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ORIGINAL PAPER

Fermentation and evaluation of *Klebsiella pneumoniae* and *K. oxytoca* on the production of 2,3-butanediol

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Abstract *Klebsiella* is one of the genera that has shown unbeatable production performance of 2,3-butanediol (2,3-BD), when compared to other microorganisms. In this study, two *Klebsiella* strains, *K. pneumoniae* (DSM 2026) and *K. oxytoca* (ATCC 43863), were selected and evaluated for 2,3-BD production by batch and fed-batch fermentations using glucose as a carbon source. Those strains' morphologies, particularly their capsular structures, were analyzed by scanning electron microscopy (SEM). The maximum titers of 2,3-BD by *K. pneumoniae* and *K. oxytoca* during 10 h batch fermentation were 17.6 and 10.9 g L^{-1}, respectively; in fed-batch cultivation, the strains showed the maximum titers of 50.9 and 34.1 g L^{-1}, respectively. Although *K. pneumoniae* showed higher productivity, SEM showed that it secreted large amounts of capsular polysaccharide, increasing pathogenicity and hindering the separation of cells from the fermentation broth during downstream processing.

Keywords 2,3-Butanediol · *K. pneumonia* · *K. oxytoca* · Fermentation · Capsular polysaccharide

Introduction

2,3-Butanediol (2,3-BD), also known as 2,3-butylene glycol or dimethylene glycol, is a four-carbon diol synthesized as a product of mixed-acid fermentation. There is growing interest in the production of this industrially important chemical from renewable resources by microbial fermentation because 2,3-BD can be converted to a high-heating value (27,198 J g^{-1}) liquid fuel additive, methyl ethyl ketone (MEK), and 1,3-butadiene, important in synthetic rubber production [1–4] the prices per kg of which have increased from US $ 1.67 to 2.32 and from US $ 2.20 to 3.99 per kg, respectively, during the first-half of 2011 mainly due to increasing prices of fossil raw materials.

Bacteria and yeasts have been studied for over 100 years with the aim of producing 2,3-BD by microbial fermentation [5–7]. Although many microorganisms inherently produce 2,3-BD via mixed-acid fermentation, *K. pneumoniae*, *K. oxytoca*, *Enterobacter aerogenes*, and *Bacillus polymyxa* can produce more 2,3-BD than other strains [8, 9]. Among them, *Klebsiella* species, *K. pneumonia* and *K. oxytoca*, have shown the best production of 2,3-BD by fermentation from a wide range of substrates, including pentoses (xylose and arabinose), hexoses (glucose, mannose, and galactose), and disaccharides (sucrose, lactose, cellobiose) [10–18]. Some *Klebsiella* species can also produce 2,3-BD from glycerol, one of the cheapest carbon sources in the recent days due to its surplus availability as a biodiesel byproduct [19, 20].

The production of 2,3-BD by various strains has been reported at high titer. The production of 2,3-BD using wild type and mutant strains of *K. pneumoniae* and *K. oxytoca* has been studied in various fermentation systems [21–23]. However, the merits and disadvantages of these two strains' fermentation capabilities and usabilities have not been reported in detail.

This work reports a series of batch fermentations by wild type strains of *K. pneumoniae* (DSM 2026) and *K. oxytoca* (ATCC 43863) with initial glucose concentrations

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of 100–750 mmol L^{-1}. Time course profiles of 2,3-BD and byproducts, including formic acid, acetic acid, ethanol, lactic acid, succinic acid, and acetoin, were recorded to investigate the selectivity of the strains. Fed-batch fermentations by both strains were conducted over an optimal range of glucose concentrations to assess in detail their fermentative characteristics. The morphology of the strains were analyzed by SEM and their industrial potential for 2,3-BD production was assessed.

Materials and methods

Microorganisms and media

Klebsiella pneumonia DSM 2026 and *K. oxytoca* ATCC 43863 were obtained from the German Collection of Microorganisms and the American Type Culture Collection, respectively. For seed preparation, cells from single colonies on Luria–Bertani (LB) agar (Difco Laboratories, Detroit, MI) plates were suspended in 20 mL test tubes with 5 mL complex medium and incubated at 37 °C for 5 h. 1 mL samples of culture medium were then transferred to 500 mL Erlenmeyer flasks containing 300 mL complex medium and grown to an optical density at 600 nm (OD_{600}) of 2.0. Test tube and flask cultures were conducted in a rotary shaker at 150 rpm and 37 °C. The complex medium contained per liter: 5 g Bacto Difco yeast extract (Becton–Dickinson, Le Pont de Claix, France), 0.05 g $FeSO_4{\cdot}7H_2O$, 0.001 g $ZnSO_4{\cdot}7H_2O$, 0.001 g $MnSO_4{\cdot}H_2O$, 0.001 g $CaCl_2{\cdot}2H_2O$, 0.25 g $MgSO_4{\cdot}7H_2O$, 6.6 g $(NH_4)_2SO_4$, 8.7 g K_2HPO_4, and 6.8 g KH_2PO_4.

Fermentations

Batch fermentations were performed in a 5-l BIOFLO® & CELLIGEN® 310 bioreactor (New Brunswick. Scientific Co., Edison, NJ) containing 2.7 L complex medium plus 100, 200, 300, 400, 500, or 750 mmol L^{-1} glucose. The fermentor was continuously aerated through a 0.2-μm membrane filter at a flow rate of 3.0 L min^{-1}. To minimize evaporation, the vent gas passed through a modified condenser mounted on the headplate of the fermentor. The agitation speed and temperature were maintained at 150 rpm and 37 °C, respectively, and pH was controlled at 6.5 ± 0.1 by the automatic addition of 5 N NaOH. Fed-batch fermentations were carried out by continuously feeding the fermentor with a concentrated solution containing 700 g L^{-1} glucose and 20 g L^{-1} $MgSO_4{\cdot}7H_2O$ at a predetermined flow rate using a peristaltic pump (Cole-Parmer, Vernon Hills, IL, USA). Unless otherwise noted, the conditions of fed-batch fermentation were similar to those of the batch fermentation.

Table 1 Comparison of batch fermentation by *K. pneumoniae* and *K. oxytoca* for up to 10 h or less

Initial glucose contents (mmol L^{-1})	*K. pneumoniae* DSM 2026						*K. oxytoca* ATCC 43863				
	200[a]	300[b]	400	500	750		200	300	400	500	750
Final cell density (g L^{-1})	5.49 (0.126)	6.97 (0.140)	6.00 (0.377)	5.98 (0.381)	4.56 (0.114)		4.31 (0.126)	3.82 (0.334)	3.73 (0.471)	2.96 (0.425)	2.44 (0.143)
2,3-BD titer (g L^{-1})	9.49 (0.227)	15.24 (0.553)	17.62 (0.881)	16.04 (0.530)	14.15 (0.289)		5.23 (0.260)	10.93 (0.574)	9.67 (0.385)	10.43 (0.608)	7.05 (0.336)
2,3-BD productivity (g L^{-1} h^{-1})	1.36 (0.023)	1.69 (0.055)	1.76 (0.088)	1.60 (0.053)	1.41 (0.029)		0.52 (0.026)	1.80 (0.057)	1.54 (0.039)	1.60 (0.061)	1.04 (0.034)
Glucose uptake rate (g L^{-1} h^{-1})	5.80 (0.114)	7.03 (0.187)	6.50 (0.325)	6.00 (0.145)	6.10 (0.088)		3.60 (0.079)	4.60 (0.230)	5.00 (0.255)	4.80 (0.623)	3.50 (0.509)
2,3-BD yield (g g^{-1})[c]	0.23 (0.003)	0.24 (0.011)	0.27 (0.006)	0.27 (0.007)	0.23 (0.002)		0.15 (0.002)	0.24 (0.006)	0.19 (0.011)	0.22 (0.006)	0.20 (0.014)
2,3-BD selectivity (g g^{-1})[d]	0.41 (0.007)	0.44 (0.033)	0.47 (0.017)	0.43 (0.008)	0.46 (0.007)		0.21 (0.003)	0.37 (0.002)	0.30 (0.002)	0.33 (0.007)	0.28 (0.003)
Fermentation end time (h)	7	9	12	15	40		10	13	15	21	52
2,3-BD titer (g L^{-1})	9.49 (0.227)	15.2 (0.553)	20.0 (1.057)	24.9 (0.879)	34.5 (0.581)		5.23 (0.260)	13.7 (0.764)	15.0 (0.816)	18.1 (0.432)	24.9 (0.873)

Numbers in parentheses indicate 95% confidence intervals

Fermentation completed by [a] 7 and [b] 9 h

[c] g 2,3-BD produced g^{-1} glucose consumed

[d] g 2,3-BD produced g^{-1} total products, including 2,3-BD, formic acid, acetic acid, ethanol, lactic acid, acetoin, succinic acid formed

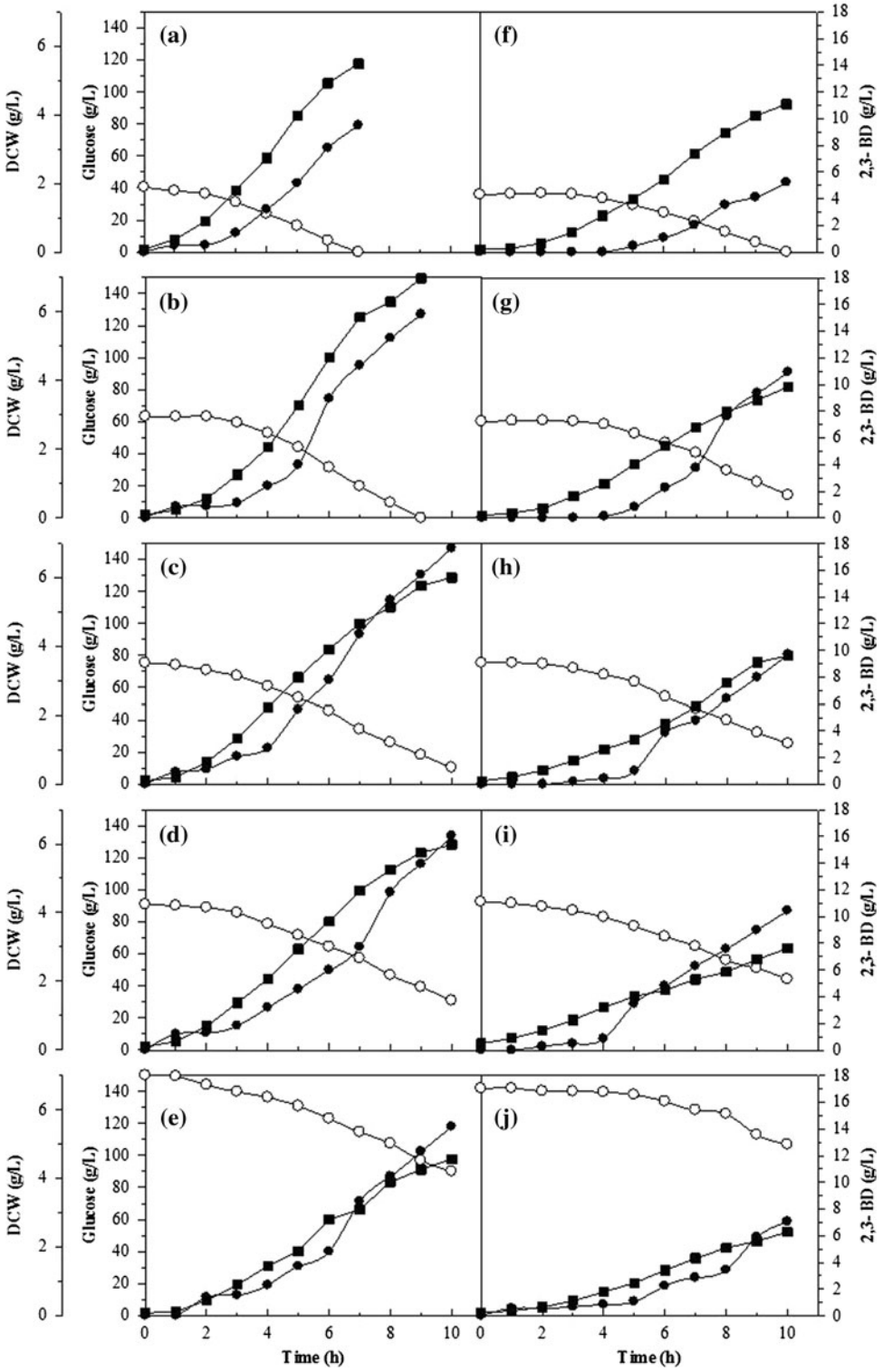

Fig. 1 Batch fermentation profiles of *K. pneumoniae* (**a**–**e**) and *K. oxytoca* (**f**–**j**) in media with different initial glucose concentrations. **a**, **f** 200 mmol L^{-1}, **b**, **g** 300 mmol L^{-1}, **c**, **h** 400 mmol L^{-1}, **d**, **i** 500 mmol L^{-1}, **e**, **j** 750 mmol L^{-1}. *Open circle* glucose, *closed square* cell growth, and *closed circle* 2,3-BD

Analytical procedures

The concentrations of glucose and metabolites, including 2,3-BD, formic acid, ethanol, acetic acid, lactic acid, succinic acid, and acetoin, were analyzed using a high-performance liquid chromatograph equipped with a refractive index detector (Agilent 1200 series, Agilent Technologies, Waldbronn, Germany). An Aminiex HPX-87H column (300 mm × 7.8 mm, Bio-Rad, Hercules, CA, USA) was isocratically eluted with 0.01 N H_2SO_4 at a flow rate of 0.6 mL min^{-1}. The column oven temperature was 80 °C. Cell growth was monitored by measuring OD_{600} by UV–vis

spectrophotometry (DR5000, Hach Company, CO). Cell concentration, dry cell weight (DCW), was determined from the standard curve relating OD_{600} to DCW per liter of culture broth (g L^{-1}). DCW was measured by filtering the culture broth through a 0.45-μm membrane and washing it twice with equal volumes of deionized distilled water. The filtered cells were dried in an oven at 80 ± 5 °C overnight, cooled to room temperature in a desiccator and weighed.

For SEM, cells were fixed in a mixture of 2.5% glutaraldehyde and 2.0% paraformaldehyde solution buffered in 0.1 M sodium cacodylate (pH 7.2) at 4 °C for 2 h. They were then immobilized on a glass slide coated with poly-L-lysine. Secondary fixation was conducted in a mixture of 1.5% potassium ferricyanide and 1.0% osmium tetroxide. Prior to dehydration with hexamethyl disilizane, samples were pre-dehydrated using ethanol at 50, 60, 70, 80, 90, and 95% in series. All chemicals used in SEM preparation were of reagent grade and from Sigma-Aldrich. The dehydrated samples were sputter coated with gold at 30 mA plasma current under argon gas for 150 s. Microscopic examinations were carried out using a SEM (Quanta™ 250FEG, FEI Company, Hillsboro, OR) at an acceleration voltage of 10 kV.

Results and discussion

Batch fermentation

The efficiency of 2,3-BD production by *K. pneumoniae* and *K. oxytoca* was assessed by cultivating the strains under bioreactor conditions in a complex medium containing various initial glucose concentrations (100–750, 100 mmol L^{-1} glucose results not shown). Fermentation was continued until the glucose was completely exhausted (Table 1). For clarity, the time course profiles (Fig. 1) represent only a maximum of 10 h. The final 2,3-BD titer by *K. pneumoniae* (Fig. 1a–e) in all conditions was between 50 and 100% higher than the corresponding *K. oxytoca* equivalent (Fig. 1f–j). Since *K. oxytoca* showed a lower rate of glucose consumption, its fermentations took longer than those by *K. pneumoniae* (Table 1). 2,3-BD yield was highest with initial glucose concentrations of 300–400 mmol L^{-1} (Table 1). Glucose added at 500 mmol L^{-1} and above decreased the rates of cell growth, 2,3-BD production, and glucose consumption through substrate inhibition.

K. pneumoniae produced more 2,3-BD than *K. oxytoca*, mainly due to the nature of the mixed-acid fermentation. The anaerobic conversion of glucose to 2,3-BD occurs by the following equation: glucose → 2,3-BD + $NADH_2$ + 2ATP + $2CO_2$ [24]. Production of 2,3-BD is likely to continue only if NAD^+ is regenerated. During fermentation, the excess $NADH_2$ is oxidized by producing reduced metabolites such as lactic acid and ethanol, with aeration reducing the demand for the production of excess metabolites [22]. The two strains showed differing metabolites profile (Figs. 1, 2). Lactic acid was the major byproduct from *K. oxytoca*, it was produced between two and four times more than by *K. pneumoniae*. Twice as much formic acid, the other acidic metabolite, was produced by *K. pneumoniae* than by *K. oxytoca*. The formic acid flux of *K. pneumoniae* indicates that most acetyl-CoA formed through pyruvate formate lyase (PFL), with *K. oxytoca* having used both PFL and pyruvate dehydrogenase complex (PDHc). *K. pneumoniae* produced twice as much ethanol, a reduced neutral metabolite, as *K. oxytoca*. This favoring of ethanol production over lactic acid contributed to the consumption of 2 mol of NADH, which was favorable to the redox balance for enhanced 2,3-BD production. Acetoin in the *K. oxytoca* culture was much higher than in the *K. pneumoniae* culture, which also suggests the large accumulation of NADH (Fig. 2). Acidic metabolites, potential inhibitors of cell function and 2,3-BD formation [25],

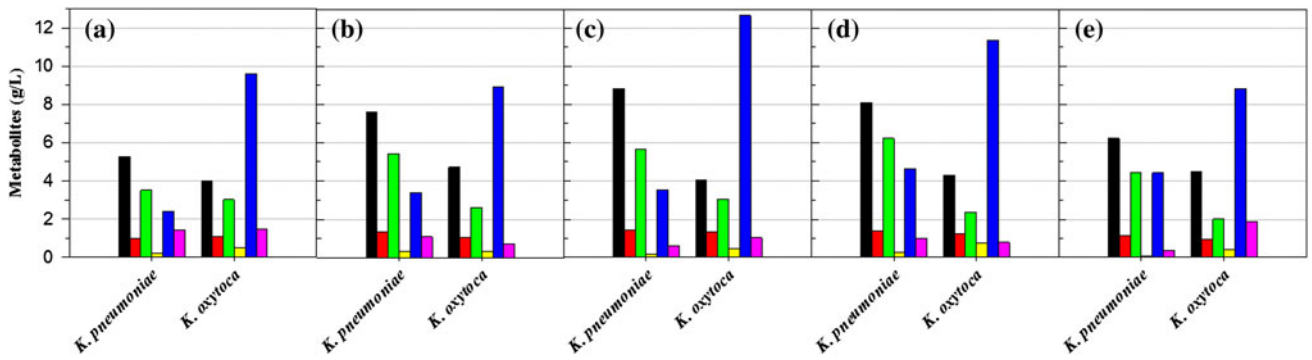

Fig. 2 Metabolite distributions of *K. pneumoniae* and *K. oxytoca* in media with different initial glucose concentrations. **a** 200 mmol L^{-1}, **b** 300 mmol L^{-1}, **c** 400 mmol L^{-1}, **d** 500 mmol L^{-1}, **e** 750 mmol L^{-1}. *black* ethanol, *red* succinic acid, *green* formic acid, *yellow* acetoin, *blue* lactic acid, and *pink* acetic acid

were produced much more in the fermentation broth of *K. oxytoca*.

Fed-batch fermentation

Fed-batch fermentation by *K. pneumoniae* for 100 h produced a maximum titer of 51 g L^{-1} 2,3-BD, about 30% higher than that from comparable fermentation by *K. oxytoca* (Table 2). In the initial 25 h of fermentation, glucose uptake and 2,3-BD production rates were comparable to those of batch fermentation. NAD^+ regeneration by *K. pneumoniae* appeared to be through ethanol production pathway during the early period of fermentation, while *K. oxytoca* used lactic acid production pathway to regenerate NAD^+. The selectivity of 2,3-BD production over other metabolites and the yield over glucose during initial *K. oxytoca* fermentation were much lower than during *K. pneumoniae* fermentation. Later during fermentation, the increased accumulation of metabolites in the fermentation broth would have limited glucose uptake, decreasing the rate of 2,3-BD production.

Table 2 Comparison of fed-batch fermentation by *K. pneumoniae* and *K. oxytoca*

Parameters	Fermentation time			
	K. pneumonia DSM 2026		*K. oxytoca* ATCC 43863	
	Phase I (0–50 h)	Phase II (51 h–end)	Phase I (0–50 h)	Phase II (51 h–end)
Biomass (g L^{-1})	4.97 (1.241)	−0.71 (0.130)	3.96 (1.372)	−0.14 (0.079)
2,3-BD titer (g L^{-1})	37.5 (3.250)	13.4 (1.273)	21.2 (2.204)	12.9 (1.189)
2,3-BD productivity (g L^{-1} h^{-1})	0.76 (0.092)	0.28 (0.035)	0.43 (0.022)	0.25 (0.056)
2,3-BD yield (g g^{-1})	0.22 (0.015)	0.28 (0.017)	0.13 (0.011)	0.32 (0.030)
Glucose consumed (g L^{-1})	170 (11.30)	0.48 (0.109)	160 (13.30)	39.2 (6.320)
Glucose uptake rate (g L^{-1} h^{-1})	3.54 (0.561)	1.02 (0.097)	3.28 (0.730)	0.77 (0.153)
Selectivity (g g^{-1})	0.39 (0.027)	0.42 (0.031)	0.25 (0.030)	0.40 (0.028)

Numbers in parentheses indicate 95% confidence intervals

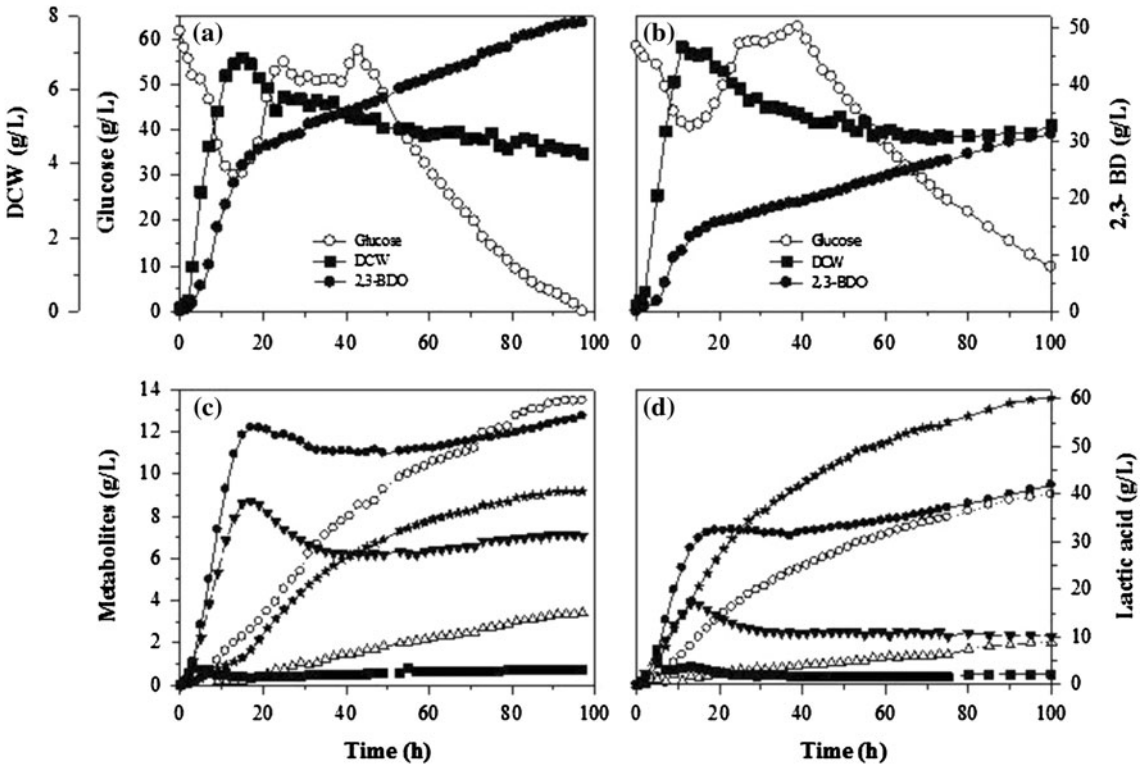

Fig. 3 Fed-batch fermentation profiles of *K. pneumoniae* (**a**) and *K. oxytoca* (**b**) during the production of 2,3-BD. Metabolites of *K. pneumoniae* (**c**) and *K. oxytoca* (**d**). *Closed star* lactic acid, *closed circle* ethanol, *open circle* succinic acid, *closed triangle down* formic acid, *open triangle up* acetoin, and *closed square* acetic acid

Each strain's metabolite profile shows similar trends to their batch results. Both strains showed similar total molar concentrations of acidic metabolites, including formic, acetic, lactic, and succinic acids. However, *K. oxytoca* required twice as much base solution (5 N NaOH) to maintain pH 6.5 during the whole period of the fermentation. Similar to batch fermentation, acidification in the *K. oxytoca* culture broth was much higher than in the *K. pneumoniae* culture broth, mainly due to the higher production of 2,3-BD and ethanol by *K. pneumoniae*. The high pK_a values of 2,3-BD (14.7 and 15.0) and ethanol (16.0) may have reduced the amount of the base solution needed for neutralization [26]. Furthermore, the pK_a of lactic acid (3.86), the main byproduct by *K. oxytoca* fermentation, was much lower than those of the other acidic byproducts (acetic acid 4.75, succinic acid 4.16 and 5.61) except formic acid (formic acid 3.75). Less alkalinization and more acidification of *K. oxytoca* fermentation were the reason for requiring more NaOH, and the resulting high salt concentrations in fermentation broths normally inhibit cell growth and significantly adversely affect downstream processing and also wastewater treatment [27]. In particular, sodium from salt can degrade soil.

Microscopic analysis

Plate and broth cultures of both strains were examined. The high viscosity of *K. pneumoniae* in the broth and its stickiness on the agar plate indicated the presence of mucoid-like substances. When the culture broth was centrifuged at high speed (15,000×*g*), cell pellet was not formed (images not shown) at the bottom of the tube. Such behavior was not shown by *K. oxytoca*.

Cells' morphological differences were observed by SEM. 50,000× and 100,000× magnification images clearly show the presence of CPS in *K. pneumoniae* but not in

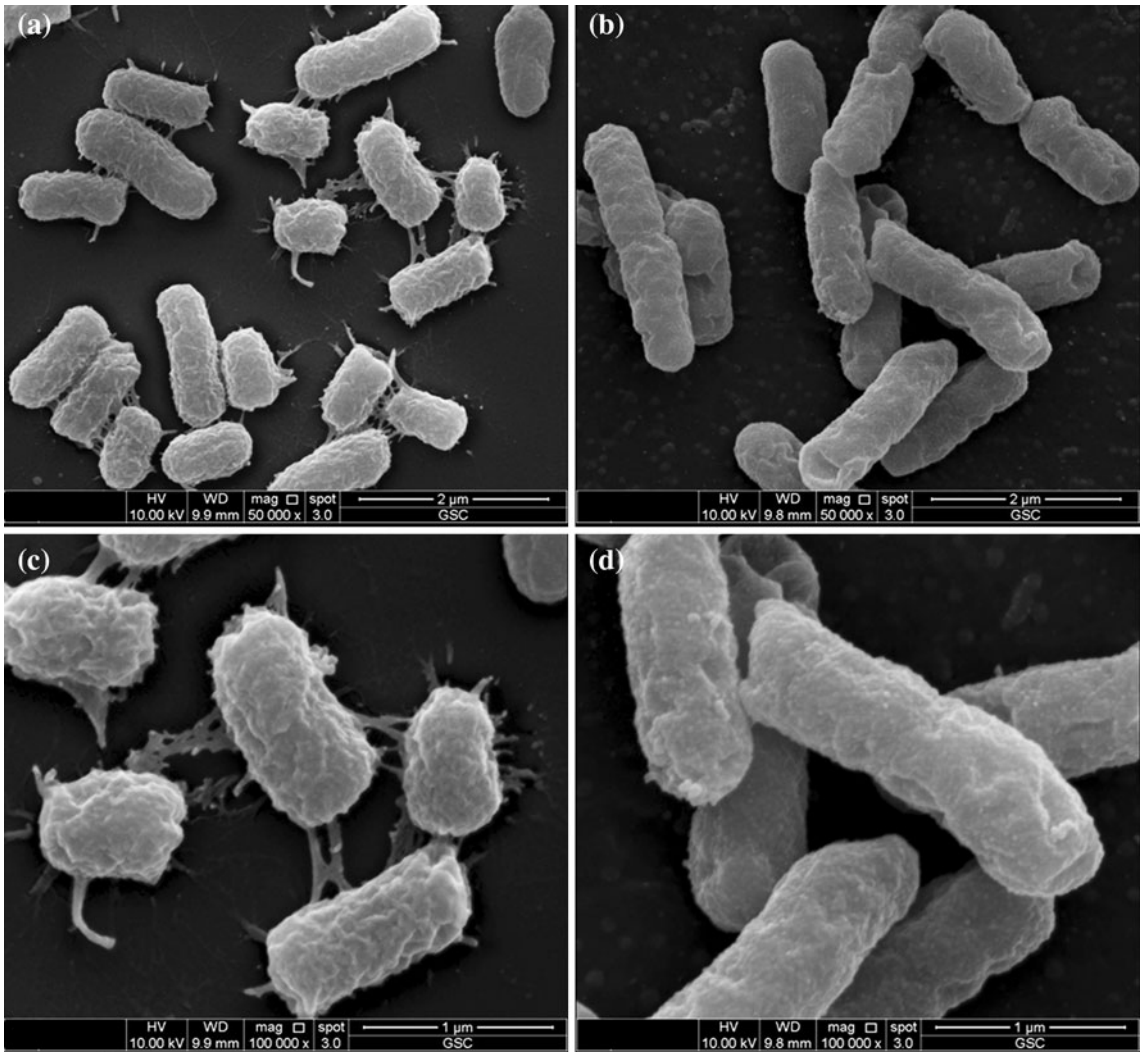

Fig. 4 Scanning electron microscopic images of *K. pneumoniae* (**a**, **c**) and *K. oxytoca* (**b**, **d**) at different magnification: **a**, **b** ×50,000 and **c**, **d** ×100,000

K. oxytoca (Fig. 3). A dense layer of CPS in *K. pneumoniae* has been reported to be pathogenic when tested in several animal models, causing pneumonia [28]. Capsulated *K. pneumoniae* cells were difficult to separate from the fermentation broth, which would hamper downstream processing. Such properties do not favor using *K. pneumoniae* in commercial 2,3-BD fermentation at large scale. However, given its superior 2,3-BD production, engineering *K. pneumoniae* for reducing CPS production is beneficial. Lin et al. [29] mutated certain genes responsible for CPS production to significantly reduce its production. Similarly, certain open reading frames responsible for CPS production in *K. pneumoniae* have been inactivated, allowing the viscosity of the fermentation broth to be reduced by up to 27% [30]. Therefore, *K. pneumoniae* with mutated genes responsible for CPS production remains potentially applicable industrially (Fig. 4).

Conclusions

2,3-BD production by two *Klebsiella* strains and their cultural behaviors during fermentation were studied. *K. pneumoniae* showed a titer of 2,3-BD production 1.5-fold that of *K. oxytoca*. However, it also produced CPS that would hinder its industrial use. Therefore, disrupting the genes responsible for CPS synthesis to obtain non-pathogenic, non-CPS forming strain that would not obstruct downstream purification appears potentially beneficial. Engineering the metabolic pathways of *K. oxytoca* to reduce production of acidic metabolites could also lead to a strain suitable for 2,3-BD production.

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