Klebsiella pneumoniae – a useful pathogenic strain for biotechnological purposes: 1,3-propanediol biosynthesis

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Abstract

Background Despite being a well-known human pathogen, *Klebsiella pneumoniae* plays a significant role in the biotechnology field, being considered as a microbial cell factory in terms of valuable chemicals biosynthesis. In fermentation processes, *K. pneumoniae* is mostly used to biosynthesize 1,3-propanediol (PDO), a very important intermediate compound utilized in the biodegradable bioplastics industry. This strain owns the ability to metabolize glycerol and to produce PDO under both aerobic and anaerobic circumstances, and under low oscillations of pH. **Results** In this work, we investigated the *K. pneumoniae* strain DSMZ 2026 in batch bioreactor cultivation. The bacterial strain was inoculated in 2 L culture broth containing 43.5 g·L⁻¹ main substrate and the pH was adjusted at 7 at the beginning of the process. After 12 h of batch cultivation, the pH values decreased to 4.77, and we obtained 51 g·L⁻¹ wet biomass, 17 g·L⁻¹ PDO, while the starting substrate reached 15 g·L⁻¹. The morphology of *K. pneumoniae* cells was analyzed on both solid and liquid media, by being identified large mucoid colonies on Columbia agar, and individual and grouped cells were observed by methyl blue staining. **Conclusion** From this study it can be concluded that *K. pneumoniae* can grow successfully in mineral broth under anaerobic environment and low decrease of pH, and can biosynthesize valuable chemicals like PDO.

Background

Among Enterobacteriaceae, *Klebsiella spp.* are recognized mostly in the medical field as famous opportunistic germs associated with pathogenic infections [1-3]. *Klebsiella pneumoniae* represents a saprophytic pathogen that might affect both plants [4], animals [4, 5] and humans [6-8], but which can be successfully used for biotechnological applications [9, 10]. In medical terms, *K. pneumoniae* is a pathogenic strain responsible for multiple nosocomial infections including pneumonia, urinary tract and soft tissue infections, and septicemias [6, 7, 11]. *K. pneumoniae* cells are able to spread very quickly especially in hospital environment, the main cause being the unclean hands of personnel [11]. The pathogenic potential of the Gram-negative *K. pneumoniae* is mainly due to the external cell structure [4, 9]. The main virulence factor is the outer cell membrane that consist of capsule, lipopolysaccharides (recognized as endotoxins in humans), siderophores, and pili [1, 11]. The capsular polysaccharides are attached to the peripheral membrane, and play a major role in capsular antigen synthesis and export [12].

In the biotechnology field, pathogenic bacteria like *Klebsiella*, *Clostridium*, *E. coli* or *Bacillus* have been studied for more than 100 years to produce important chemical compounds such as propanediols (1,2-propanediol, 1,3-propanediol), organic acids (lactic, acetic, citric, succinic, pyruvic) or alcohols (ethanol, 1-butanol) [13-15]. *K. pneumoniae* is a representative strain used at large scale for the biosynthesis of PDO by means of fermentation processes at neutral

pH values, under both aerobic and anaerobic conditions, starting from glycerol as the main nutrient substrate [16-18]. *K. pneumoniae* is mostly used for PDO synthesis at large scale because of its natural ability to produce the B12 co-enzyme, a very important factor for microbial PDO synthesis [19]. The biotechnological production of PDO is extensively studied by multiple research groups because of the

major development of market demands worldwide for PDO [20-22]. PDO plays an important role in the synthesis of biodegradable plastics, namely for the poly(trimethylene-terephthalate) (PTT) production [18, 23].

Considering the microbial potential of the pathogenic strains like *K. pneumoniae* in biotechnology field, in this study was used *K. pneumoniae* DSMZ 2026 (risk group: level 2) [24, 25] for PDO production in bioreactor batch fermentation, in anaerobic conditions and different values of pH. The biomass evolution, pH oscillation, PDO biosynthesis and substrate consumption were monitored during 12 h of fermentation. The bacterial morphology on both solid and liquid media was analyzed.

Results And Discussions

The biomass evolution, pH decrease, substrate consumption and PDO biosynthesis were monitored during 12 h of batch fermentation, and the results are presented in Table 1:

Table 1 Results obtained after 12 h of fermentation by using the pathogenic *K. pneumoniae* DSMZ 2026.

Time (h)	pH	Wet biomass g·L ⁻¹	Substrate (glycerol) g·L ⁻¹	PDO g·L ⁻¹
0	6.91	12	44	0.00
2	5.91	19	40	10.44
4	5.48	30	34	13.81
6	5.10	35	30	14.16
8	5.01	37	27	14.62
10	4.86	42	17	15.30
12	4.77	51	15	16.55

After 12 h of batch cultivation the pH decreased from 7 to 5, while the wet biomass increased from 12 g·L⁻¹ to 51 g·L⁻¹ (Figure 1). More than a half of the initial concentration of the substrate was consumed during 12 h of cultivation, reaching 15 g·L⁻¹. The final concentration of PDO obtained after 12 h was 16 g·L⁻¹ (Figure 2).

Figure 1: Wet biomass and pH variations during 12 h of cultivation using *K. pneumoniae* DSMZ 2026

Figure 2: Substrate consumption and PDO production during 12 h of fermentation using *K. pneumoniae* DSMZ 2026.

PDO is a three carbon diol with important contribution in ecological materials development, such as polymers, polyesters, composites, coatings, etc. [19]. Considering the results obtained for PDO (Table 1),

these are similar to those reported by literature. Cheng et al. [26] employed a *K. pneumoniae* strain M5a1 in batch cultivation at bioreactor level and achieved 18 g·L⁻¹ PDO after 18 h, while the pH was maintained at 6.8 through automatic addition of NaOH [26]. Da Silva et al. [27] obtained similar results on *K. pneumoniae* strain GLC29 in batch trials where pH values were maintained between 6.9 and 7.1. They achieved a final PDO concentration of 20 g·L⁻¹ after 9 h of fermentation [27]. Kumar et al. [28] tested two different mutant strains of *K. pneumoniae* DSMZ 2026 and *K. pneumoniae* J2B at shake-flask level, in anaerobic conditions, and after 12 h they achieved maximum concentrations ranging between 33 g·L⁻¹ and 54 g·L⁻¹ of PDO. For the same study, the pH values were kept at 7 [28]. Higher PDO concentrations in batch trials were obtained by Zhao et al. [29] who used microencapsulated *K. pneumoniae* type ZJU 5205. They achieved 63 g·L⁻¹ after 11 h of cultivation starting from high initial glycerol concentration of 120 g·L⁻¹ [29].

Placed on solid media and incubated at 37°C for 24 h, *Klebsiella* cells developed large (>1 mm), opaque, cream-colored and glistening mucoid colonies (Figure 3, left). Under microscope light, individual cells surrounded by a thin halo could be observed (Figure 3, right), structure which constitutes the capsule of the bacteria. According to Evrad et al. [8], the voluminous capsular layer is made of polysaccharides that covers the entire bacterial surface, and its role is to protect the bacteria cell against macrophage phagocytosis in animal and human models [8]. In biotechnological processes instead, large amounts of capsular polysaccharides induce mucoviscosity [13] and obstruct the separation of bacteria cells from fermentation media during the downstream process [9].

Figure 3: The *K. pneumoniae* DSMZ 2026 colony appearance on Columbia solid media (left), and the microscopic examination of bacterial cells after methylene blue staining (right). The red arrow indicates the cell capsule.

Conclusion

The pathogenic strain, *K. pneumoniae* DSMZ 2026, was used in batch fermentation process at bioreactor level to biosynthesize PDO. *K. pneumoniae* grew successfully in mineral broth under anaerobic environment, and low decrease of pH. Compared to literature where the pH was maintained to 7, in this work we investigated the influence of pH lowering over the PDO synthesis by *K. pneumoniae* DSMZ 2026. *K. pneumoniae* was cultivated in 2 L of fermentation broth containing 43.5 g·L⁻¹ glycerol as the main substrate, and maintained at 37°C for 12 h at 200 RPM. The pH was adjusted at 7 at the beginning of the process, but was not maintained at a specific value during the fermentation. After 12 h of batch cultivation we obtained 51 g·L⁻¹ wet biomass, 15 g·L⁻¹ unconsumed substrate, 17 g·L⁻¹ PDO, and the pH values reached almost to 5. On solid media after 24 h of incubation at 37°C, *K. pneumoniae* developed large, opaque, cream-colored and glistening mucoid colonies. Under microscope examination was observed individual cells surrounded by light halo constituting the bacterial capsular layer.

Methods

Microorganism and culture conditions

In this study, we used *K. pneumoniae* DSMZ 2026 obtained from the German Collection of Microorganisms and Cell Culture (DSMZ, Braunschweig, Germany); the cultivation conditions and fermentation broth components were similar to those used by Menzel et al. [30].

Firstly, the bacterial strain was pre-cultured in a mineral broth with the following composition: glycerol 43.5 g·L⁻¹; K₂HPO₄ 3.4 g·L⁻¹; KH₂PO₄ 1.3 g·L⁻¹; (NH₄)₂SO₄ 2.0 g·L⁻¹; MgSO₄ x 7 H₂O 0.2 g·L⁻¹; yeast extract 1.0 g·L⁻¹; CaCO₃ 2.0 g·L⁻¹; FeSO₄ x 7 H₂O 5.0 mg·L⁻¹; CaCl₂ 2.0 mg·L⁻¹; ZnCl₂ 0.14 mg·L⁻¹; MnCl₂ x 4 H₂O 0.2 mg·L⁻¹; H₃BO₃ 0.12 mg·L⁻¹; CoCl₂ x 6 H₂O 0.4 mg·L⁻¹; CuCl₂ x 2 H₂O 0.04 mg·L⁻¹; NiCl₂ x 6 H₂O 0.05 mg·L⁻¹; Na₂MoO₄ x 2 H₂O 0.07 mg·L⁻¹.

Secondly, the bacterial strain was cultured in bioreactor batch culture containing: glycerol 43.5 g·L⁻¹, KCl 0.75 g·L⁻¹, NaH₂PO₄ x H₂O 1.38 g·L⁻¹, NH₄Cl 5.35 g·L⁻¹, Na₂SO₄ 0.28 g·L⁻¹, MgCl₂ x 6 H₂O 0.26 g·L⁻¹, CaCl₂ x 2 H₂O 0.29 g·L⁻¹, citric acid x H₂O 0.42 g·L⁻¹, yeast extract 1.0 g·L⁻¹, ZnCl₂ x 6 H₂O 3.4 mg·L⁻¹, FeCl₂ x 6 H₂O 27 mg·L⁻¹, MnCl₂ x 4 H₂O 10 mg·L⁻¹, CuCl₂ x 2 H₂O 0.85 mg·L⁻¹, CoCl₂ x 6 H₂O 2.35 mg·L⁻¹, H₃BO₃ 0.5 mg·L⁻¹, Na₂MoO₄ x 2 H₂O 25 µg·L⁻¹. All components were of analytically grade, and the pH of the culture broth was adjusted to 7 before sterilizing at 121°C for 15 min.

Batch cultivation at bioreactor level

Initially, 200 mL of pre-culture broth was inoculated with a 24 h colony of 10⁸ CFU·mL⁻¹. The pre-culture was maintained at 37°C for 24 h and 200 RPM. The experiment was performed using a 5 L bioreactor (B. Braun Biotech International) filled with 2 L of culture broth, where the inoculum was added in sterile conditions. The fermenter was fitted with temperature, pH, and rotation speed control. Temperature was maintained at 37°C and rotations were maintained at 200 RPM. pH was not kept constant, in order to observe its influence on PDO production. The batch trial run for 12 h anaerobically. Samples were collected every 2 h during 12 h for specific tests.

Testing methods

The wet biomass quantity was determined by weighing 10 mL of sample centrifuged at 7000 RPM for 15 min, washed twice with double distilled water, and removing the supernatant.

The glycerol consumption was determined by using the enzymatic test purchased from Sigma Aldrich, Glycerol Assay Kit.

PDO production was measured by HPLC after a sample derivatization process [31]. The HPLC unit (Agilent 1200) was equipped with quaternary pump, solvent degasser, auto-sampler, UV-Vis photodiode detector (DAD) coupled with single quadrupole mass detector (MS, Agilent 6110), equipped with electrospray ionization source (ESI) (Agilent Technologies, CA, USA), and controlled by Agilent ChemStation software. The ESI detection was performed by following the work conditions: capillary voltage 3100 V, 350°C, nitrogen flow 7 L·min⁻¹, m/z 100-500 full-scan. The compounds separation was

done with an Eclipse XDB C18 column (5 μ m, 4.6x150 mm I.D.) (Agilent Technologies, CA, USA) using the 20 mM NH₄HCO₂ mobile phase (A), pH 2.8, and (B) ACN/A (90/10, v/v) at a flow rate of 0.3 mL·min⁻¹ at 25°C.

1 mL of fermentation sample was diluted six times in sterile saline solution (0.85% NaCl) and spread (100 μ L) on Columbia agar plates in order to observe the colonies aspect after 24 h of incubation at 37°C (32). Microscopic analysis consisting in simple staining was applied in order to observe the bacterial cell morphology and appearance. A loop of fermentation sample collected after 12 h of cultivation was stained with methylene blue [33] and examined under microscope at 400x magnification.

List Of Abbreviations

PDO – 1,3-propanediol; PTT- polytrimethylene-terephthalate; CFU – colony forming units; RPM – rotations per min; HPLC – high performance liquid chromatography; DAD - diode array detector; MS – mass spectrometer; ESI - electrospray ionization.

Declarations

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

Not applicable.

Competing interest

The authors declare that they have no competing interests.

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Authors' contributions

LM and DV conducted the experiment in the laboratory. LM, MT, AR and DV outlined and assembled the manuscript. All authors read and approved the final manuscript.

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Figures

Figure 1

Wet biomass and pH variations during 12 h of cultivation using *K. pneumoniae* DSMZ 2026.

Figure 2

Substrate consumption and PDO production during 12 h of fermentation using *K. pneumoniae* DSMZ 2026.

Figure 3

The *K. pneumoniae* DSMZ 2026 colony appearance on Columbia solid media (left), and the microscopic examination of bacterial cells after methylene blue staining (right). The red arrow indicates the cell capsule.