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Isolation and partial characterization of a new strain of *Klebsiella pneumoniae* capable of high 1,3 propanediol production from glycerol

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ABSTRACT: Glycerol is a promising feedstock for microbial cultivation and production of 1,3 propanediol (1,3 PDO). Here we report a newly isolated bacterial strain BA11 from soil, capable of fermenting glycerol to 1,3 PDO, and has been identified to be a strain of *Klebsiella pneumoniae.* Strain BA11 was fast growing showing peak 1,3 PDO production in 6 h of cultivation with productivity of 1.2 g/L-h without the addition of Vitamin B_{12}. Based on the optimum glycerol utilization (75%) and 1,3 PDO production (8.3 g/L) and yield (0.56 mol/mol glycerol utilized), the most appropriate glycerol concentration for cultivation was 20 g/L. The strain BA11 could tolerate the pH range of 6 to 8.5 as no inhibitory effects were seen on growth as well as 1,3 PDO production. Strain BA11 was most active and could produce high 1,3 PDO in the incubation temperature range of 25 to 40 ^{o}C. The production of 1,3 PDO was maximum (9.3 g/L) under aerobic condition with 95.8% glycerol utilization. Addition of glucose to the glycerol fermentation led to increased cell mass but no improvement in the 1,3 PDO production.

Keywords: *Anaerobic enrichment, Aerobic fermentation, Isolation, Molar yield, Productivity, pH tolerance*

INTRODUCTION

With the increase in global biodiesel production, there is a tremendous surplus of glycerol resulting in a dramatic decrease in its prices. The excess glycerol generated may become an environmental problem in future and a daunting challenge to the biodiesel industry. For every 100 kg of biodiesel produced, 10 kg of glycerol is produced (da Silva *et al.*, 2009). In addition, waste streams containing high levels of glycerol are generated in almost every industry that uses animal fats or vegetable oils as starting material. In 2010, the annual biodiesel production in the US and Europe were 6.96 and 11.2 million tons, respectively (Khanna *et al.,* 2012). At the current international price (US $ 0.21–0.23/lb), glycerol can offer an opportunity to synthesize a large array of products via microbial fermentation (Khanna *et al.,* 2012; Saxena *et al.,* 2009). In the last few decades, the low-price of raw glycerol obtained from various industrial processes was evaluated for the bioconversion to 1,3-propanediol by different workers (Biebl, 2001; Chatzifragkou *et al.,* 2011; Cheng *et al.*, 2006; Forsberg, 1987; González-Pajuelo *et al.*, 2005). Papanikolaou and Aggelis, (2003) evaluated raw glycerol for the production of 1,3 propanediol and found similar or even better results when compared with the commercial glycerol. It has been shown that the effect of crude glycerol (from biodioesel production) on the growth of *Clostridium butyricum* was minimal and it also did not interfere with 1,3 propanediol production (González-Pajuelo *et al.*, 2004; Papanikolaou *et al.,* 2004). Same investigation had also been done for the most potent microorganism *Klebsiella pneumoniae* (Mu *et al.*, 2006). The authors showed the microbial production of 1,3 propanediol by *K. pneumoniae* by using crude glycerol as the sole carbon source. When the effect of impurities of the industrial feedstock was investigated, no adverse effect on microbial growth and metabolism of *C. butyricum* was observed (Papanikolaou *et al.,* 2008). The production of 1,3-propanediol by *C. butyricum* using raw glycerol as a sole carbon and energy source was also found feasible (Asad ur *et al.*, 2008).

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In this study, we report the glycerol bioconversion to 1,3 PDO by a newly isolated strain *K. pneumoniae* BA11 from soil sample collected in the vicinity of an oil mill. The partial characterization and optimal bioconversion potential of the new isolate *K. pneumoniae* BA11 under various cultivation conditions has been investigated in this study.

MATERIAS AND METHODS

Enrichment, isolation and screening of the 1,3 PDO producing cultures

For enrichment and isolation of bacteria, the medium used contained following composition; (per litre) $(NH_4)_2SO_4$:5g, K_2HPO_4: 7g, KH_2PO_4: 3g, NaCl: 5g, Yeast extract: 1g, Peptone: 1g, $MgCl_2.6H_2O$:0.1g, trace element stock: 1mL, Cysteine HCl: 0.5g, Resazurin: 1 mL. pH was adjusted to 7. No vitamins solution was added to the medium. To maintain anaerobic condition, media was boiled and then poured into serum vials followed by flushing with nitrogen gas for 5 min and sealing with butyl ruber stopper and alumunium crimp seal. Pure glycerol (Qualigen ExcelR grade, 99.5% pure) was added at concentration of 10 g/L (w/v). For isolation same medium with 20 g/L agar was used. Samples collected from 12 different sites which includes effluents from anaerobic digesters set using various wastes, soil samples from various places and sediment samples from different lakes, were used for setting up the enrichments. One g of each sample was added to 9 mL of saline and it was flushed with nitrogen gas. Two mL of this diluted sample was inoculated in 18 mL minimal medium containing 10 g/L pure glycerol. Enrichment was set up by transferring the grown culture to 65 mL serum bottles which were incubated at 37 °C for two days. The enriched culture was further sub-cultured repetitively by transferring to fresh medium. The glycerol utilization and 1,3 PDO production ability was checked at regular intervals.

Enrichments showing higher glycerol utilization and 1,3 PDO production were selected for isolation by streaking on agar plate. Isolation was carried out using anaerobic techniques described by Holdeman and Moore (Holdeman and Moore, 1975), using anaerobic glove box (Thermo Forma Scientific, USA). Gas mixture of $N_{2:}H_{2:}CO_2$ in the ratio (85:10:5) was used as a gas phase inside the glove box to maintain anaerobic condition. The isolates were screened for their glycerol utilization and 1, 3 PDO production ability. Screening was performed under anaerobic, static conditions in 65 mL serum bottles with a final volume of 20 mL. Inoculum density was kept constant and 10% inoculum volume was used. Incubation was carried out at 37 °C with an initial pH of 7 under static condition. Isolate purity was checked by repeated streaking on agar plate.

Growth characteristics and identification of the high yielding isolate BA11

The morphology, Gram character, nature of the organism was studied by routine microbiological processes. Biochemical characterization of the isolate was done according to Bergey's manual of Systematic Bacteriology (Brenner *et al.*, 2005). Molecular identification of the isolate was carried out by 16S rRNA gene sequence analysis. Total genomic DNA isolation was carried out from freshly grown cultures using standard phenol: chloroform method (Sambrook and Russell, 2001) which was followed by 16S rRNA gene amplification using Universal primer set 27F (5'-CCAGAGTTTGATCGTGGCTCAG-3') and 1488R (5'-CGGTTACCTTGTTACGACTTCACC-3'). The PCR reactions were carried out in a total volume of 25 µl. The reaction constituted 1X standard Taq Buffer, 200 nM dNTPs, 0.4 µM of each primers, 0.625U Taq Polymerase (Banglore Genei, Banglore, India) and 20 ng of template DNA. All PCR were performed for 35 cycles. Purified PCR products were sequenced using Big Dye Terminator Cycle Sequencing Ready Reaction Kit v 3.1 in an automated 3730xl DNA analyzer (Applied Biosystems Inc.). BLAST was performed for the sequence using BLAST algorithm (http://blast.ncbi.nlm.nih.gov). The Genebank sequence accession number for the strain BA11 is JX421757.1. Phylogenetic analyses were conducted in MEGA4 (Tamura *et al.,* 2007).

Biochemical behaviour of isolate BA11 under various cultivation conditions

Substrate concentration, pH and temperature

The effect of glycerol concentration, pH and temperature on the growth of the newly isolated strain BA11 was studied and the optimum values of these variables were obtained for maximal1,3 PDO yield. The experiments were carried out in 130 mL serum bottles with a working volume of 50 mL. Inoculum density (10%) and volume were kept constant in all the experiments. Substrate concentrations was taken in the range of 10 to 60 g/L with the initial pH at 7 and temperature of 37 °C. pH in the range of 6 to 8.5 were studied at an interval of 0.5 pH units with the same incubation conditions as

above. Initial glycerol concentration was kept at 10 g/L for the pH optimization runs. Temperatures range of 25 to 45 °C were studied at initial pH 7 and glycerol concentration of 10 g/L.

Mode of cultivation

Three different modes of cultivation were studied for the newly isolated strain BA11 to evaluate their effect on the growth and metabolism and 1,3 PDO production. Strict anaerobic, partial anaerobic and aerobic conditions were maintaned during the cultivation. Partial anaerobic conditions were maintained by sealing the bottles without any addition of reducing agent and N_2 flushing. While for aerobic condition similar bottles with a cotton plug were used with no addition of reducing agent. Batch cultivation was carried out in 130 mL serum bottles with a final volume of 50 mL. Initial pH was set at 7 and incubation was carried out at 37 °C with glycerol at a concentration of 20 g/L as the sole carbon source. Growth, metabolites and 1,3 PDO were measured at every 24 h interval.

Addition of co-substrate

Glucose as a co-substrate was used to evaluate the effect of co-fermentation on the growth, metablism and 1,3 PDO production. Glucose at a concentration of 0.2, 0.4 and 0.6% was added to the cultivation medium containing 2, 4 and 6% glycerol, respectively. Glucose and glycerol alone as the sole carbon source were kept as the control. All the fermentations were done at pH 7 and incubation temperature of 37 °C. Sample were collected every 24 h interval and checked for cell mass and metabolites.

Analytical methods

Growth was measured by monitoring optical density at 600nm on spectrophotometer (Shimadzu UV 1601, Japan). The concentration of glycerol, 1,3 PDO, succinic acid, lactic acid, formic acid, acetic acid, butyric acid, ethanol, butanol and 2,3 butanediol was determined by a high-performance liquid chromatography system (Dionex, USA) using Aminex HPX-87H, Biorad column, equipped with refractive index (RI) detector. The operating conditions were: 5mM H_2SO_4 as a mobile phase with a flow rate of 0.7 mL/min and 40 °C as the column temperature. Concentration of gas in the headspace of the bottle was determined by a gas chromatograph (PerkinElmer) equipped with thermal conductivity detector (TCD), Porapak Q column (SS, 1/800 x 80 mesh) with argon as carrier gas (flow rate :40 mL/ min). Temperature settings for TCD analysis were as follows: oven at 40 °C, injector and detector at 70 °C, and injector at 100 °C. All experiments have been carried out atleast in duplicates and mean values are reported.

RESULTS AND DISCUSSION

Screening for high 1,3 PDO producers

From 12 samples used for enrichments, a total of 78 glycerol utilizing and 1,3 PDO producing anaerobic organism were obtained. After primary screening, only eleven strains were found to be high 1,3 PDO producers (yield >0.5 mol/mol glycerol utilized). One of the isolate named BA11 isolated from soil sample collected near an oil mill was selected for further studies and characterization.

Biochemical and molecular characterization of strain BA11

Isolate BA11 is found to be a facultative Gram negative anaerobic, short thick rods occurring mostly singly, average size of 2.24µm x 1.075µm, non-motile, spore former, capsulated. The biochemical tests showed strain BA11 as lactose fermenting, urease negative, citrate positive, indole negative, methyl red positive and VP positive. Using the biochemical and morphological characteristics of the organism and according to Bergey's manual of systematic bacteriology the organism was identified as *Klebsiella* sp. BA11. From the growth pattern of *Klebsiella* sp. BA11 (Fig. 1), the isolate was found to be fast growing with a specific growth rate of 0.59 per hour and a doubling time, T_d, equal to 1.17 h. From Fig. 1, it can be seen that the strain BA11 is a fast growing bacteria giving peak 1,3 PDO production in 6 h of cultivation. The 1,3 PDO productivity of the strain at 10 g/L glycerol concentration was calculated to be 1.2 g/L-h. The 16S gene sequence analysis of the isolate *Klebsiella* sp. BA11 further confirmed the phylogeny of the isolate, and closely related to *K. pneumoniae*. The isolate has been named as *K. pneumoniae* BA11, and showed 99% sequence homology with *K. pneumoniae* strain DSM 30104.

Effect of glycerol concentration, pH and temperature on growth and metabolism of Klebsiella pneumoniae BA11

The results for growth, metabolic products and 1,3 PDO production at different glycerol concentrations are shown in Fig. 2. The concentration of 1,3 PDO increased nearly 2-fold, from 4.2 g/L to 7.5 g/L when the glycerol concentration was increased from 10 g/L

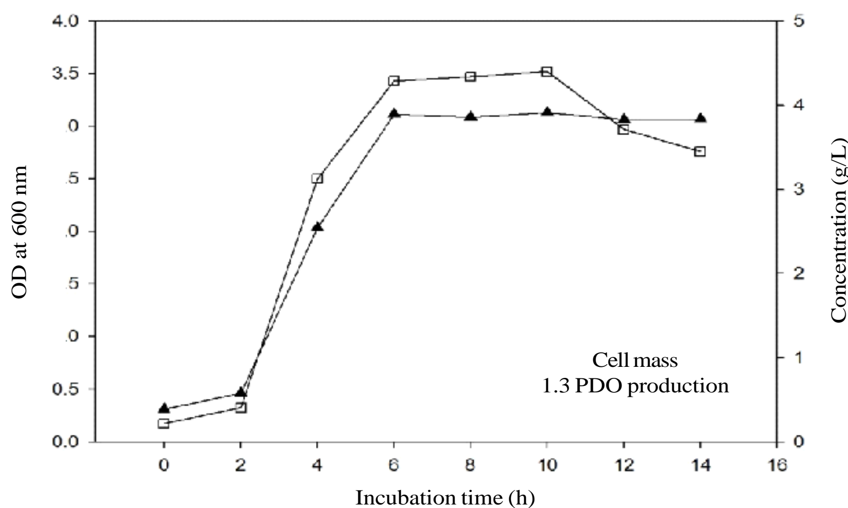

Fig. 1: Time course of growth and 1,3 propanediol production by Klebsiella pneumoniae BA11

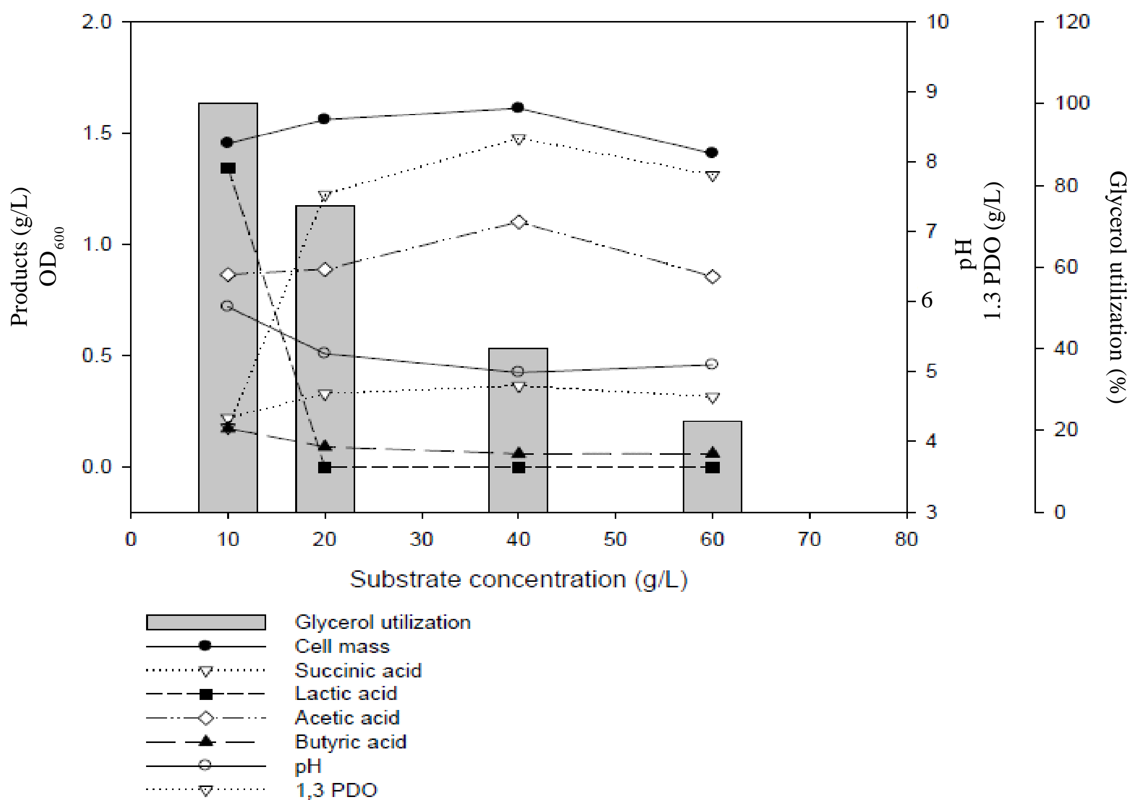

Fig. 2: Growth and metabolic profile of *Klebsiella pneumoniae* BA11 under different concentration of glycerol as carbon source

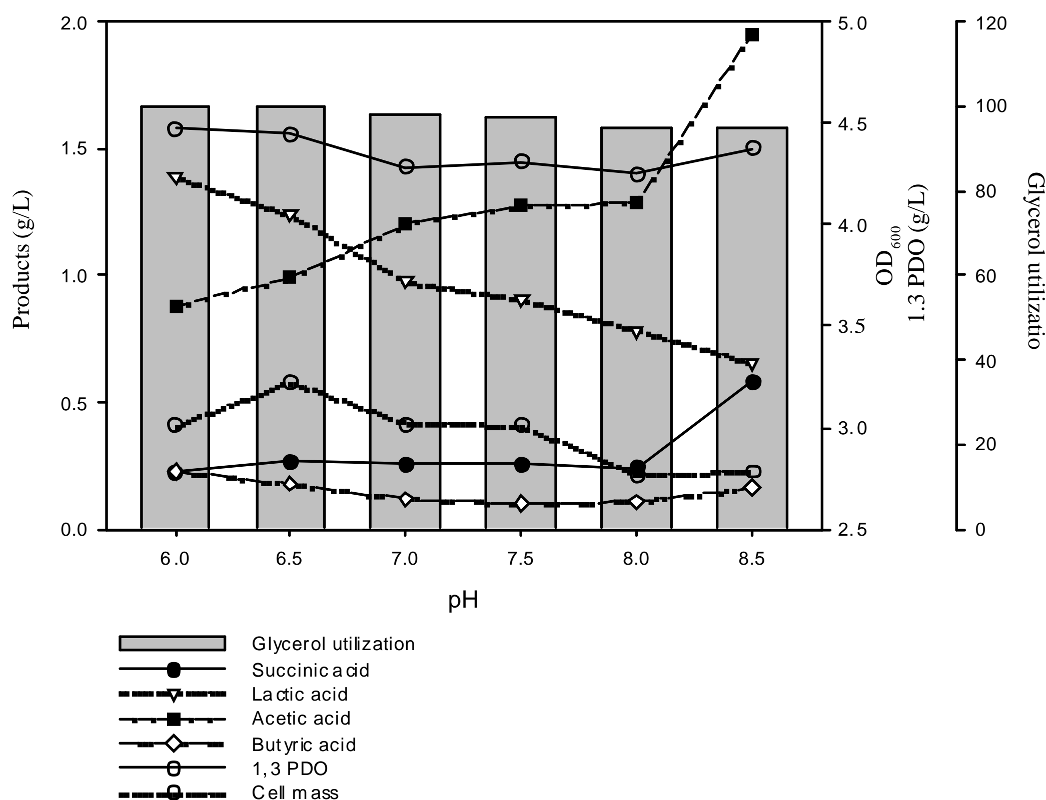

Fig. 3: Growth and metabolic profile of *Klebsiella pneumoniae* BA11 under different initial pH

to 20 g/L. However, further increase of glycerol concentration beyond 20 g/L did not show a marked increase in 1,3 PDO production. The glycerol utilization was 100% at 10 g/L and decreased drastically to 22% at 60 g/L of glycerol. The peak concentration of 1,3PDO from batch glycerol fermentation was found to be 8.3 g/L with 40 g/L initial glycerol. The maximum 1,3 PDO yield was found at 60 g/L initial glycerol concentration. However, based on the optimum glycerol utilization (75%) and 1,3 PDO production (8.3 g/L) and yield (0.56 mol/mol glycerol utilized), the most appropriate glycerol concentration for cultivation by the strain BA11 was 20 g/L. Previous study also showed 20 g/L as the optimum glycerol concentration for *K. pneumoniae* strain (Zhang et al., 2007). At 40 g/L glycerol the cell mass (OD_{600}), succinic acid and acetic acid were maximum with values of 1.6, 0.36 g/L and 1.1 g/L, respectively. Cell growth was not inhibited at higher concentrations of glycerol and glycerol concentration upto 60 g/L did not prove toxic to cells. Lactic acid production was noted at only 10 g/L glycerol. At 20 g/L initial glycerol, the metabolism of strain BA was mainly directed towards production of 1,3 PDO as evident from the minimal production of other products such as lactic acid and succinic acid.

The effect of different pH on the glycerol fermentation by *K. pneumoniae* BA11 was studied and the results are shown in Fig. 3. There was nearly 100% utilization of glycerol in all the pH values from 6 to 8.5 with about 4.4 g/L of 1,3 PDO production. However, the pH variation led to shifts in the metabolism. At pH 8.5, succinic acid and acetic acid peaked to 0.6 g/L and 1.9 g/L, respectively, whereas lactic acid and butyric acid peaked at pH 6 to 1.4 g/L and 0.23 g/L, respectively. The cell mass remained in a nearly constant level of OD_{600} 3 in the pH range 6 to 7.5, but dropped slightly to 2.7 in the pH of 8 and 8.5. Thus, the results above indicate that the strain BA11 could tolerate the tested pH range (6 to 8.5) as no inhibitory effects were seen on growth as well as 1,3 PDO production.

The result of temperature effect on growth and 1,3 PDO production and yield is shown in Fig. 4. The strain BA11 was found to be metabolically active with high 1,3 PDO production up to a temperature of 40 ºC. At 40 ºC, succinic acid and lactic acid showed peak production whereas acetic acid and butyric acid was highest at 25 ºC. There was a remarkable change in the production of 1,3 PDO when the incubation temperature was varied in the range of 25 to 40 ºC. However, a drastic drop in cell mass and metabolism was noted at incubation temperature of 45 ºC. This shows that the strain BA11 remain active and could produce high 1,3 PDO in the incubation temperature range of 25 to 40 ºC and is therefore considered a mesophilic culture.

Effect of different mode of cultivation on the production of 1,3 PDO by Klebsiella pneumoniae BA11

The results of the effect of strict anaerobic, partial anerobic and aerobic fermentative pathways on the growth and metabolism of strain BA11 is shown in Fig. 5. Remarkable differences in the utilization of glycerol was noted when the growth condition was changed from strict anaerobic to aerobic condition. At initial glycerol concentration of 20 g/L the highest glycerol utilization (96%) was found under aerobic condition followed by partial anerobic (80%) and strict anaerobic (75%). The final pH of the cultivation medium dropped to pH 5.0 under aerobic condition, whereas remained at pH 5.3 under partial anaerobic and anaerobic conditions. It was also noted that under strict anaerobic condition acetic acid (0.9 g/L) was major metabolite next to 1,3 PDO, whereas succinic acid (0.38 g/L) and lactic acid (3.6 g/L) were major ones in partial anaerobic and aerobic conditions. In fact under aerobic condition there was a steep increase in the lactic acid production to 3.6 g/L over time.

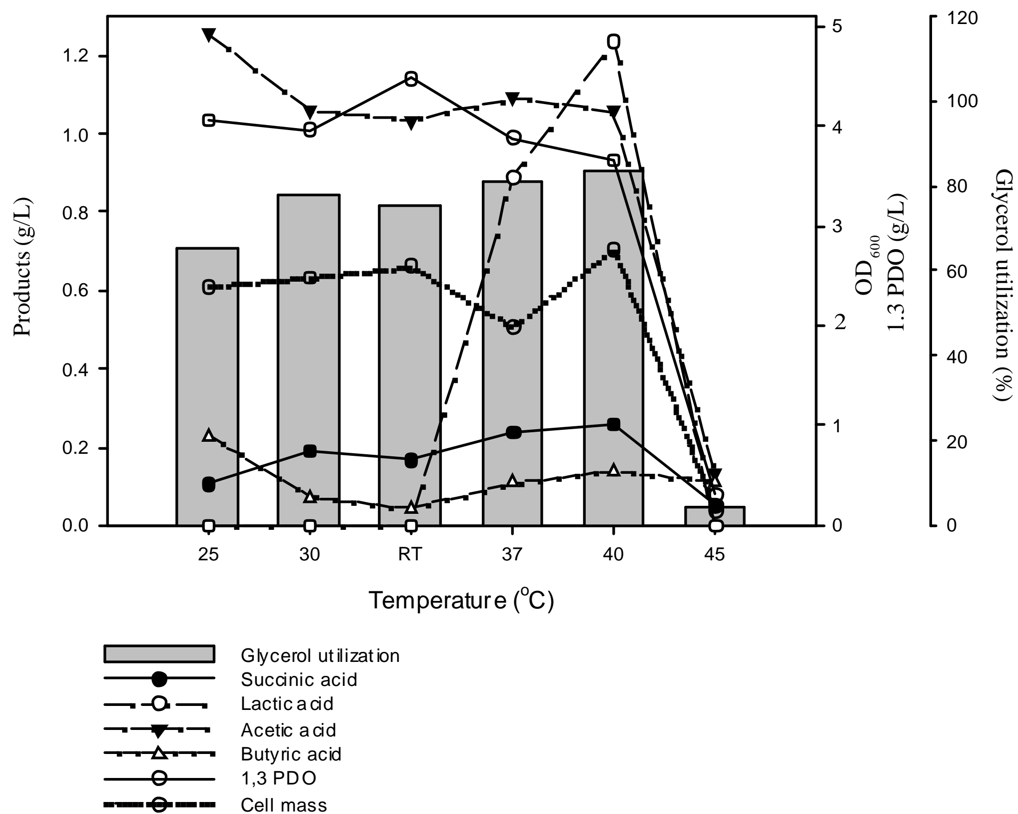

Fig. 4: Growth and metabolic profile of *Klebsiella pneumoniae* BA11 under different incubation temperatures. 'RT' indicates room temperature

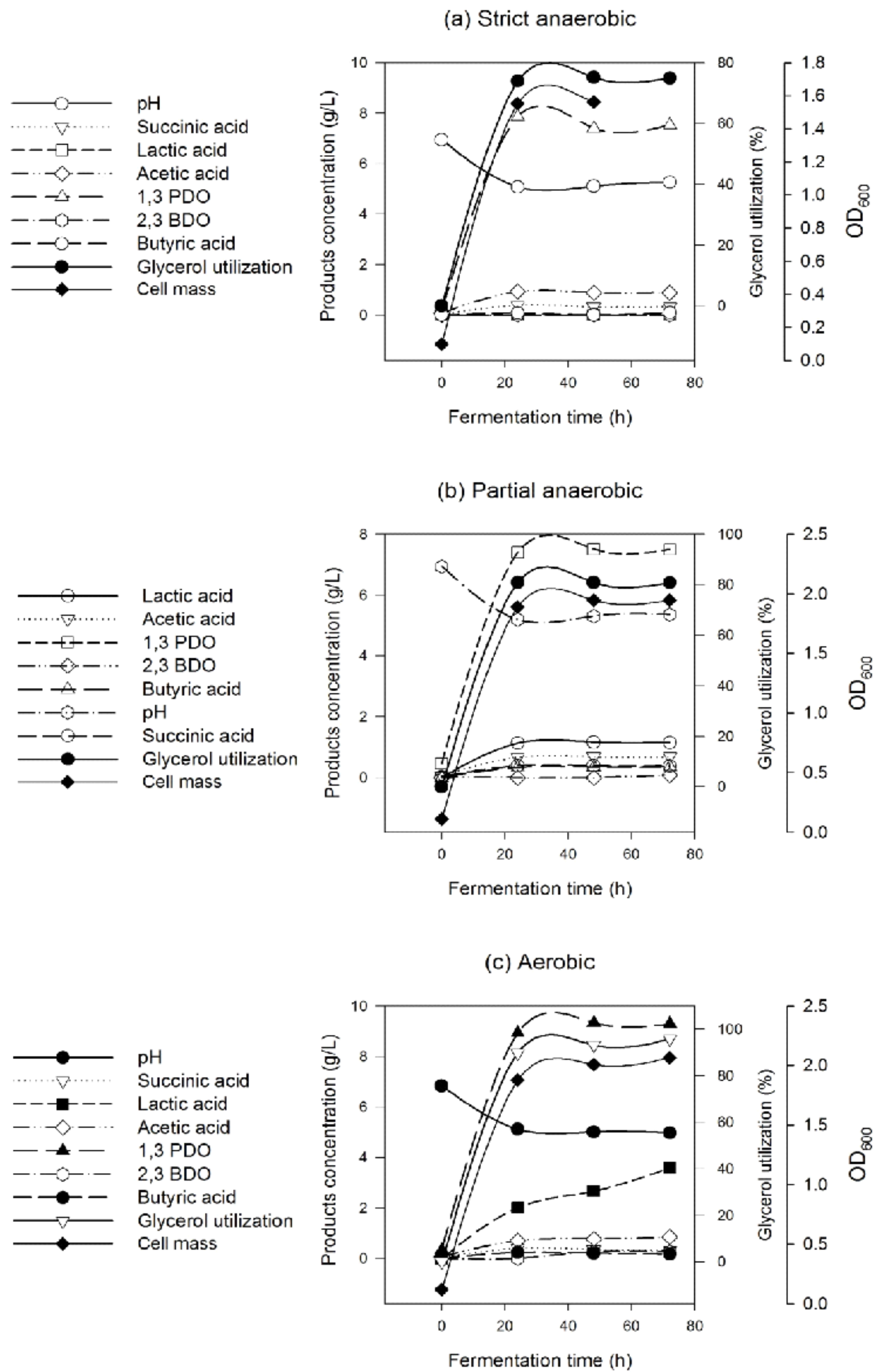

Fig. 5: Effect of different mode of cultivation on the growth and metabolic profile of *Klebsiella pneumoniae* BA11

Effect of addition of glucose as co-substrate on the growth and 1,3 PDO production by Klebsiella pneumoniae BA11

In the present study glucose was added to increasing glycerol concentration at a 1:10 g/g ratio. The results as shown in Fig. 6 indicate that glucose could increase the cell mass but did not have any positive effect on the 1,3 PDO production. Moreover, addition of glucose alone did not show any production of 1,3 PDO. This suggests that the strain BA11 was not capable of converting glucose to 1,3 PDO. At 2% glycerol, glucose lowered both the 1,3 PDO production and glycerol utilization. However, at 4% glycerol, glucose addition showed increase in glycerol utilization, which indicated that the amount of $NADH_2$ generated from 0.4% glucose was adequate to support the reduction of glycerol to 1,3 PDO via 3-hydroxy propionaldehyde. At higher glycerol concentration of 6%, glucose addition did not prove beneficial. However, at 1:10 ratio of glucose to glycerol, there was no improvement in the 1,3 PDO production except the increase in cell mass.

Glycerol is abundant in nature, since it is the structural component of many lipids. Due to its ample occurrence in nature, many known microorganisms can naturally utilize glycerol as a sole carbon and energy source (Temudo *et al.,* 2008). These microorganisms have attracted attention to the potential use in bioconversion of abundant glycerol produced from biodiesel. Anaerobic fermentation of glycerol produces several important chemicals and bio-based materials (da Silva *et al.*, 2009). The major products which could be obtained from glycerol fermentation are 1,3 propanediol, dihydroxyacetone, succinic acid, propionate, ethanol, citric acid, pigments, polyhydroxyalkanoates and biosurfactants (Dobson *et al.*, 2012). Production of value-added products from glycerol would not only reduce the production cost but also improve the economic viability of biodiesel production. The use of glycerol as feedstock in fermentation processes has yet another advantage: i.e., given the highly reduced nature of carbon atoms in glycerol, fuels and reduced chemicals can be produced from it at higher yields than those obtained from common sugars such as glucose or xylose (Clomburg and Gonzalez, 2013). To fully realize these advantages, the use of anaerobic fermentations by new bacterial isolates is highly desirable. The newly isolated and

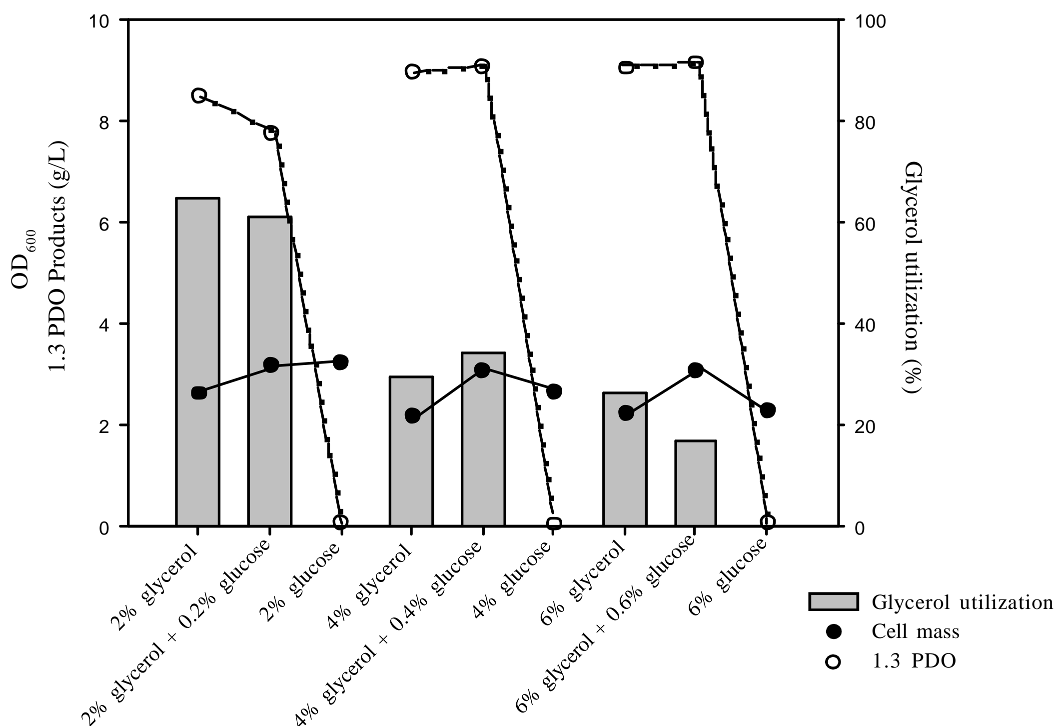

Fig. 6: Effect of addition of glucose as co-substrate on the glycerol fermentation by *Klebsiella pneumoniae* BA11

identified strain *K. pneumoniae* BA11 was studied for its ability to produce 1,3 PDO via glycerol fermentation. The strain was found to be closely related to *K. pneumoniae* strain DSM 30104. The strain BA11 is a fast 1,3 PDO producer and can give maximum 1,3 PDO in 6 h of cultivation. Earlier study on *K. pneumoniae* strain has shown 8 h for peak 1,3 PDO production (Zhang *et al.,* 2007).

The optimum glycerol concentration for strain BA11 to produce peak yield 1,3 PDO was 20 g/L without any addition of vitamin B_{12}. Previous study also showed 20 g/L as the optimum glycerol concentration for *K. pneumoniae* strain (Zhang *et al.*, 2007). Moreover, the strain is able to tolerate a wide range of pH and temperature. *Klebsiella* spp. are facultative anaerobes and therefore show different behaviour and metabolic activity when there is a shift in the mode of fermentation from strict anaerobic to aerobic conditions (Chen *et al.*, 2003). It is therefore important to study the effect of the change in cultivation mode on the production of 1,3 PDO to obtain the most optimum growth condition. Interestingly, this new strain of *K. pneumoniae* showed high 1,3 PDO yield and could utilize glycerol completely when grown under aerobic shaking conditions. Previous study have shown that microaerobic cultivations are favorable for cell growth, reduction of culture time and ethanol formation, and enhancement of volumetric productivity of 1,3 PDO (Xiu *et al.,* 2007). In our study the production of 1,3 PDO was maximum (9.3 g/L) under aerobic condition probably as a result of high glycerol utilization and high cell mass. Therefore, our results indicate that aerobic mode of cultivation could lead to maximum 1,3 PDO production. But, in terms of 1,3 PDO yield, strict anaerobic mode show highest value of 0.6 mol/mol glycerol utilized. On the other hand aerobic condition could give 1,3 PDO yield of 0.5 mol/mol glycerol utilized. A high lactic acid concentration under aerobic condition in the present study might be a result of increased flow of metabolic flux of glycerol towards glycerol dehydrogenase rather than PDOR. A previous study has shown that deletion of 1,3 propanediol oxidoreductase (PDOR) leads to increased lactate secretion (Xu *et al.,* 2011). Another possibility could be differential affinity of the PDOR and lactate dehydrogenase towards oxygen. However, this requires further studies to establish the affinities of the two enzymes towards oxygen.

Previous studies on glucose-glycerol co-fermentation have shown positive effect on the 1,3 PDO production and increased flow of glycerol to 1,3 PDO formation (Abbad-Andaloussi *et al.*, 1998; Baeza-Jimenez *et al.*, 2011; Biebl and Marten, 1995). Glucose addition leads to increased generation of $NADH_2$ which is then consumed by glycerol to produce 1,3 PDO (Biebl and Marten, 1995). Most studies were done using *Clostridium* sp., however, it is not extensively validated in other genera like *Klebsiella*. In the present study *K. pneumoniae* strain BA11 was unable to produce 1,3 PDO from glucose alone and addition of glucose to glycerol fermentation could only enhance the cell mass concentration. Therefore, addition of glucose to glycerol fermentation needs to be suitable measured to show any positive effect. Further studies on the metabolic regulation could provide detailed information on this wild strain. This strain could be an excellent candidate for genetic engineering and subsequent application in large scale 1,3 PDO production owing to its unique metabolic properties.

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