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Aerobic Conversion of Glycerol to 2,3-Butanediol by a Novel *Klebsiella variicola SRP3* Strain

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Abstract

The manufacture of biodiesel generates about 10% crude glycerol as a core by- product. This research is to identify novel bacterial strains which are capable of efficiently converting glycerol aerobically, and improve the strains for large scale production of value-added products. The production of a major product 2,3-butanediol (2,3-BD) from glycerol as a sole carbon source by the newly isolated *Klebsiella variicola* SRP3 are reported in a series of batch processes under aerobic condition. This study also compares the bacterial cell biomass, bio-products and glycerol dehydrogenase (GDH) enzyme production of *K. variicola* SRP3 isolated from paper mill waste when grown in aerobic condition. The incubation temperature, pH, glycerol concentration and nitrogen sources are the most important factors ruling the glycerol dehydrogenase. Acidic initial pH (pH 5.0) led to enhanced GDH activity (558.2 µmol/min/mg protein), yielding 25.33 g/L 2,3-BD, 7.6 g/L 1,3-PDO and 2.2 g/L acetoin from 50.0 g/L glycerol. In our aerobic batch culture the mutated strain *K. variicola* SRM3 exhibited 1.3 fold increased GDH activity of 721.5 units/mg protein from 558.2 units/mg protein, yielding 29.87 g/L 2,3-BD, 7.08 g/L 1,3-PDO and 2.02 g/L acetoin from 50.0 g/L glycerol. In our report, the optimal conditions for maximal GDH enzyme activity were defined, and 0.79 g/g product yield was achieved by the muted strain *K. variicola* SRM3, which is the highest amount obtained from glycerol as a sole carbon source until now. The research has for the first time proved that this *K. variicola* species can efficiently convert glycerol.

Keywords: Glycerol dehydrogenase; Glycerol; 2,3-Butanediol; 1,3-Proponediol; *Klebsiella variicola*

Introduction

Increasing demand and the rising cost of fossil fuels, as well as a concern for global climate change have shifted global efforts to utilize renewable resources for the production of a 'greener' energy replacement [1]. One major obstacle facing the development of biodiesel is the high volume of crude glycerol (10% v/v) generated from transesterification process. Generally speaking, there is a lack of microorganisms which can produce sufficient amounts of glycerol degrading enzymes to efficiently convert crude glycerol to value-added bio-products. The new isolate of bacteria permits screening, isolation and over expression of enzyme to help overcome these challenges. Several microorganisms have been found to produce a variety of enzymes for the biodegradation of glycerol [2]. Primarily, glycerol dehydrogenase (GDH) is an important cell-bound (intracellular) enzyme of bacteria that can convert glycerol to dihydroxyacetone (DHA) and other products under aerobic condition [1,3,4]. *Klebsiella pneumoniae* has well developed metabolic pathways for glycerol metabolism. In aerobic condition, *K. pneumoniae* utilizes glycerol through complex oxidative and reductive pathways (Figure 1) [5]. In the oxidative pathway glycerol is dehydrogenated by an NAD^+-dependent glycerol dehydrogenase to dihydroxyacetone (DHA) by generating reducing equivalent $NADH_2$. Afterwards, DHA is then further metabolized to various products viz., 2,3-BD, ethanol, acetic acid through pyruvate [6,7]. The reducing equivalent NADH generated from oxidative pathway by GDH directly influenced on the production of 1,3-PDO under aerobic condition of glycerol metabolism [2,6]. However, in the parallel reductive branch, glycerol is 1st dehydrated to 3-hydroxypropionaldehyde (3-HPA) by glycerol dehydratase, and it is then reduced to 1,3-PDO by NADH-linked 1,3-PDO dehydrogenase, thereby regenerating NAD^+ [4,6,7]. In *K. pneumoniae,* the generation of NAD^+ from the reductive pathway is utilized by the 1st enzyme GDH of the oxidative pathway [2].

Acetoin and 2,3-BD are two significant biorefinery platform chemicals. As an important physiological metabolic product acetoin can be produced by fermentation of sugar and other carbon sources [8,9]. Acetoin is widely used in food, flavour, cosmetics, and chemical synthesis [10]. The 2,3-butanediol, a reduced form of acetoin, is widely

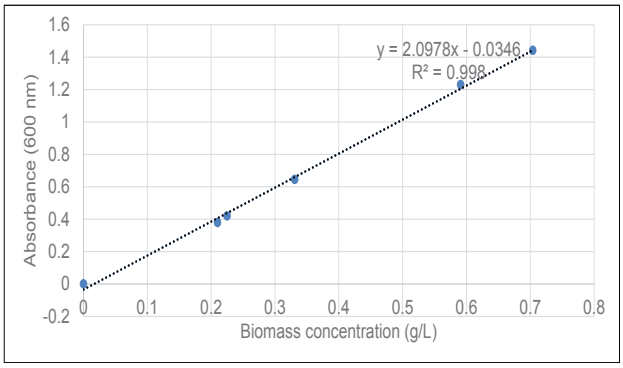

Figure 1: Standard curve for conversion of absorbance to dry cell weight (DCW) of bacteria.

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used as an antifreeze agent, lubricant, liquid fuel or fuel additive and a precursor of many synthetic materials including polymers and resins [11]. It can also be used for the manufacturing of printing ink, perfumes and fumigants, polymer, pharmaceutical carrier, moistening and softening agents, and reagent in different asymmetric chemical synthesis [12]. The 2,3-BD is a physiological metabolic product of the acetoin metabolic pathway in bacteria, and can be transformed to each other by 2,3-butanediol dehydrogenase in cells [10]. There are three key enzymes comprising catabolic α-acetolactate synthase, α-acetolactate decarboxylase, and 2,3-butanediol dehydrogenase, also called acetoin/diacetyl reductase are liable for the 2,3-BD biosynthesis [13,14].

A simple organic chemical 1,3-PDO obtained from microbial fermentation of glycerol is one of the high value products that has several interesting applications. The potential uses of this chemical are in the preparation of plastic, laminates, UV cured coating, adhesives material, anti-freeze, and it is also used as a solvent. The 1,3-propanediol-based polymers possess some better features than that generated from 1,2-PDO, butanediol or ethylene glycol. Now, 1,3-PDO is used to produce poly-trimethylene terephthalate (PTT), a biodegradable polyester which is widely used in carpet and textile manufacturing [15]. The precursor 1,3-PDO of PTT is produced through chemical synthesis [16] and fermentatively from glucose by microbes [17]. The joint venture company DuPont and Tate & Lyle, Loudon, Tennessee, USA have developed a proprietary process to produce 1,3-PDO using corn glucose instead of petroleum-based feed stocks. Nowadays, the anaerobic fermentation is the most promising option for bioconversion of glycerol by *Klebsiella*, *Citrobacter*, *Clostridium, Lactobacillus* and *Bacillus* [18].

Several *Klebsiella* strains except *K. variicola* have already been isolated which are able to ferment glycerol and the main product was 1,3-propanediol (1,3-PD), while 2,3-BD was in minor quantity along with other products [15, 19-21]. In our research article, we deal with the metabolic aspects of aerobic batch fermentation of glycerol by a novel strain *K. variicola*, and the effect of fermentation kinetics, and the conditions providing the gain of over expression of GDH. In this study, we report a major product of 2,3-BD and turning 1,3-PDO into minor from glycerol as a sole carbon source by an isolate SRP3 identified as *K. variicola* and its mutant SRM3. The fermentation kinetics of batch culture processes was studied in detail, and the conditions providing the gain of increased activity of GDH were evaluated. As a result, the growth rate, glycerol uptake rate, and the product concentrations were greatly enhanced in *K. variicola* and its mutant.

Materials and Methods

Isolation and identification of bacterial strain

The paper mill waste samples for isolation of glycerol-degrading bacteria were obtained from a paper mill in Thunder Bay, Ontario, Canada. About 5g of sample was inoculated into 100 ml minimal salt (MS) broth medium supplemented with 100 g/L glycerol in a 250 ml Erlenmeyer flasks, and incubated at 35 °C with shaking (200 rpm) for 48 h. MS medium containing pure glycerol as a sole carbon source and comprised of (per L): glycerol (100 g, 99.0% analytical grade, Sigma), $NaNO_3$ (0.1 g), K_2HPO_4 (0.1 g), KCl (0.1 g), and $MgSO_4$.$7H_2O$ (0.05g). Following incubation, inoculum from flasks showing growth was plated onto MS agar plates containing 5% analytical grade glycerol, and pure isolates were preserved at 4°C.

The greatest glycerol utilizing (GDH enzyme producing) isolate SRP3 was identified using 16S rRNA sequencing confirmed by its phenotypic and physiological characteristics. To amplify the 16S rDNA fragments universal primers designed within conserved regions of the 16S rDNA for Eubacteria were used: HAD-1 (5′-GACTCCTACGGGAGGCAGCAGT) and E1115R (5′-AGGGTTGCGCTCGTTGCGGG), they amplified a 796 bp fragment. The PCR product was purified with Clean-up kit (FroggaBio, Canada) and sequenced (Eurofins MWG Operon, US). Sequencing result was inputted online in the nucleotide blast tool through the NCBI database (http://blast.ncbi.nlm.nih.gov/) to identify the possible genus. The 16S rRNA sequence of isolated strain has been submitted to the GenBank under accession numbers KR092086. To identify the species of the genera the morphological and physiological characteristics were determined [22-24].

Fermentation medium and culture conditions

The strain was maintained at 4°C (sub-cultured every month) and one set stored at -80°C with 20% (w/w) glycerol added. The inoculum cultures were grown at 35°C and at 200 rpm under aerobic conditions in the Luria-Bertani (LB) broth medium (yeast extract, 5g/L; peptone 10 g/L; pH 7.0). In case of enzyme assay, different pH, incubation temperatures (25,30,35 and 40 °C), and concentrations of glycerol, yeast extract and peptone were maintained in the culture medium. When indicated, the medium was supplemented with specified concentration of different nitrogen sources. The pH of the medium was adjusted with 1 M NaOH or 1 M HCl, depending on the experiment. Batch fermentations were carried out in 125 ml Erlenmeyer flasks containing 50 ml fermentation medium, with 50 µl of 20 hours culture, and incubated at 35°C under aerobic condition at 200 rpm using rotary shaker (New Brunswick Scientific, C25 incubator shaker, NJ, USA). The fermentation medium containing glycerol as a sole carbon source and comprised of (per L): $NaNO_3$ (0.1 g), K_2HPO_4 (0.1 g), KCl (0.1 g), and $MgSO_4$.$7H_2O$ (0.05 g), glycerol (50.0 g), yeast extract (2.5 g) and peptone (5.0 g).

Optimization of fermentation process and medium components

Cells from slant cultures were inoculated into a LB broth medium to prepare the seed culture. After 20h incubation at 35°C under aerobic condition at 200 rpm using rotary shaker, 50 µl of grown seed culture was inoculated into appropriate medium for optimization growth conditions including incubation temperature and time, medium initial pH, glycerol concentration, and nitrogen sources. All the optimization parameters were performed in triplicates.

Enzyme activity assay and protein determination

Cells from 1 ml of aerobic cultures (OD_{600} of ~0.8) were harvested by centrifugation (3 min, 15,000xg), washed twice with 100 mM potassium phosphate buffer (pH 8.0). Cells were resuspended in 100 mM potassium phosphate buffer containing 50 mM KCl and sonicated at 4°C for 2 minutes (10 sec at a time, and until 2 min). After centrifugation (3 min,15000x*g*) the supernatant was kept at low temperature (4 °C) or in ice. The catalytic activity of intracellular GDH was determined at room temperature by measuring the reduction of NAD^+ to the substrate-dependent absorbance change of NAD(H) at 340 nm (ε340 = 6.22 mM−1 cm−1) using the method described by Ahrens et al. [6], Raj et al. [3], Gonzalez et al. [4] and Ashok et al. [2] with slight modifications. The enzyme assay was performed in triplicates, and the 1 ml reaction mixture contains 50 mM potassium phosphate buffer (pH 8.0), 30 mM ammonium sulfate, 0.2 M glycerol and 1.2 mM NAD. The assay was initiated by adding 50 µl of cell extract

in 250 µl reaction mixture, and the absorbance increase (NADH) was followed with a spectrophotometer for 3-5 minutes. One unit of activity is the amount of enzyme required to reduce 1µmole of substrate per minute. The specific activity of GDH is expressed as µ moles of substrate/minute/mg of cell protein and represent averages for at least three cell preparations. The protein concentration was determined by using the Bradford method [25], and bovine serum albumin served as the standard protein.

Adapted mutant development

The selected strain SRP3 was used to progressively develop adapted mutant strains that withstood 100, 125, 150, 175 and 200 g/L glycerol concentrations. The six-tube subculture-generations of evolutionary technique was used to achieve mutant [26]. This adaptive evolutionary technique involved sub-culturing the organism six consecutive times in tubes of MS medium containing 2.5 g/L yeast extract, the same glycerol at the same concentration and culturing conditions. The glycerol concentration was increased, and the subculture generations were repeated. The adapted strain labeled as SRM3 was obtained that grew in 200 g/L glycerol by increasing the concentration of glycerol and repeating the six subculture generations.

Analytical methods

Cell growth and GDH enzyme activity were measured as optical density (OD) at 600 and 340 nm respectively using microplate spectrophotometer (EPOCH, BioTek). The cell dry weight was calculated from the optical density (OD_{600}) using a standard curve (calibration curve) for this bacterial strain.

The pH values of the culture broth were determined using AB15 pH meter (Fisher Scientific). After confirming the identity of the products by GC-MS (Varian 1200 Quadrupole GC/MS using helium as the carrier gas), the concentrations (g/L) of glycerol, 2,3-BD, 1,3-PDO, acetic acid, acetoin etc. were determined using gas chromatograph GC 14A (Shimatzu Corp., Kyoto, Japan) equipped with a flame ionization detector and DB-WAXetr column. The column temperature was set to range from 45 °C to 240 °C; whereas, the injector and detector temperature was 250 °C. The carrier gas was nitrogen. About 1.5 ml of culture broth (fermented broth) was taken after 0, 24, 48, 72, 96, 120 and 144h of incubation. All the samples tested herein were purified immediately by centrifugation at 15,000xg at 4 °C for 5 min (accu Spin Micro 17, Fisher Scientific). The supernatants were filtered through membrane filter (0.45 µm pores size), and frozen for later GC-FID analyses. The purified samples except 0h incubation were diluted 5-10 times with distilled (Milli Q) water, and then injected 1 µl into GC. The results are presented as the means of three independent experiments.

Gene Bank accession No: The gene Bank accession number of the sequence reported in this paper is KR092086

Results

Strain isolation and identification

A number of bacterial strains were isolated from soil and paper mill waste samples with the goal of isolating strains able to utilize glycerol as a sole carbon source to produce GDH enzyme under aerobic condition. Under our experimental conditions the strain SRP3 (*K. variicola*) isolated from paper mill waste displayed significant GDH activity. This isolated strain SRP3 was identified using 16S rRNA gene sequencing. Sequence alignment in NCBI revealed a 99% similarity to the sequence of the strains *K. variicola* DSM 15968 or *K. variicola* KSM-005 or *K. variicola* MMUST-005, and then the species *K. variicola* (strain *K. variicola* SRP3) was identified by its morphological and physiological properties (Table 1). The strain was found to grow well on LB agar, yielding large mucoid colonies. Light microscopy revealed rod-shaped and encapsulated cells, arrange singly/ in pair/ in short chain, 0.3-1.0 µm in diameter and 0.6-6.0 in length. The very strain reported in this paper has been designated as *Klebsiella variicola* SRP3, and its GenBank accession No. was released as KR092086.

Effect of temperature, incubation time, medium ingredient and pH on enzyme activity

Glycerol dehydrogenase (GDH) is a key enzyme in oxidative pathway for aerobic bioconversion of glycerol. To optimize the incubation temperature and time for maximum enzyme activity, experiments were performed in batch fermentation processes without pH control (starting pH 7.0) at 25, 30, 35 and 40 °C. The MS medium containing glycerol (50 g/L) and yeast extract (2.5 g/L) was used for growth of the bacterium. The maximum enzyme activity (347.33 units/ mg protein) was attained at 35 °C and 72h incubation time (Figure 2).

In our study glycerol is only the substrate and yeast extract is the nitrogen source of GDH enzyme production. For optimization of glycerol and yeast extract concentrations, three different concentrations (20 g/L, 50 g/L and 75 g/L) of glycerol, and three different concentrations (1.0 g/L, 2.5 g/L and 5g/L) of yeast extract were used respectively. The results showed that 50 g/L glycerol and 2.5 g/L yeast extract were the favorable carbon and nitrogen sources for enzyme activity at 35 °C after 24, 48 and 72h of incubation (Figure 3).

The influence of pH on GDH and cell growth in the batch bioprocess without pH control was significant (Table 2 and Figure 4). For optimization of pH, the initial (starting) pH values of the culture medium were adjusted to set point by adding 1M NaOH/ HCl before autoclaving. The time profiles of pH changes, and the production of cell biomass with different starting pH are shown in Table 2. However, the results of enzyme activity under shake-flask fermentation with

Characters	Results	Expected results for *K. variicola*	Results related to the sp. of *Klebsiella*
Gram staining	Gram -	+	All species
Capsule	+	+	All species
Indole production	-	-	All sp. except *K. oxytoca*
H_2S production	-	-	All species
Methyl red	-	-	*K. oxytoca* and *K. pneumoniae*
Voges-Proskauer	+	+	*K. oxytoca, K. planticola* and *K. pneumoniae*
Citrate	+	+	*K. oxytoca, K. planticola* and *K. pneumoniae*
Urea hydrolysis	+	+	*K. oxytoca* and *K. planticola*
Gas/acid from Lactose	+	+	*K. oxytoca* and *K. pneumoniae*
Gas/acid from glucose	+	+	*K. pneumoniae* and *K. oxytoca*
Lysine decarboxilate	+	+	*K. oxytoca, K. planticola* and *K. pneumoniae*
Oxidase	-	-	All species
Catalase	+	+	All species
Adonitol fermentation	-	-	*K. oxytoca, K. planticola* and *K. pneumoniae*
Rhamnose fermentation	+	+	Negative to all other species

Table 1: Morphological and physiological characters of strain K. variicola SRP3

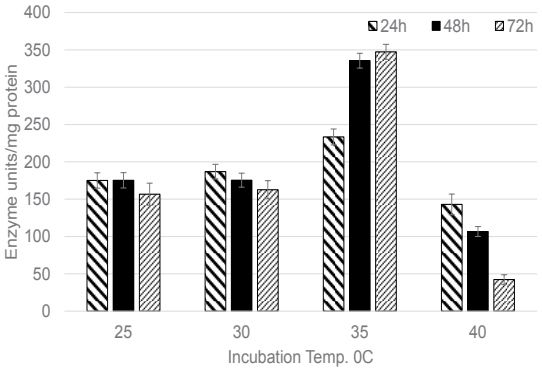

Figure 2: Effect of incubation temperature on specific activity of enzyme of strain SRP3 in batch process, minimal salt (MS) medium supplemented with yeast extract 0.25% and glycerol 5.0%, stating pH 7.0 and incubation temperature 35 °C.

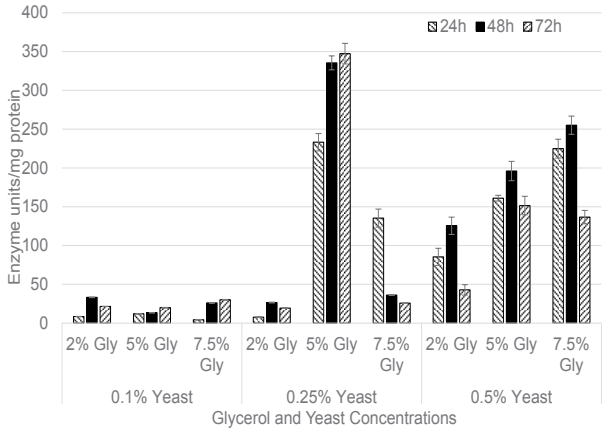

Figure 3: Effect of glycerol and yeast extract concentration on enzyme activity of SRP3 strain, minimal salt (MS) medium supplemented with different concentrations of glycerol and yeast extract, stating pH 7.0 and incubation temperature 35 °C.

various pHs ranging from 4.0 to 9.0 are presented in Figure 4. Low and higher pH inhibited cell growth and GDH production. However a pH higher than 7.0, a similar phenomenon was observed. At a pH 5.0, the cell growth and enzyme production were increased. As shown in Figure 4 and Table 2, the maximum enzyme activity and cell growth were obtained at pH 5.0. Interestingly, the pH of the medium was sharply dropped from starting pH (5.0, 6.0, 7.0, 8.0 and 9.0) after 24h of incubation and reached values between 4.48 and 4.68. On the other hand, the medium pH was increased from pH 4.0 to 4.39. This incubation period is the exponential growth phase of the batch culture and the pH was dropped due to the production of acetate in the culture medium.

After optimized yeast extract and glycerol concentrations, the effects of five other nitrogen sources (5 g/L) on GDH activity were also investigated in this study, which included peptone, malt, $(NH_4)_2SO_4$, NH_4Cl and NH_4NO_3. MS medium with supplementary carbon and nitrogen sources was used throughout the experiments. As shown in Figure 5, peptone and malt extract showed the highest enzyme activity compared to that of other nitrogen sources. Although, the price of peptone is too high for bulk fermentative products, it is extensively used as an ideal medium for bacterial growth. That is why here we investigated the influence of peptone concentration on GDH enzyme. In case of peptone, the maximum enzyme activity (421.82 units/mg protein) was obtained at a concentration of 5 g/L after 72h (Figure 6).

Batch fermentation under optimized conditions

After optimized conditions for maximizing GDH activity, batch fermentations were performed in fermentation medium at pH 5.0, temperature 35°C, glycerol 50g/L, yeast extract 2.5 g/L and peptone 5 g/L for quantification of bio-products. Under the above fermentation conditions, two major products viz., 2,3-BD and 1,3-PDO, and some minor products including acetoin, acetate, lactate and succinate were obtained after 24h of incubation. The product 2,3-BD obtained from optimized condition was a mixture of meso-2,3-BD and SS-2,3-BD.

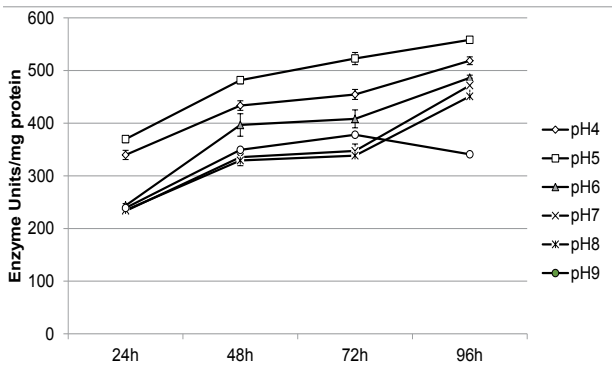

Figure 4: Influence of initial pH on enzyme activity of SRP3 without pH control, incubation temperature 350C, minimal salt (MS) medium supplemented with glycerol 5.0% and yeast extract 2.5%, and incubation temperature was 35 °C.

Initial pH	Biomass (OD_{600nm}) of the Culture broth				*Final pH of the fermented broth			
	24h	48h	72h	96h	24h	48h	72h	96h
4.0	0.747	0.855	1.021	1.137	4.39	4.45	4.47	4.58
5.0	0.789	0.994	1.126	1.314	4.48	4.47	4.52	4.78
6.0	0.713	0.973	1.073	1.222	4.57	4.52	4.56	4.77
7.0	0.780	0.919	1.071	1.202	4.61	4.61	4.76	4.84
8.0	0.746	0.933	1.123	1.198	4.57	4.57	4.64	4.72
9.0	0.766	0.916	1.131	1.197	4.68	4.68	4.73	4.62

Table 2: Effect of pH on cell growth after 24, 48, 72 and 96 hours of incubation at 35 °C of the bacterial strain *K. variicola* SRP3.

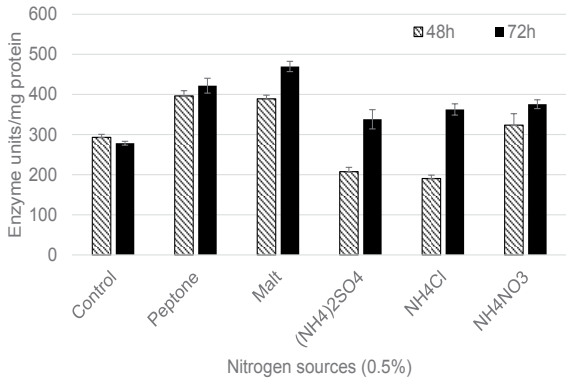

Figure 5: Effect of nitrogen sources on enzyme activity of strain SRP3, minimal salt (MS) medium supplemented with glycerol 5.0% and yeast extract 0.25%, stating pH 7.0 and incubation temperature 35 °C.

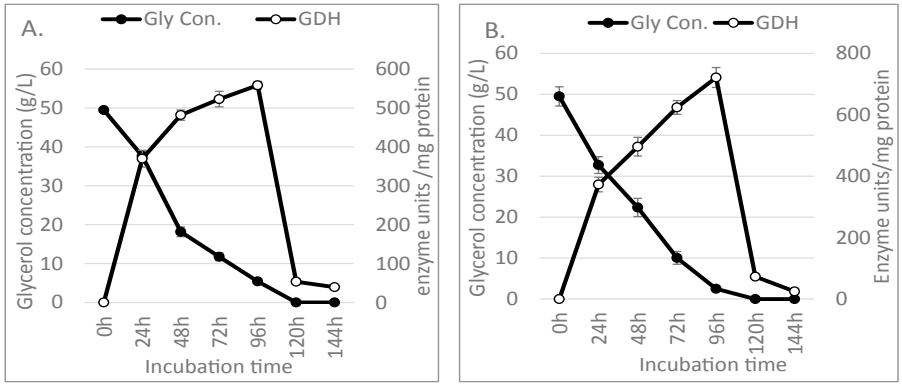

Figure 6: Glycerol consumption and specific activity of GDH by SRP3 (A) and SRM3 (B) in fermentation medium at initial pH 5.0 and incubation temperature 35 °C.

Figure 7 shows a typical profile for the fermentation of glycerol by the wild type strain SRP3 at different incubation time and accumulation of major products. The maximum product concentrations were achieved from 96 to 120h incubation. After 120h incubation the glycerol (50 g/L) was completely consumed, and the concentration of major principal product 2,3-PDO was 25.07±0.60 g L^{-1}. The other bio-products determined in the culture broth were 1,3-BD, acetoin and acetic acid, with concentrations, 7.62 ± 0.75 g L^{-1}, 2.81 ± 0.23 g L^{-1} and 0.82 ± 0.09 g L^{-1} respectively. The highest concentration of 2,3-PDO obtained after 96h was 25.33 ± 0.90 g L^{-1}. However, the highest concentration of 1,3-PDO, acetoin, acetic acid, lactic acid, succinic acid and oxalic acid obtained after 144h were 7.82± 0.83 g L^{-1}, 8.47 ± 0.23 g L^{-1}, 0.85 ± 0.07 g L^{-1}, 0.77 ± 0.04 g L^{-1}, 0.98 ± 0.06 g L^{-1} and 0.56 ± 0.04 g L^{-1} respectively. The productivity of the whole process for the four major products (2,3-BD, 1,3-PDO, acetoin and acetate) was about 0.37 g L^{-1} h^{-1} after 96h. The main bio-products of SRP3 were 2,3-BD, 1,3-PDO and acetoin, with extraordinarily little acetic acid, succinate, lactate and oxalic acid accumulation. Nevertheless, the Figure 8 shows that *K. variicola* SRM3, the adapted mutant strain of SRP3 reported this article, used 100% glycerol within 120h, producing 23.36 ± 1.04 g L^{-1} 2,3-BD, 7.52 ± 0.96 g L^{-1} 1,3-PDO, 10.19 ± 0.76 g L^{-1} acetoin, 1.06 ± 0.06 g L^{-1} acetate, 0.96 ± 0.05 g L^{-1} lactate and 0.88 ± 0.06 g L^{-1} succinate. The highest concentration of 2,3-BD obtained after 96h incubation was 29.87 ± 1.54 g L^{-1}. However the yields of 2,3-BD, 1,3-PDO and acetoin after 96h incubation were 0.6, 0.15 and 0.04 g/g respectively (Table 3). This adapted strain is capable to grow in a very high glycerol concentration, up to 200g/L. This demonstrated that the mutated strain is better adapted to utilize glycerol and effectually convert it to 2,3-BD, 1,3-PDO and acetoin than the wild strain.

We tried to keep the pH stable and neutralize acid in the culture medium during fermentation process. The citrate buffer (pH 4.8) and $CaCO_3$ (2% v/w) were used in the culture medium to keep the pH stable and neutralize acid respectively (Table 3). The Comparison between the final concentrations of major products and conversion yields of glycerol utilization by wild type *K. variicola* SRP3 and its adapted mutant SRM3, obtained in batch process is shown in Table 3. The adapted mutant strain *K. variicola* SRM3 utilized 97.5% glycerol within 96h incubation.

Discussion

Anaerobically many microorganisms are able to utilize glycerol as a sole carbon source, and the use of these microorganisms has increased attention for the bioconversion of glycerol [27]. Due to lake of external electron acceptor microbial growth is hampering in anaerobic condition. To date, several attempts have been made to biotechnologically produce value-added products viz., 1,2-PDO, 1,3-PDO and H_2 from low priced glycerol using anaerobic or microaerophilic fermentation process [28,29] but very little work have been done on aerobic process. Microaerophilic *Klebsiella pneumoniae* strains are also practical candidates for bio-fermentation process to produce bio-products [19,21]. Considering the aerobic bioconversion of glycerol to biofuels and value-added bio-products, our aim was to isolate novel strains for efficient product yield. Because, there were no available effectual strains designated nor was there rationality about the strain's requirement for successful production of 1,3-PDO and 2,3-BD from glycerol under aerobic process.

Recently, it was reported that *Klebsiella* could produce 1,3-PDO anaerobically [30] and 2,3-BD aerobically [12]. Still now there is no any report for the bioconversion of glycerol by *K. variicola*. Our new isolated strain *K. variicola* SRP3 could utilize glycerol as a sole carbon and energy source for their growth, and produce significant amount of numerous industrially important products under aerobic condition through GDH dependent oxidative pathway. Our report indicated that the batch fermentation with initial glycerol 50 g/L was the optimal concentration for maximum GDH activity which is the highest glycerol concentration in batch culture till now (Figure 2). Recently, Petrov and Petrova [12] reported the highest product yield 48.47% of 2,3-BD in feed batch fermentation. They also claimed that this amount was the highest reported till now. Furthermore, under optimized conditions in our study, the product yield 59.8% (29.9 g/50.0 g glycerol) of 2,3-BD was achieved by a adapted novel strain *K. variicola* SRM3 was the highest amount and reported till now. The results reported in Figure 6, which supported that the GDH activity and fermentation process were decreased after 96 h due to some product inhibition as well as depletion of glycerol concentration in the medium in aerobic batch fermentation condition. The strain *K. variicola* SRP3 reported herein exhibited 100% of glycerol consumption after 120 h. This research viewed that the higher glycerol concentration, up until about 50 g/L of feedstock concentration showed maximum production of reported end products which could be the new prospect of glycerol bioconversion field.

Our current study indicates that the GDH enzyme played a vital role to catalyze the primary step of glycerol oxidation which is responsible for glycerol utilization in *K. variicola*. This GDH enzyme is a key component in oxidative pathway of glycerol metabolism, and 2,3-BD, 1,3-PDO and acetoin formation in *K. variicola*. These findings recommended that the GDH enzyme might have dynamic

physiological consequence to the microbes when glycerol as carbon source was used. In addition, the role of proper environmental conditions like incubation temperature, nitrogen source, pH etc. in this strain might also contribute to the GDH over expression and favor the survival of this strain on glycerol by shifting glycerol metabolism from DHA to the formation of other compounds. Our results also suggested the establishment of optimal conditions for 2,3-BD, 1,3-PDO and acetoin synthesis must include enhancement of the GDH activity through oxidative pathway of the bioconversion of glycerol. In oxidative pathway, glycerol is dehydrogenated, phosphorylated, and directed towards the glycolysis, generating a variety of end products, including 2,3-BD, ethanol, DHA, acetate and acetoin. However, we attained 2,3-BD as a final major product, and 1,3-PDO, acetoin and acetic acid as the minor products.

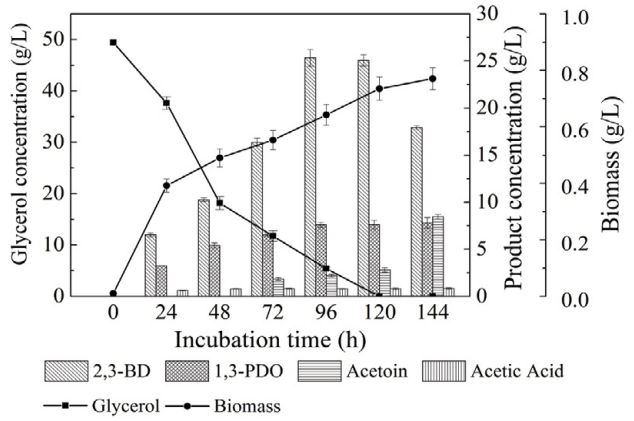

Figure 7: Production kinetics of major products obtained by *K. variicola* SRP3 in fermentation medium without pH control, starting pH 5.0 and incubation temperature was 35 °C.

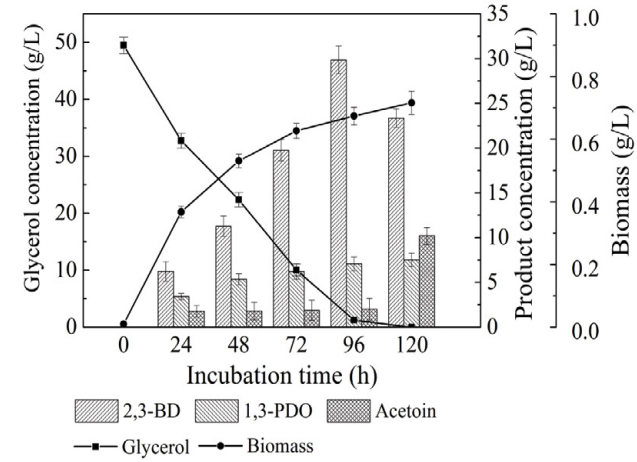

Figure 8: Production kinetics of major products obtained by adapted strain *K. variicola* SRM3 in fermentation medium without pH control, started at pH 5.0, incubation temperature 35 °C.

Our research paper reported a role of GDH involved in oxidative pathway of glycerol metabolism and some important metabolites (2,3-BD, 1,3-PDO and acetoin) formation in a novel bacterial strain *K. variicola* SRP3. The GDH was over expressed, and this new strain promises to be a better organism for the bio-conversion of glycerol to value-added products.

For scale up 2,3-BD product concentration up to industrial level we need to develop the strain and optimize fermentation conditions. Crude glycerol (biodiesel waste) contains methanol, salts, soaps, non-glycerol organic matter, and catalysts as the main impurities which can negatively influence the bioconversion process. Moreover, some acid byproducts like acetate, lactate and succinate inhibit of 2,3-BD production [31]. To overcome this problem, the strain should be developed by mutagenesis [32]. Thus, further work is required to obtain the highly efficient strain by mutagenesis for the utilization of crude glycerol and yield of the end product 2,3-BD by this *K. variicola* SRP3 strain. Bioreactor and optimized process parameters should be used to scale up product concentration for economic feasibility for mass bio-production of 2,3-BD from biodiesel waste glycerol. The strain *K. variicola* produced 2,3-BD, along with a number of by-products included some organic acids. 2,3-BD can be recovered from the fermentation broth using alcohol precipitation and vacuum distillation process proposed by Jeon et al. [33]. Briefly, the cells are removed from the fermentation broth by centrifugation and filtration. The cell-free fermented broth is concentrated to around 500 g/ L of 2,3-BD by vacuum evaporation at 50°C and 50 mbar vacuum pressure. For precipitation of organic acids and inorganic salts, concentrated solution is further treated with light alcohols like methanol, ethanol, and isopropanol. At the last step, a vacuum distillation process empowered the recovery of 76.2% of the treated 2,3-BD, with 96.1% purity.

Strains (Medium)	Initial pH	Final pH	Biom. (g/L)	Glycerol utilized (%)	2,3-BD		1,3-PDO		Acetoin	
					Concen. (g/L)	Yield[a] (g/g)	Concen. (g/L)	Yield[a] (g/g)	Concen. (g/L)	Yield[a] (g/g)
SRP3 (M)	5.0	4.84	0.64	88.0	25.3±0.90	0.58	7.6±0.26	0.17	2.2±0.15	0.05
SRP3 (MC)	7.0	5.46	0.64	84.0	23.1±1.02	0.55	7.1±0.34	0.17	1.8±0.2	0.04
SRP3 (MCB)	4.8	4.94	0.61	79.0	26.0±0.98	0.59	6.9±0.44	0.17	2.5±0.31	0.06
SRM3(M)	5.0	4.90	0.67	97.5	29.9±1.54	0.60	7.08±1.22	0.15	2.0±0.78	0.04

All experimental points presented are mean values from triplicate experiments;
Biom. Dry weight of biomass
Concen. Concentration
M Minimal salt medium with glycerol 5%, yeast extract 0.25% and peptone 0.5%
MC M with $CaCo_3$ 2%
MCB M with citrate buffer
[a]Product (g) obtained from bioconversion of per gram glycerol in batch fermentation

Table 3: Comparison between the final concentrations of major products and conversion yields of glycerol utilization at initial concentration 50 g/L under un-controlled pH condition by wild type SRP3 and its mutant SRM3, obtained in batch process after 96h incubation at 37 °C.

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