on 2,3-BDO production as it was found to play important role in 2,3-BDO production [30]. Maximum 2,3-BDO production was achieved by the ICGEB2008 strain when culture pH was set at 6.3 (Fig. 1b). Higher cultivation pH favoured ethanol production (Supplementary Fig. 2).

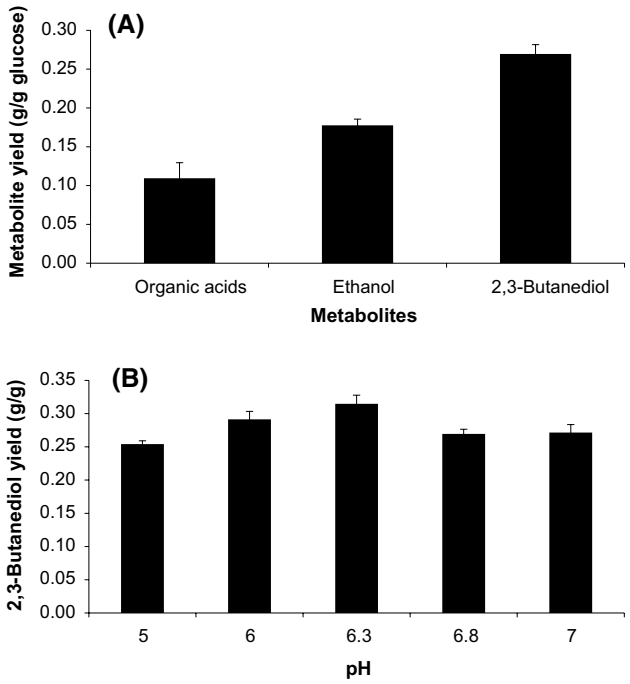


**Fig. 1** 2,3-Butanediol production by *Paenibacillus polymyxa* ICGEB2008. **a** Typical product profile of ICGEB2008 strain grown under anaerobic condition in YEPD medium. **b** Impact of culture pH on production of 2,3-BDO

### Media optimization to enhance 2,3-BDO production

The influence of media components as independent variables on the production of 2,3-BDO from *Paenibacillus* sp. ICGEB2008 was determined using Plackett–Burman screening design [20]. Recent studies have indicated importance of Mn, K and Mg salts for 2,3-BDO production [5, 12]. In this study, along with these micronutrients, we have also investigated the role of $FeSO_4$ and $NH_4Cl$ on 2,3-BDO production. Significance of each variable was determined using Student *t* test. Out of 9 variables studied, three variables (yeast extract, KCl, $MnSO_4$) had significant influence on 2,3-BDO production as evident by their *t* value ($t > 0$ and $p < 0.05$) obtained from the regression analysis (Table 1). Different media composition resulted in significant variation in 2,3-BDO production ranging from 2.71 to 3.82 g/L (Supplementary Table 1). On analysis of the regression coefficients of the 9 variables, yeast extract, $K_2HPO_4$, KCl, $KH_2PO_4$ and $MnSO_4$ showed positive effect on 2,3-BDO production whereas $FeSO_4$, $NH_4Cl$, sodium acetate and $MgSO_4{\cdot}7H_2O$ contributed negatively (Table 1; Fig. 2). These finding illustrates the order of significance of the variables affecting the 2,3-BDO production as: yeast extract > $KH_2PO_4$ > $K_2HPO_4$ > $MnSO_4$ > KCl [20]. Various concentrations of yeast extract, KCl and $MnSO_4$ were

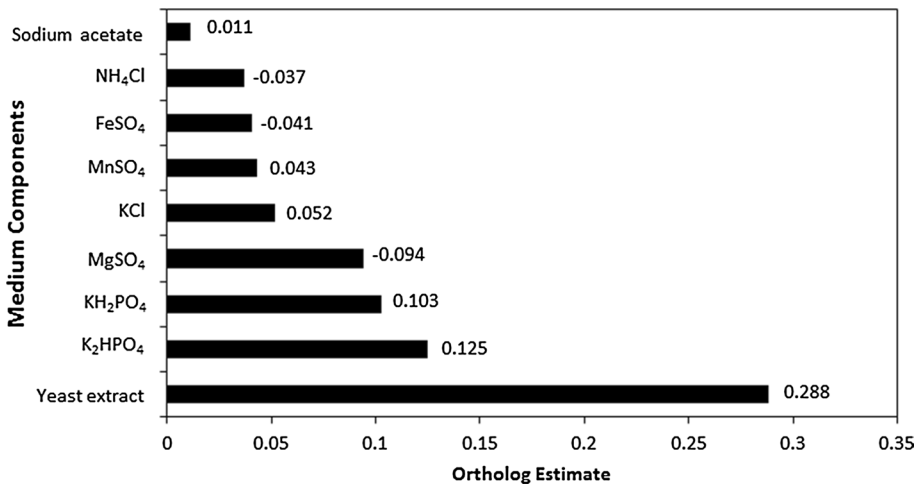


**Fig. 2** Pareto chart representing the effect of variables in Plackett-Burman design on 2,3-butanediol production by *Paenibacillus polymyxa* ICGEB2008

considered for the next stage of standardization using response surface optimization technique. $KH_2PO_4$ (0.2 g/L) and $K_2HPO_4$ (2 g/L) were retained as the buffering components in the medium.

Further media optimization was done using Box–Behnken design as mentioned in the materials and methods section. The adequacy of the model was checked using analysis of variance (ANOVA), which was tested using Fisher's statistical analysis (Table 2). The ANOVA showed that this regression model was highly significant ($p < 0.01$) with $F$ value of 33.52 (Table 2). The fitness of the model was further confirmed by a satisfactory value of determination coefficient ($R^2$), which was calculated to be 0.9328, indicating that 93.28 % of the variability in the response could be predicted by the model. The fitted response for the above regression model is plotted in Fig. 3a in which $MnSO_4$ shows hyperbolic response, i.e., increasing levels of $MnSO_4$ support high 2,3-BDO levels till its concentration reaches 0.0225 g/L and then subsequent higher $MnSO_4$ concentration results in decline in 2,3-BDO production. On the other hand, higher levels of the 2,3-BDO were attained with increasing the concentration of yeast extract in the medium (Fig. 3a). The addition of KCl as supplement to yeast extract also showed positive impact on 2,3-BDO production (Fig. 3b). It is clear from the cube plot (Supplementary Fig. 3) that neither KCl nor yeast extract alone had any major impact 2,3-BDO production but significant increase in 2,3-BDO was observed when yeast extract and KCl were supplemented together as media components. Further increase was observed when $MnSO_4$ was added to the final media.

The optimal levels of the three components as obtained from the maximum point of the polynomial model were estimated as- KCl, 0.3 g/L; $MnSO_4$, 0.0225 g/L and yeast extract, 15 g/L with a predicted 2,3-BDO production of 3.51 g/L (Supplementary Fig. 4). The 2,3-BDO concentration under optimal medium condition was 30 % higher than the non-optimal medium condition, reflecting the value of optimization process.

**Table 2** Anova for 2,3-butanediol production according to response surface quadratic model

| Factors | Statistics | | | | |
|---|---|---|---|---|---|
| | Sum of squares | $df^a$ | Mean square | $F$ ratio | Prob > F |
| Model | 0.39868036 | 9 | 0.044298 | 33.5202 | <0.0001 |
| Yeast extract | 0.09150625 | 1 | 0.09150625 | 69.2428 | <0.0001 |
| KCl | 0.02975625 | 1 | 0.02975625 | 22.5166 | 0.0002 |
| $MnSO_4$ | 0.009025 | 1 | 0.009025 | 6.8292 | 0.0176 |
| Yeast extract × KCl | 0.0153125 | 1 | 0.0153125 | 11.587 | 0.0032 |
| Yeast extract × $MnSO_4$ | 0 | 1 | 0 | 0 | 1 |
| KCl × $MnSO_4$ | 0.00005 | 1 | 0.00005 | 0.0378 | 0.848 |
| Yeast extract × yeast extract | 0.0000225 | 1 | 0.0000225 | 0.017 | 0.8976 |
| KCl × KCl | 0.0000025 | 1 | 0.0000025 | 0.0019 | 0.9658 |
| $MnSO_4$ × $MnSO_4$ | 0.2356225 | 1 | 0.2356225 | 178.2955 | <0.0001 |

$R^2 = 0.943$, $R^2(\text{adj.}) = 0.915$

[a] Degree of freedom

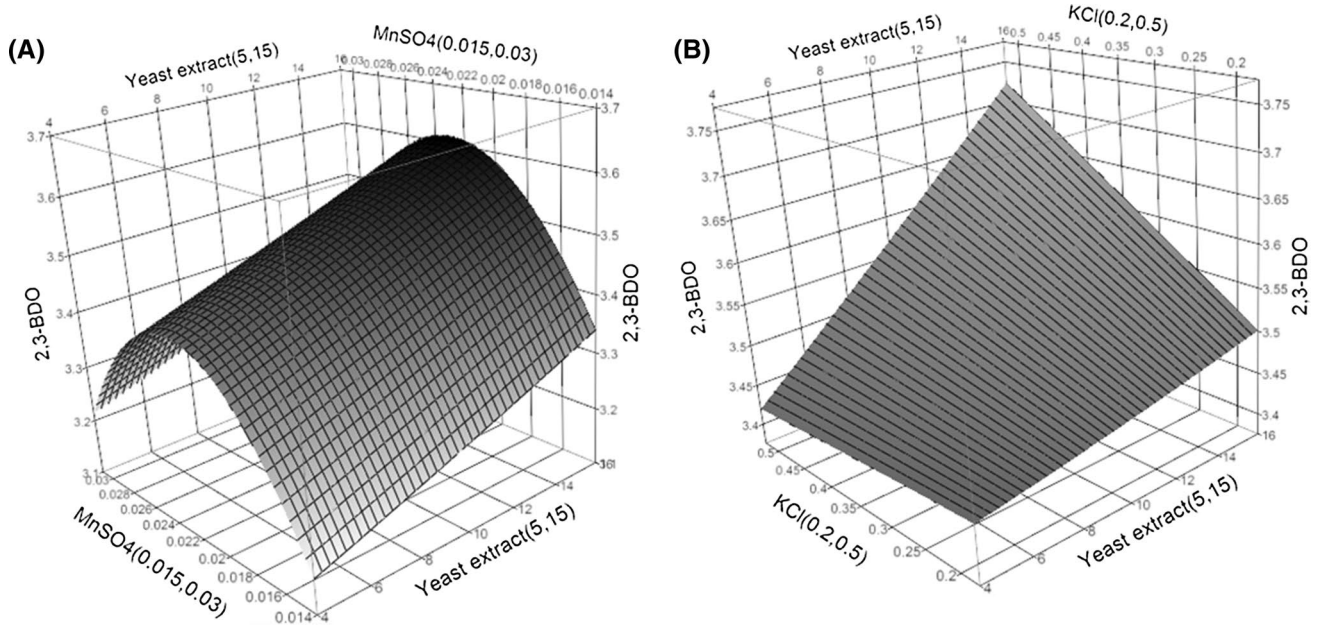


**Fig. 3** The response surface plot showing the effects of **a** $MnSO_4$ and **b** KCl on 2,3-butanediol production by *Paenibacillus polymyxa* ICGEB2008

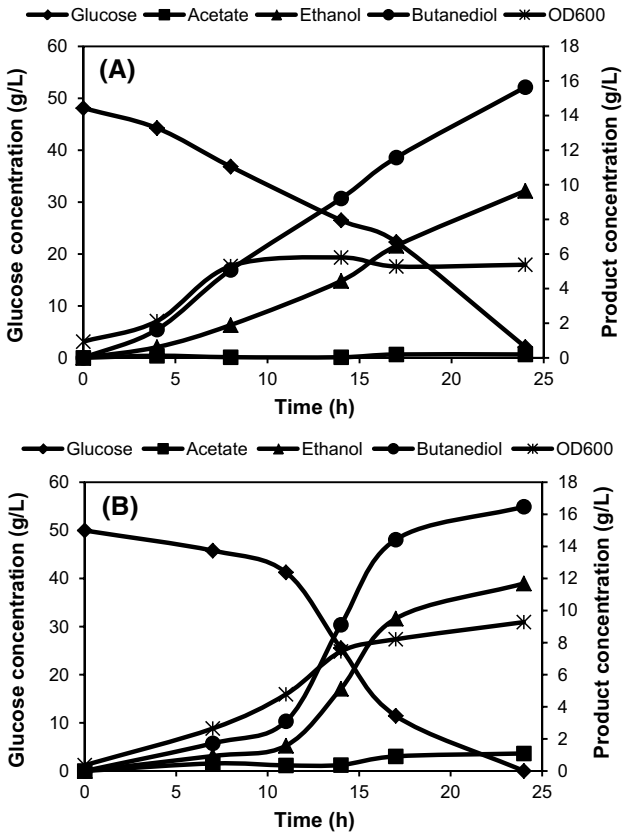


**Fig. 4** Fermentation profile of *Paenibacillus polymyxa* ICGEB2008 using **a** complex YEPD and **b** optimized medium containing 50 g/L glucose as carbon source

## 2,3-BDO production in the bioreactor

Validation of the model was carried out in the bioreactor using media components obtained after optimization process. A medium of following composition was used for 2,3-BDO production—yeast extract, 15 g/L; $KH_2PO_4$, 0.5 g/L; $K_2HPO_4$, 2 g/L; $MnSO_4$, 0.0225 g/L; and KCl, 0.3 g/L. The yield of 2,3-BDO in this medium was 0.33 g/g glucose, which was similar to that obtained in the YEPD medium with 50 g/L glucose (Fig. 4; Table 3). Longer lag phase was observed in the optimized medium followed by steep exponential curve, whereas YEPD (50) medium showed faster initial growth owing to presence of highly complex nitrogen source, i.e., 20 g/L soya peptone and 10 g/L yeast extract. However, the optimized media showed 2.3-fold higher maximum volumetric productivity of 2,3-BDO, i.e., 2.01 g/L/h as against 0.86 g/L/h in case of YEPD (50) medium. This was the highest maximum volumetric productivity and average volumetric productivity (0.95 g/L/h) of single (R,R)-2,3-BDO isomer reported so far in the literature for any native microbe [15].

## Use of biomass hydrolysate as substrate

Along with optimized media components, there is an emergent requirement to explore an alternative, abundant and less expensive carbon source to make the production process cost-effective. At present, cellulosic biomass is considered to be environmentally sustainable resource for producing liquid transportation fuels to meet the goals. Most

**Table 3** Comparative analysis of production of 2,3-butanediol using different nitrogen and carbon sources

| S. no. | Media | C-source | Complex N-source | 2,3-butandiol production | | | 2,3-BDO + ethanol yield (g/g sugar) |
|---|---|---|---|---|---|---|---|
| | | | | Maximum volumetric Productivity (g/L/h) | Average volumetric Productivity (g/L/h) | Yield (g/g sugar) | |
| 1. | YEPD media | Glucose (50 g/L) | Yeast extract (10 g/L) soya peptone (20 g/L) | 0.86 (±0.03) | 0.67 (±0.012) | 0.34 (±0.019) | 0.55 (±0.024) |
| 2. | Optimized media | Glucose (50 g/L) | Yeast extract (15 g/L) | 2.01 (±0.07) | 0.95 (±0.011) | 0.33 (±0.012) | 0.56 (±0.028) |
| 3. | Optimized media | Hydrolysate (65 g/L sugars) | Yeast extract (15 g/L) | 1.16 (±0.05) | 0.31 (±0.032) | 0.30 (±0.021) | 0.50 (±0.018) |
| 4. | Optimized media | Hydrolysate (65 g/L sugars) | Corn steep liquor (15 g/L) | 0.70 (±0.06) | 0.27 (±0.021) | 0.33 (±0.09) | 0.53 (±0.020) |
| 5. | Optimized media | Biomass (57 g/L) + cellulolytic enzymes (100 FPU/g) | Corn steep liquor (15 g/L) | ND | 0.35 (±0.014)* | 0.30 (±0.07)* | 0.34 (±0.05)*,§ |

* Include values for 2,3-BDO and acetoin together in 3:2 ratio

§ More acetate instead of ethanol was detected during SSF

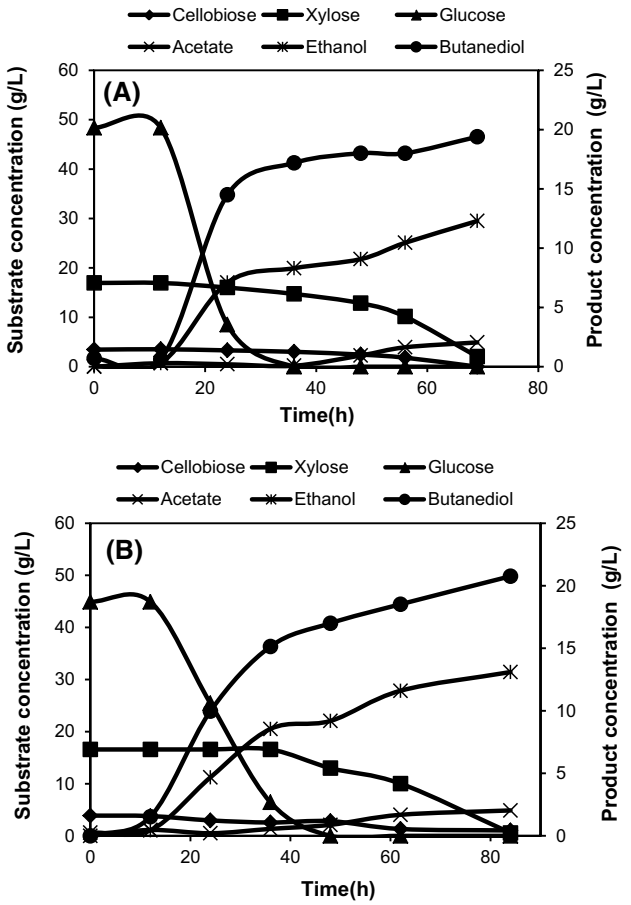


**Fig. 5** Fermentation profile of *Paenibacillus polymyxa* ICGEB2008 using optimized media containing yeast extract (**a**) and corn steep liquor (**b**) as nitrogen source with biomass hydrolysate being used as carbon source

of the cellulosic biomass is composed of hexose and pentose sugars in various proportions. We have used ammonia pretreated biomass for this study, which upon enzymatic saccharification gives primarily glucose, xylose and cellobiose. We evaluated utilization of different simple and complex sugar by ICGEB2008 and found that it has inherent potential to metabolize wide array of substrates including cellobiose (data not shown). We, therefore, replaced the carbon source from glucose to biomass hydrolysate in the growth media and performed fermentation study in the bioreactor (Fig. 5a). ICGEB2008 strain could effectively metabolize complete biomass hydrolysate, including 45 g/L glucose, 16.5 g/L xylose and 3.9 g/L cellobiose, and produce 19.4 g/L of 2,3-BDO with yield comparable with that obtained with glucose as the carbon source. The maximum and average productivity obtained with the hydrolysate were 1.16 and 0.31 g/L/h, respectively.

The use of expensive yeast extract in the fermentation process for 2,3-BDO production makes the process cost-ineffective. Few earlier reports suggested use of corn-steep liquor (CSL), instead of yeast extract, as low-cost nitrogen source [9]. Therefore, we tested effect of different CSL concentration (ranging from 5 to 20 g/L) in the media and found that 15 g/L CSL was optimal N-source required for 2,3-butanediol production (Supplementary Fig. 4). Henceforth, we replaced yeast extract in the optimized media with 15 g/L CSL for next batch of fermenter and obtained 21 g/L 2,3-BDO with the yield of ~0.33 g/g and maximum and average productivity of 0.70 and 0.27 g/L/h, respectively (Fig. 5b).

The optimized fermentation condition offers economical platform for cellulose-based 2,3-BDO production since the yield of 2,3-BDO obtained did not change with changing the substrate from pure glucose to cellulosic hydrolysate or upon replacing yeast extract with CSL (Table 3). We also recovered rest ~33 % of the carbon in the form of ethanol. This is the first report where we have produced pure R,R form of 2,3-BDO from an agricultural waste using CSL as nitrogen source. Earlier reports indicated production of mixed isomers of 2,3-BDO using corncob molasses [29], corncob hydrolysate [7] and Jerusalem artichoke stalk [23]. Pure R,R-2,3-BDO was reported to be produced from food material such as Jerusalem artichoke tuber [11]. Another important observation was that 2,3-BDO was produced throughout the cultivation of ICGEB2008 strain on glucose, xylose and cellobiose (Fig. 5a, b). The metabolite patterns during fermentation indicated that significant portion of xylose was converted to 2,3-BDO. This finding was contrary to the earlier observation that polymyxa strains cannot produce 2,3-BDO from xylose or its polymers [14].

## Simultaneous saccharification and fermentation (SSF)

Since SSF offers economical solution by saving time and resources by combining cellulosic saccharification and fermentation in single step, we used ICGEB2008 strain as a model organism to develop platform for SSF to produce 2,3-BDO. Further advantage in using this strain was that it could utilize cellobiose and cello-oligosachharides [2], thereby minimizing the product inhibition to the cellulase enzymes. The fermentation experiment was carried out in the bioreactor with 57 g/L of dry biomass supplemented with 100 FPU cellulolytic enzymes per gram biomass and 10 g/L CSL under microaerobic condition as mentioned in material and method section. The analysis of SSF products revealed formation of acetoin along with 2,3-BDO in the ratio of 2:3. Total C4 products obtained were ~17 g/L in 48 h with yield and productivity similar to that obtained when hydrolysis was done in a separate vessel (Table 3). Only 2.5 g/L of ethanol and significant quantity of acetate (8.6 g/L) were formed during the process. Formation of more oxidized products, such as acetoin and acetate, signifies the microaerobic environment maintained during SSF to accelerate the hydrolysis process.

## Conclusions

*P. polymyxa* ICGEB2008 was found to be an efficient producer of single enantiomer of 2,3-BDO. Optimization of media compositions using Plackett–Burman and response surface methodology led to 2.3-fold improvement in maximum volumetric productivity of 2,3-BDO as compared to that in rich media without compromising the yield. Further, we used biomass hydrolysate as carbon source and CSL as nitrogen source and found efficient production of 2,3-BDO with maximum volumetric productivity close to 0.70 g/L/h. Moreover, ICGEB2008 strain was also found to be an excellent organism for producing 2,3-BDO in simultaneous saccharification and fermentation mode.

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