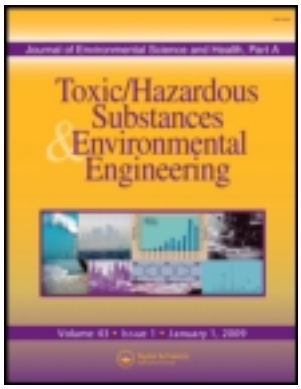

Journal of Environmental Science and Health, Part A: Toxic/Hazardous Substances and Environmental Engineering

Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/lesa20

Biodegradation of glycerol using bacterial isolates from soil under aerobic conditions

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Published online: 11 Oct 2013.

To cite this article: Kerisha Raghunandan, Siphesihle Mchunu, Ashwani Kumar, Kuttanpillai Santhosh Kumar, Algasan Govender, Kugen Permaul & Suren Singh (2014) Biodegradation of glycerol using bacterial isolates from soil under aerobic conditions, Journal of Environmental Science and Health, Part A: Toxic/Hazardous Substances and Environmental Engineering, 49:1, 85-92, DOI: 10.1080/10934529.2013.824733

To link to this article: http://dx.doi.org/10.1080/10934529.2013.824733

Journal of Environmental Science and Health, Part A (2014) **49**, 85–92

ISSN: 1093-4529 (Print); 1532-4117 (Online)
DOI: 10.1080/10934529.2013.824733

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Biodegradation of glycerol using bacterial isolates from soil under aerobic conditions

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Glycerol, a non-biodegradable by-product during biodiesel production is a major concern to the emerging biodiesel industry. Many microbes in natural environments have the ability to utilize glycerol as a sole carbon and energy source. The focus of this study was to screen for microorganisms from soil, capable of glycerol utilization and its conversion to value added products such as ethanol and 1,3-propanediol (1,3-PDO). Twelve bacterial isolates were screened for glycerol utilization ability in shake flask fermentations using M9 media supplemented with analytical grade glycerol (30 g/L) at various pH values (6, 7 and 8) and temperatures (30°C, 35°C and 40°C). Among these, six bacterial isolates (SM1, SM3, SM4, SM5, SM7 and SM8) with high glycerol degradation efficiency (>80%) were selected for further analysis. Highest level of 1,3-PDO production (15 g/L) was observed with isolate SM7 at pH 7 and 30°C, while superior ethanol production (14 g/L) was achieved by isolate SM9 at pH 8 and 35°C, at a glycerol concentration of 30 g/L. The selected strains were further evaluated for their bioconversion efficiency at elevated glycerol concentrations (50–110 g/L). Maximum 1,3-PDO production (46 g/L and 35 g/L) was achieved at a glycerol concentration of 70 g/L by isolates SM4 and SM7 respectively, with high glycerol degradation efficiency (>90). Three isolates (SM4, SM5 and SM7) also showed greater glycerol tolerance (up to 110 g/L). The isolates SM4 and SM7 were identified as *Klebsiella pneumoniae* and SM5 as *Enterobacter aerogenes* by 16S rDNA analysis. These novel isolates with greater glycerol tolerance could be used for the biodegradation of glycerol waste generated from the biodiesel industry into value-added commercial products.

Keywords: Glycerol dehydratase (GD), 16S rDNA, 1,3-propanediol, (1,3-PDO), ethanol.

Introduction

Biodiesel is an alternative fuel that is chemically produced by reacting a vegetable oil or animal fat with an alcohol (methanol or ethanol) in the presence of an alkali catalyst. Despite the rapid development and commercialization of biodiesel, there are several key challenges that must be addressed. One such challenge is the production of glycerol as a co-product of biodiesel production during transesterification and esterification of vegetable oil, which will result in the accumulation of massive quantities of glycerol into the market in the near future.[1,2] In biodiesel production processes, about 10% of crude glycerol is liberated and most of the unutilized glycerol is discharged as waste into the environment.[3] This warrants the urgent need to develop novel methods to utilize the waste glycerol as a raw material for the production of economically value-added products. Utilization of glycerol as a feedstock for other commercial applications will also enhance the economic viability and sustainability of the biodiesel industry.

Several microorganisms capable of growing anaerobically and utilize glycerol as a sole carbon and energy source have been previously reported.[4–6] Studies have also indicted the potential of these isolates to covert glycerol to value added products such as 1,3-PDO in micro-aerobic conditions.[4,7–9] This include strains such as *Klebsiella pneumoniae, Klebsiella oxytoca, Klebsiella aerogenes, Enterobacter agglomerans, Enterobacter aerogenes, Citrobacter freundii, Lactobacillus reuteri, Lactobacillus buchnerii, Lactobacillus collinoides, Pelobacter carbinolicus,* and *Rautella planticola*.[4,7,8]

Glycerol is a highly reduced carbon source and theoretically has higher product yield than glucose for the production of chemicals of a reduced nature.[3,10] Being a simple molecule, glycerol can be easily taken up into the microbial cell by facilitated diffusion and get converted to different metabolic intermediates (1,3-propanediol, succinate, dihydroxyacetone, propionic acid and pigments) and biofuels (ethanol and butanol) using various metabolic

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Received March 24, 2012.

pathways.[4] During bioconversion process, glycerol is dehydrogenated to dihydroxyacetone, and can be converted to pyruvate. In another pathway, reductive glycerol conversion involves a vitamin B12-mediated dehydration to 3-hydroxypropionaldehyde and a reduction of the aldehyde to 1,3-PDO.[7] The fate of pyruvate is different in different bacteria strains.[11]

In enterobacterial fermentation, acetic acid is formed from acetyl-CoA, yielding extra ATP as well as ethanol, although in sugar fermentation, pyruvate can also be condensed to acetolactate to give acetoin and 2,3-butanediol. Recently, production of 1,3-PDO has emerged as a commodity chemical as it can be utilized as an essential monomer to synthesize a new type of polyester polytrimethylene terephthalate (PTT), which is an important constituent in polymers, cosmetics, lubricants, medicines and foods.[5,12] PTT is mainly used for carpet and textile fiber production.[13] *Clostridium butyricum* and *Klebsiella pneumoniae*, are best known organisms for producing 1,3-PDO and both can achieve high yields and productivities.[7,14] It is estimated that 10^5 tons of 1,3-PDO are produced annually, primarily by chemical processes,[15] which requires high temperature, high pressure and expensive catalysts resulting in the discharge of toxic intermediate compounds.[16,17]

Unlike the chemical conversion of glycerol into value-added products, bioconversion could provide an efficient solution for the sustainable management of glycerol, which would be beneficial to improve the economics of biodiesel industries.[18] Recent studies have proven that the most efficient way of isolating bacteria is from waste sample itself or from environmental samples such as nutrient-rich soils capable of converting wastes.[19] Therefore, this study was aimed at isolating bacteria from soil, capable of utilizing pure glycerol as the sole carbon and energy source to produce value-added compounds such as ethanol and 1,3-propanediol.

Materials and methods

Isolation of glycerol-degrading bacteria

Soil samples were collected from the Durban Botanical Gardens, Durban, South Africa and inoculated (5% inoculum) into 250-mL Erlenmeyer flasks containing M9 media and incubated at 37°C with shaking (180 rpm) for 48 h. M9 media containing pure glycerol as a sole carbon source and comprised of (per L) commercial glycerol (30 g, 99.0% analytical grade, Sigma), NH_4Cl (1 g), KH_2PO_4 (3 g), Na_2HPO_4 (6 g), NaCl (1 mM), $MgSO_4$ (0.1 mM) and $CaCl_2$ (0.1 mM). The pH of the medium was adjusted with 1 M NaOH or 1 M HCl.[20] Batch fermentations were carried out in 250-mL Erlenmeyer flasks (100 mL of M9 media) under different pH conditions (6, 7 and 8) and at different temperatures (30°C, 35°C and 40°C) for 48 h. Following incubation, inoculum from flasks showing growth was plated onto M9 agar plates containing 1% analytical glycerol. Positive isolates were characterized by morphological features and Gram staining. Pure isolates were preserved or maintained on M9 media containing glycerol at 4°C.

Glycerol utilization and its bioconversion

Isolates were further screened for glycerol utilization and bioconversion efficiency by re-inoculating into M9 media containing glycerol. The isolates were further grown on M9 media shake flasks with increased glycerol concentrations (50–70 g/L). These flasks were then incubated at 37°C for 5 days shaking at 180 rpm. The isolates that showed high glycerol utilization were inoculated onto fresh M9 medium with elevated concentrations of glycerol (90–110 g/L) and analyzed for breakdown products.

Quantification of 1,3-propanediol and ethanol by HPLC

Glycerol, 1,3-PDO, and ethanol were measured by HPLC (Merck-Hitachi Lachrom, Dallas, TX, USA) equipped with a Refractive Index Detector. Separation was performed on a Hi-Plex H Fast Acid Column (100 × 7.7 mm, Polymer labs, Shropshire, UK) using 0.01 M sulphuric acid as mobile phase with a flow rate of 0.5 mL min^{-1} and column temperature of 55°C. Pure standards were purchased from Sigma (St. Louis, MO, USA) and samples were filtered through a 0.2 μM polyethersulfone membrane prior to analysis.[21]

16S rDNA partial sequencing and genetic analysis of selected isolates

Genomic DNA was extracted from the selected bacterial isolates using ZR Fungal/Bacterial DNA MiniPrep Kit (Zymo Research, Irvine, CA, USA). The extracted DNA was further amplified using universal bacterial primer pairs 63F (5′-CAG GCC TAA CAC ATG CAA GTC-3′) and 1387R (5′-GGG CGG WGT GTA CAA GGC-3′). The PCR mixture contained 1 μL DNA, 5 μL dNTPs (10 mM), 5 μl 63F primer, 5 μL 1387R primer (10 mM), 3 μL $MgCl_2$, 5 μL Taq Buffer (Fermentas), 1 μL Taq polymerase (Fermentas) and 25 μL MilliQ water. The PCR reactions were performed in an automated thermal cycler (PTC-200, Biorad, Hercules, CA, USA) with the following conditions: an initial denaturation at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 1 min and extension at 72°C for 2 min, and a final extension at 72°C for 10 min. The PCR products were purified using QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Selected PCR products were sequenced using an ABI Big Dye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Grand Island, NY, USA) and the sequences were read on an Applied Biosystems 3130 genetic analyzer (Applied Biosystems). Sequence analysis was performed using Sequence Scanner v1.0 software, and then compared with

sequences in the GenBank databases using BLAST search (http://www.ncbi.nlm.nih.gov/genbank/).

MEGA 3.1 [22] software (The Biodesign Institute, Tempe, AZ, USA) was used for phylogenetic analysis after multiple alignments of sequence data using CLUSTAL_X [23] Distances were calculated using distance options according to Kimura's two-parameter model [24] and clustering was performed with the neighbour-joining method.[25] Maximum-likelihood [26] and maximum-parsimony [27] trees were generated using the tree making algorithms contained in the PHYLIP package (University of Washington, Seattle, WA, USA). Bootstrap analysis was used to evaluate the tree topology by means of 1,000 re-samplings.[28] The evolutionary relationships between known glycerol utilizing bacteria were established.

Results and discussion

Isolation and screening of bacteria for glycerol utilization and its bioconversion

The isolation procedure yielded 12 bacterial isolates and selection of colonies was primarily based on tolerance and ability to degrade glycerol as the sole carbon source (30 g/L). Isolated colonies were further screened on M9 media containing elevated concentrations of glycerol at different temperatures (30°C, 35°C and 40°C) and pH (6, 7 and 8) for 48 h.

Of the bacterial isolates screened, six isolates (SM1, SM3, SM4, SM5, SM7 and SM8) exhibited high levels of glycerol degradation (>80%) at 35°C and pH 7 (Fig. 1), whereas other isolates SM2, SM6, SM9, SM10, SM11 and SM12, showed significantly lower levels of glycerol degradation (data not shown).

Apart from glycerol degradation, isolates (SM1, SM3, SM4, SM5, SM7 and SM8) also showed relatively higher amounts (>10 g/L) of 1,3-PDO production (Fig. 2). Among these, isolate SM7 recorded the highest 1,3-PDO production (15.6 g/L) after 48 h incubation, followed by isolates SM4 and SM3 (14 g/L) (Fig. 2). Production of 1,3-PDO was higher at 30 and 35°C at all the evaluated pH values. Along with 1,3-PDO, ethanol was also produced by few isolates (SM3, SM4, SM9, SM10 and SM11) at temperatures 30 and 35°C, but failed to produce ethanol at 40°C (Fig. 3).

In a similar study, Hao et al.[29] isolated eight bacterial strains from soil samples for 1,3-PDO (11 g/L) and ethanol (2.5 g/L) production in shake flask cultivation using 30 g/L of pure glycerol. Recently, Rossi et al.[30] reported complete degradation of glycerol and production of 22.8 g/L 1,3-PDO using environmental consortia. In the same study, *Klebsiella pneumoniae* strain BLh-1 produced 1,3-PDO (9.4 g/L) and ethanol (6.1 g/L) under anaerobic conditions. In contrast to these studies, our results showed production of 15 g/L of 1,3-PDO and 14 g/L of ethanol (Figs. 2 and 3) after 48 h, at a glycerol concentration of 30 g/L.

Glycerol utilization and bioconversion

Isolates (SM1, SM3, SM4, SM5, SM7 and SM8) capable of producing high levels of 1,3-PDO (>10 g/L) at 30 g/L glycerol were further evaluated at higher concentrations (50–70 g/L) of glycerol (Table 1). For this, we selected best growth conditions (35°C and pH 7). Among these, isolate SM3 and SM8 showed a lower glycerol degradation efficiency (<60%) at 50 g/L glycerol, whereas, isolates

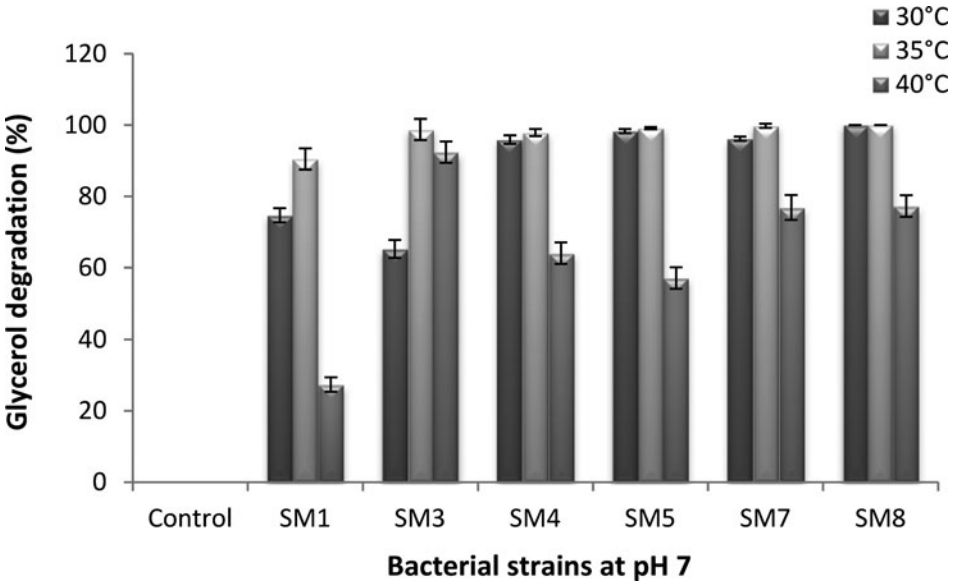

Fig. 1. Glycerol degradation (initial glycerol concentration of 30 g/L) by selected bacterial isolates in shake flask fermentations (180 rpm) at various temperatures (30, 35 and 40°C) at pH 7.

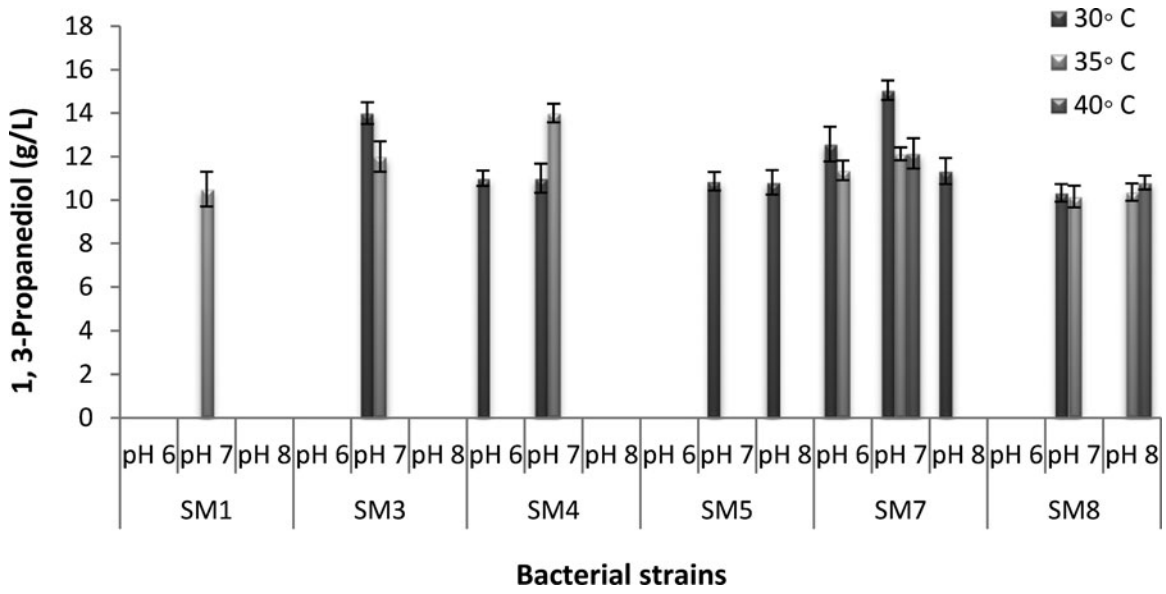

Fig. 2. 1,3-PDO production (Only data with production above 10 g/L are shown) by bacterial isolates in shake flask fermentations (180 rpm) using 30 g/L glycerol at various pH values and temperatures (30, 35 and 40°C) after 48 h.

SM1, SM4, SM5 and SM7 showed relatively higher degradation efficiency after 48 h of incubation (Table 1). A further increase of fermentation time to 120 h under similar conditions resulted in the complete degradation (100%) of glycerol (50 g/L) by isolate SM5 and SM7 followed by isolate SM4 (98%).The isolates also showed a higher degradation efficiency (>90%) even at elevated glycerol concentrations of up to 70 g/L (Table 1).

Bioconversion efficiency of these isolates at elevated concentrations of glycerol was analyzed using HPLC. The results indicated that at higher concentrations of glycerol (50–70 g/L), the bioconversion efficiency of the isolates (SM1, SM4, SM5, SM7 and SM8) were limited to 1,3-PDO with no ethanol formation (Table 1). Among the isolates tested, SM4 produced the highest amount of 1,3-PDO (46 g/L) at 70 g/L glycerol (Table 1). Zhuge et al.[31] reported 1,3-PDO production (18.3 g/L) from a *K. pneumoniae* strain with *dhaT* and *yqhD* gene expression for the conversion of 3-HPA to 1,3-PDO. Zhang et al.[32] also reported 12.2 g/L of 1,3-PDO using another *K. pneumoniae* strain isolated from soil and was able to produce significant amount of 1,3-PDO using analytical grade glycerol

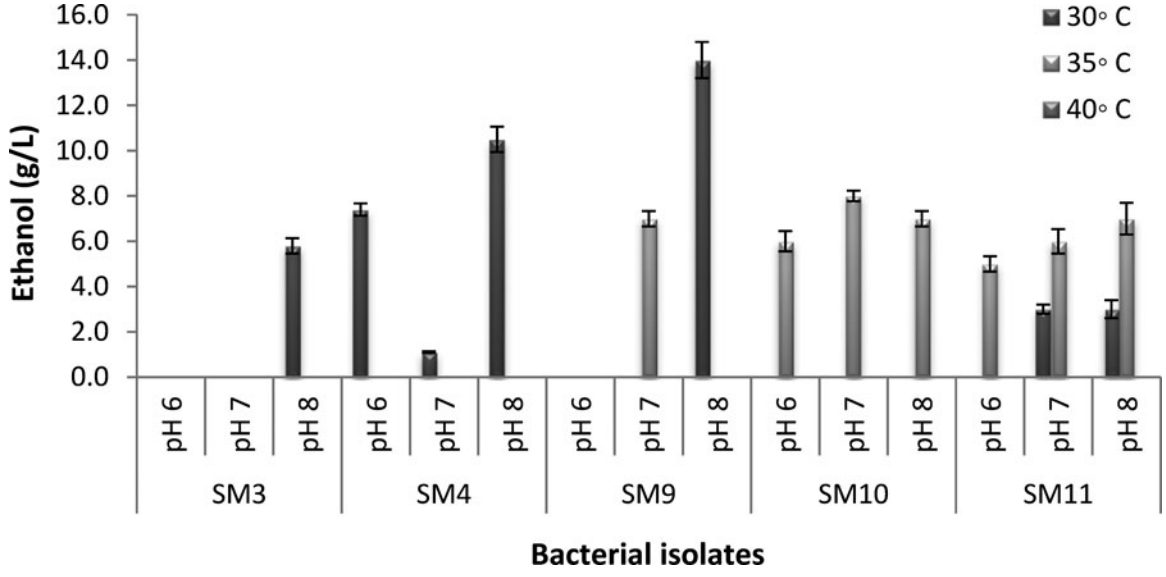

Fig. 3. Ethanol production by bacterial isolates in shake flask fermentations (180 rpm) using 30 g/L glycerol at various temperatures (30, 35 and 40°C) and pHs (6, 7 and 8).

Table 1. Glycerol breakdown (%) and its bioconversion into 1,3-PDO at increased glycerol concentrations (50–70 g/L) in shake flask fermentations at 35°C and pH 7 and 180 rpm.

Bacterial strain	*Glycerol conc. (g/L)*	*Glycerol degradation after 48 h (%)*	*Glycerol degradation after 120 h (%)*	*1,3-PDO production after 120 h (g/L)*
SM1	50	66.8 ± 0.3	78 ± 0.52	28 ± 0.3
	60	57 ± 0.41	68 ± 0.9	33 ± 0.32
	70	40 ± 0.8	64 ± 0.32	29 ± 0.5
SM3	50	26.8 ± 0.43	66.2 ± 0.63	0
	60	20 ± 0.57	61 ± 0.46	0
	70	19 ± 0.73	59 ± 0.32	0
SM4	50	86.2 ± 0.87	98 ± 0.3	36 ± 0.5
	60	72 ± 0.8	92 ± 0.3	38 ± 0.32
	70	68 ± 0.3	90 ± 0.5	46 ± 0.23
SM5	50	69.6 ± 0.32	100	26 ± 0.4
	60	58 ± 0.54	97 ± 0.45	33 ± 0.3
	70	51 ± 0.74	93 ± 0.43	31 ± 0.5
SM7	50	96.6 ± 0.4	100	12 ± 0.43
	60	80 ± 0.4	99 ± 0.4	20 ± 0.23
	70	71 ± 0.65	99 ± 0.4	35 ± 0.17
SM8	50	38 ± 0.78	72.4 ± 0.87	13 ± 0.54
	60	33 ± 0.71	70 ± 0.56	15 ± 0.45
	70	21 ± 0.23	34 ± 0.45	13 ± 0.63

(20 g/L). Hao et al.[29] reported on *K. pneumoniae* and *C. freundii* isolated from soil to produce a maximum of 11 g/L of 1,3-PDO from 30 g/L analytical grade glycerol in aerobic shake-flask cultivations. In microorganisms, glycerol is first converted to 3-hydroxypropionaldehyde (3-HPA) by a coenzyme B12-dependant glycerol dehydratase (Dhab), which is then reduced to 1,3-PDO by a reduced nicotinamide adenine dinucleotide (NADH)-dependent 1,3-PDO oxido-reductase (DhaT).[33]

Molecular identification

The bacterial isolates (SM4, SM5 and SM7) with high glycerol degradation efficiency were identified using partial sequencing of 16S rDNA. Sequence analysis of the isolates with those in GenBank using BLASTN showed 99% sequence similarity of isolate SM4 and SM7 with *Klebsiella pneumoniae* spp. rhinoscleromatis strain R-70 (Accession number-NR037084) and SM5 to *Enterobacter aerogenes* (Accession number-NR024643). The 16SrDNA sequence of SM4, SM5 and SM7 and other glycerol utilizing bacterial isolates retrieved from GenBank are presented as a phylogenetic tree, where strains SM4 and SM7 formed a lineage with the *Klebsiella pneumoniae* strains R-70 and SM5 with *Enterobacter aerogenes* JCM1235 (Fig. 4).

Glycerol degradation at an elevated range (90–110 g/L)

Due to the higher glycerol degradation potential, strains SM4, SM5 and SM7 were further screened for their bioconversion efficiency at higher glycerol concentrations of 90 to 110 g/L (Fig. 5). Strains SM4 and SM5 degraded 45% glycerol after 5 days of fermentation at 90 g/L glycerol. Interestingly, these strains survived and tolerated a glycerol concentration of up to 110 g/L, but with low glycerol degradation (SM5, 26%; SM4, 17% and SM7, 15%). This might be due to the rapid suicidal inactivation of glycerol dehydratase (GD) at high glycerol concentrations[34] and also due to increased formation of organic acids during fermentation. However, the formation of by-products and its effect on microbial growth was not evaluated in the present study.

The 1,3-PDO production was affected by an increase in glycerol concentration (90–110 g/L). Isolate SM4 showed a superior level of 1,3-PDO at 90 g/L of glycerol and further increase in glycerol concentration significantly affected its 1,3-PDO production efficiency (Fig. 5). The highest glycerol tolerance was exhibited by isolate SM5 at 110 g/L, with 1,3-PDO production of 17 g/L (Fig. 5).

Most of the previous reports on bioconversion of glycerol have shown the use of low concentrations of glycerol in either crude or pure form for the production of 1,3-PDO.[3,30,35] In this study, it was confirmed that a glycerol concentration of 70 g/L induced optimum 1,3-PDO production and glycerol degradation by *Klebsiella pneumoniae* strain (SM4). As reported in previous studies, *K. pneumoniae* has flexible regulation of the carbon and reducing equivalent fluxes under different conditions with higher product yield compared to other strains.[29,36]

Glycerol dehydratase (GD) catalyses glycerol reductive conversion to 3-hydroxypropanaldehyde (3-HPA), this being the first step required for the microbial conversion of glycerol to 1,3-PDO.[37] GD is the most important enzyme involved in converting glycerol to value-added

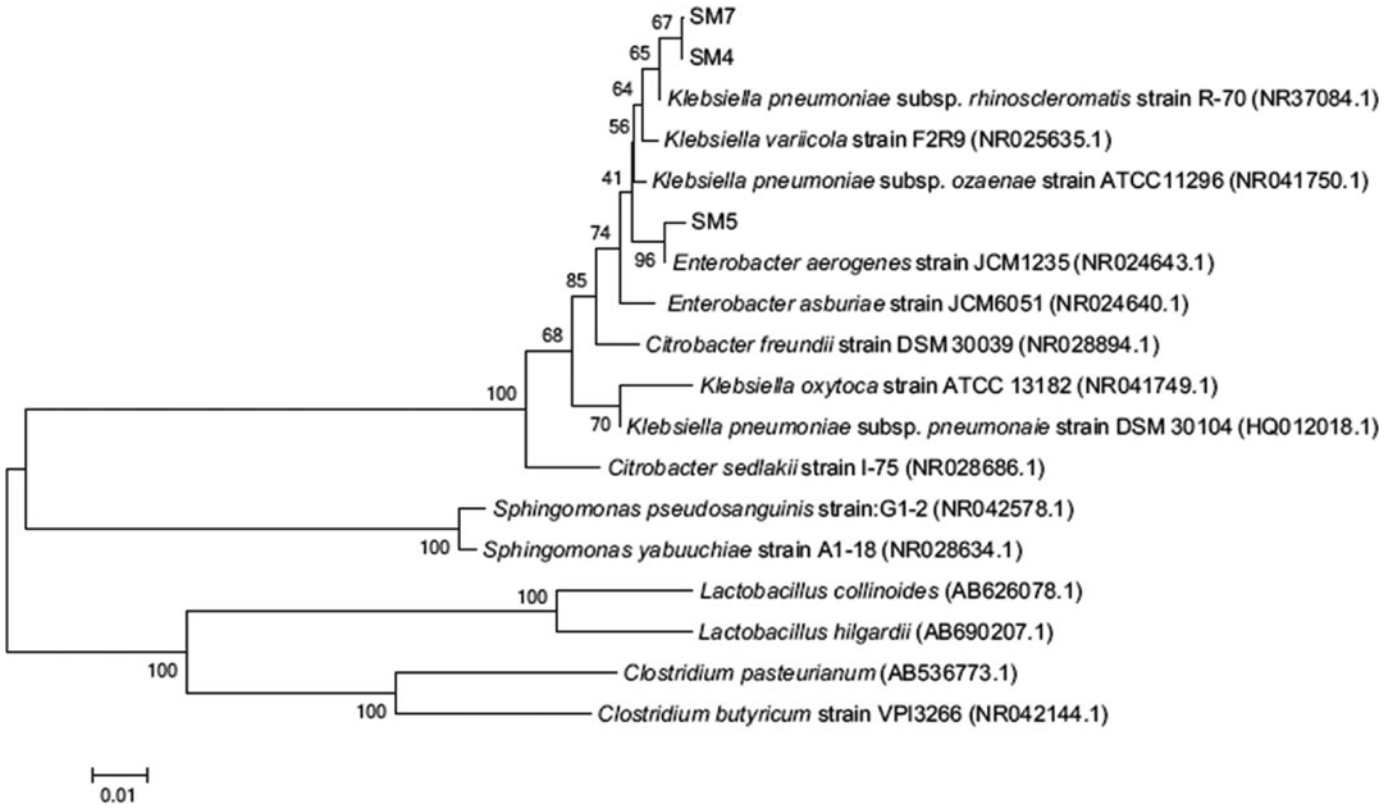

Fig. 4. Phylogenetic tree showing the phylogenetic position of *Klebsiella pneumoniae* subsp. rhinoscleromatis R-70 and related genera based on 16S rRNA gene similarity. The tree was constructed using the neighbour-joining method. Numbers at nodes represent levels of bootstrap support (%) based on analysis of 1000 replications (values ≥50 were shown). The scale bar represents 1 nucleotide substitution per 100 nucleotides of 16S rRNA sequence.

compounds such as 1,3-PDO and ethanol. However, high concentrations of 3-HPA represses glycerol uptake and cell growth.[38] The increased glycerol concentration has an inhibitory effect and causes the accumulation of 3-hydroxypropionaldehyde, a strong inhibitory compound for the growth of *K. pneumoniae* and cease the fermentation before glycerol exhaustion.[39] These results supported our findings in the present study.

The incubation period also has an impact on the degradation of glycerol. It was noticed that longer exposure of the bacterial isolates to glycerol led to a better degradation (Fig. 2 and Table 1). From this it can be concluded that at higher glycerol concentrations, GD requires an extended time of exposure to glycerol. In contrast to these results, Xu et al.[40] and Jun et al.[41] reported the high production of 1,3-PDO (102.1 g/L) by *K. pneumoniae* strain LDH526 (two stage process) and 80.2 g/L of 1,3-PDO with *K. pneumoniae* strain DSM 4799 using fed-batch cultivations, respectively. In this study, a simplified approach was employed to analyse the biodegradation of glycerol and it was observed that three bacterial strains, (SM4, SM5 and SM7) tolerated a glycerol concentration of 110 g/L and consumed up to 15–26% of glycerol.

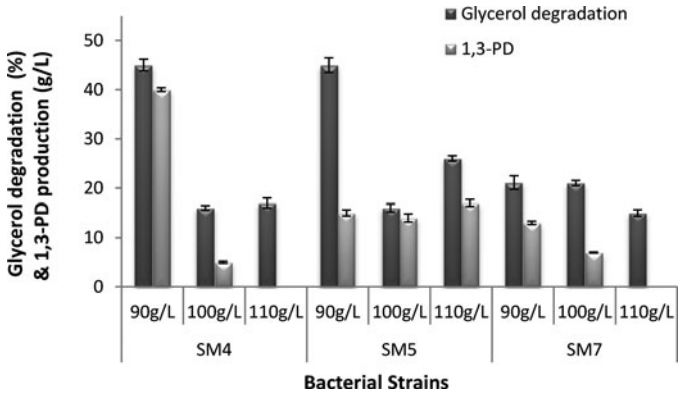

Fig. 5. Glycerol degradation (%) and 1,3-PDO production (g/L) in shake flask fermentations (180 rpm) using increased glycerol concentrations (90–110 g/L) at 35°C, pH 7 after 5 days of fermentation.

Conclusions

In summary, six isolates (SM1, SM3, SM4, SM5, SM7 and SM8) showed high glycerol degradation efficiency (>80%) at 30 g/L glycerol. Among these, three isolates (SM4, SM5 and SM7) exhibited greater glycerol tolerance up to

90–110 g/L glycerol, however, their ability to utilise glycerol was reduced considerably at this level. Maximum bioconversion efficiencies were achieved at 70 g/L glycerol concentration for SM4 and SM7 and at 60 g/L for SM5. 16S rDNA sequencing revealed that isolates SM4 and SM7 are closely related to *Klebsiella pneumoniae* and SM5 to *Enterobacter aerogenes*. This study further confirms the efficiency of bacterial strains isolated from soil for their glycerol utilization ability and bioconversion to value added products. Further optimization is required to improve the glycerol degradation and by-product formation by these potential strains.

Acknowledgments

The authors would like to thank the National Research Foundation (NRF), South Africa, and the Durbin University of Technology for providing funding for this study.

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