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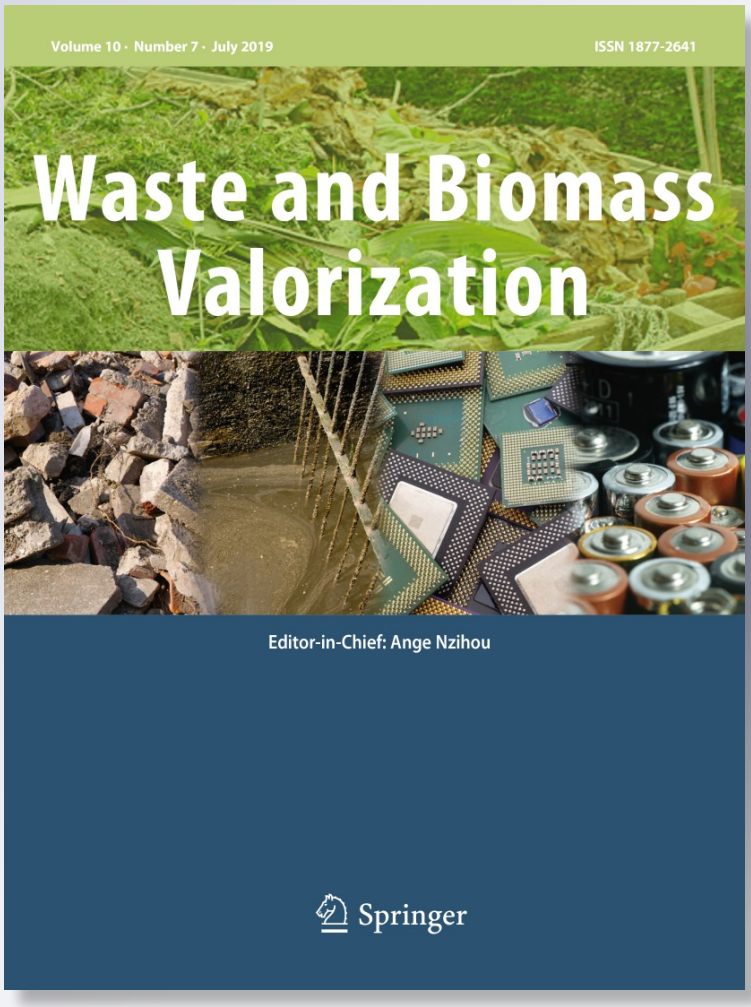

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ORIGINAL PAPER

Enzymatic Conversion of Glycerol to 2,3-Butanediol and Acetoin by *Serratia proteamaculans* SRWQ1

Iman Almuharef[1] · Md. Shafiqur Rahman[1,2] · Wensheng Qin[1]

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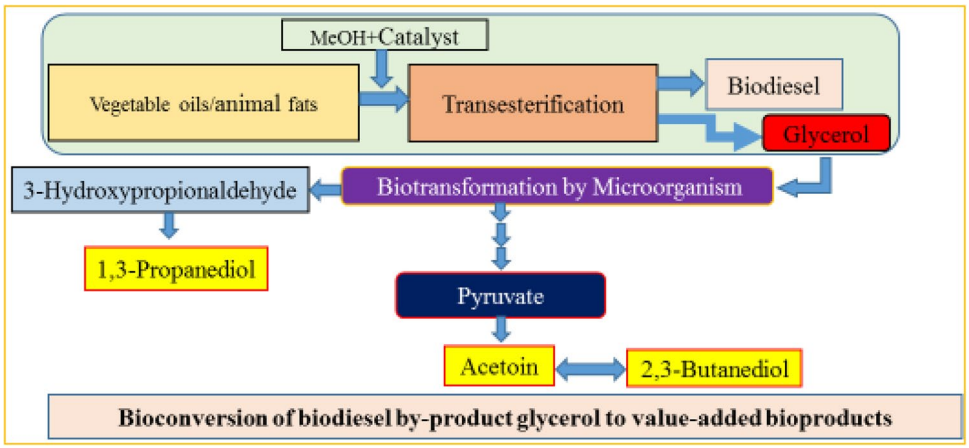
Abstract

Biodiesel, a renewable and environment friendly biofuel is produced by transesterification process using animal fats and vegetable oils. However, the flourishing of biodiesel industries has led to produce a huge amount (10% v/v) of crude glycerol as a core by-product, created an overflow problem. Therefore, biotransformation of glycerol into biofuel and other value-added products is one of the promising applications of glycerol due to its high availability at low cost. In this study, we report the capability of converting glycerol as a sole carbon source to 2,3-butanediol (2,3-BDO) and acetoin by using a newly isolated *Serratia proteamaculans* SRWQ1 strain in batch biotransformation process under aerobic condition. Strain SRWQ1 displayed a maximum up to 18.43 ± 1.55 g/L of 2,3-BDO, yielding 0.4 g/g using 49.0 g/L glycerol which was 98.0% of glycerol utilization. The strain SRWQ1 also successfully produced a significant amount of acetoin 8.38 ± 0.76 g/L with yields 0.06 g/g. Moreover, the maximum activity of glycerol dehydrogenase (GDH) which is a key enzyme of glycerol metabolisms was 408.69 ± 0.069 units/mg protein. The newly isolated strain *S. proteamaculans* SRWQ1 displayed the best ability to synthesize 2,3-BDO and acetoin using glycerol as the sole substrate, and it is the first report on biotransformation of glycerol by *S. proteamaculans*. Therefore, this aerobic conversion of glycerol to value-added green products 2,3-BDO and acetoin with potential industrial applications would represent a noteworthy alternative to add value for biodiesel production helping biodiesel industries development.

Graphical Abstract



Keywords Glycerol dehydrogenase · Bioconversion · *Serratia proteamaculans* · 2,3-Butanediol · Acetoin

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Extended author information available on the last page of the article

Introduction

Glycerol, a simple polyol, is a major by-product of biodiesel industries [1]. Typically, around 10% (w/w) of crude glycerol is produced from biodiesel synthesis process [2]. Thus, every hundred pounds of biodiesel produced ten pounds of crude glycerol as a core by-product [3]. Globally, the major producers of glycerol are USA and India, which produced 1.3 and 0.8 billion gallons respectively in 2013 [4]. Pure glycerol is used in various manufacturing industries such as pharmaceuticals, soaps, paint, toothpaste, and cosmetics [5]. However, biodiesel is an eco-friendly source of energy that recently has begun to replace the use of older sources of energy such as diesel, petrol, gasoline, and others [6]. The main reason why biodiesel is being adopted to replace these petroleum products is to promote an eco-friendly way of harnessing energy. The use of petroleum products has had a few drawbacks such as having adverse effects on the environment [7], and have created global ecological disturbances due to harmful effects on the environment in the long run.

In addition, biodiesel is a renewable fuel that can be produced from animal fats, vegetable oil, tallow and waste cooking oil [8]. The transesterification, a biodiesel production process performs a set of processes leading to the production of biodiesel and furthermore, releasing crude glycerol a core by-product [9]. This process involves a string of reactions that are catalyzed by the addition of an acid or base catalyst [10, 11]. The transesterification is often performed in large manufacturing fronts for the purpose of mass production [12]. Despite that, the transesterification ends up producing an eco-friendly fuel source called biodiesel, however; this core by-product raw glycerol produced from biodiesel synthesis is not quite the environment-friendly, because of its impurities like methanol, soap, catalysts and matter organic non-glycerol (MONG) which are negatively influence on biodegradation process, creating a substantial environmental concern. Consequently, crude glycerol in large amounts which poses a threat to the environment and needs high cost for purification. Therefore, there is a need for the conversion of this crude glycerol to value-added products that are not harmful to the environment [13]. Nevertheless, the process of glycerol bioconversion involves biotechnical processes which produce other products that have proven valuable to the industry [14]. Bioconversion of glycerol to producing value-added green products is an economic advantage for adding value to the fuel industry. In fact, the amount of crude glycerol production as a by-product of biodiesel industry is significantly increasing by increasing of biodiesel industries; therefore, it has made the purified glycerol as a cheaper product. Moreover, glycerol is regarded as an efficient and low-cost feedstock for industrial fermentation [15]. Ideally, glycerol can be converted by few microorganisms which are able to utilize glycerol as a source of carbon to produce commercially valuable chemicals, and *Serratia proteamaculans* SRWQ1 could be one of the most promising candidates for industrial bioconversion process.

Serratia proteamaculans, a facultative anaerobic bacterium that can be isolated from the soil. There is only a small fraction of the *Serratia* that has been discovered in the field of medical research [16]. *Serratia* sp. from unexplored habitats have gained considerable attention in recent years to characterize various organic acids such as malic acid, lactic acid, and acetic acid through phosphate solubilization process [17]. In this research, a novel bacterial strain *S. proteamaculans* SRWQ1 is attained through the investigation for the production of glycerol dehydrogenase (GDH) enzyme. Additionally, in this research, the bioconversion of crude glycerol to other high value chemicals by *S. proteamaculans* SRWQ1 is discussed in this research article. The focus was given on isolation and characterization of glycerol utilizing microbes, as well as the aerobic conversion of glycerol by a novel strain *S. proteamaculans* SRWQ1. Moreover, an aerobic condition was used for the large-scale production of value-added bioproducts 2,3-BDO and acetoin.

However, two significant platform chemicals acetoin and 2,3-BDO can be obtained from aerobic pathways of glycerol metabolisms of many bacteria [18, 19]. Furthermore, as a reduced form of acetoin 2,3-BDO is extensively used as a reagent, liquid fuel or fuel additive, lubricant, antifreeze agent, and in the preparation of polymer, pharmaceutical carrier, printing ink, fumigants, perfumes, softening and moistening agents [20, 21]. Nevertheless, acetoin is commonly used in food, flavor, cosmetics, and synthesis of industrially important many chemicals [22]. In the recent years, several strains of the species of *Citrobacter, Klebsiella, Clostridium* and *Enterobacter* are able to ferment glycerol, biodiesel derived raw glycerol and sugars, and the main product was 1,3-PDO, while 2,3-BDO was not reported along with other products [23, 24]. In oxidative pathway of glycerol metabolisms, GDH is an intracellular NAD^+-dependent key enzyme, can convert glycerol to dihydroxyacetone (DHA) and other metabolic products (Fig. 1). Therefore, the objective of this present work is to isolate a novel bacterial candidate from environmental samples to efficiently convert glycerol to a green product 2,3-BDO. The biotransformation kinetics of batch culture processes was studied in detail, and the novel bacterial strain providing the gain of increased 2,3-BD product yield were evaluated.

All the research works presented this study, approached challenges in the current production of value-added products including platform chemicals and liquid fuel or fuel

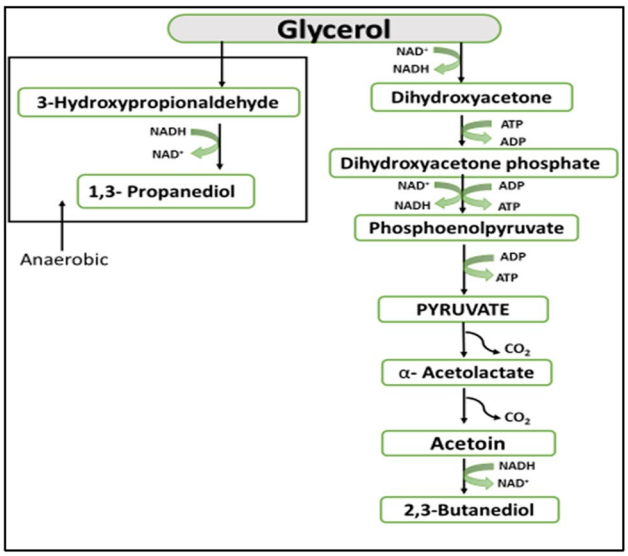

Fig. 1 Glycerol metabolic pathways to produce 1,3-propanediol, acetoin and 2,3-butanediol

additive from different perspectives through cost-effective processes with a main focus on novel bacteria strain. There were several reasons of this research, namely bacteria can be easily cultured, can be found inhabiting unlimited environments and can survive in extreme environmental stresses. These attributes lend to the potential exploitation of hardier bacterial strain for the industrial biotransformation of a large volume of glycerol or crude glycerol to biofuel and other high-value products. This research work led to contribute in the development of biorefineries and reduce industrial waste disposal. Also, this research work helps in summarizes different strategies employed to produce valuable chemicals (1,3-PDO, 2,3-butanediol, ethanol, *n*-butanol, organic acids, polyols and others). Moreover, this work can evaluate the efficiency of bioconversion of crude glycerol to bioproducts by novel bacterial strains under the aerobic conditions with potential in future studies for advancement in bioconversion processes. A very little work has been done on the aerobic process of glycerol metabolisms, and it is our first report for biotransformation of glycerol to 2,3-BDO using our newly isolated *S. proteamaculans* SRWQ1. Bioconversion of glycerol to value-added green products 2,3-butanediol (2,3-BDO) and acetoin, with potential industrial applications represents a noteworthy alternative to add values to biodiesel production and promote development of the biodiesel industry. Consequently, we report a newly isolated efficient GDH producing bacterial strain *S. proteamaculans* SRWQ1 with high yields of 2,3-BDO and acetoin. This is the first study that showed 2,3-BDO and acetoin productions by *S. proteamaculans* until now.

Materials and Methods

Growth Media

For isolation of bacterial strain, the minimal salt (MS) medium per litre composed of 0.1 g $NaNO_3$, 0.1 g K_2HPO_4, 0.1 g KCl and 0.05 g $MgSO_4 \cdot 7H_2O$ supplemented with glycerol was used. However, for GDH enzyme activity assay and optimization of bioconversion process, MS medium supplemented with glycerol, yeast extract and/peptone was used. Luria–Bertani (LB) broth medium was used for seed cultures of bacterial strains, and composed per litre: 10.0 g peptone, 5.0 g yeast extract, 5.0 g NaCl [25]. The batch cultivation medium MS-2 used for bioconversion of glycerol to value-added bioproducts, and composed (per L) of 50 g glycerol as a sole carbon source, 0.1 g $NaNO_3$, 0.1 g K_2HPO_4, 0.1 g KCl, and 0.05 g $MgSO_4 \cdot 7H_2O$, 5.0 g yeast extract and 5.0 g peptone.

Isolation of Bacterial Strains

Organic materials including paper mill waste, soil and rotting wood were collected from the forest in Thunder Bay, Ontario, Canada. Five grams of each sample was added to the flask containing 100 mL of MS medium supplemented with 100 g/L glycerol, homogenized through vortex using a magnetic stirrer, and then incubated at 30 °C in a shake flask incubator (200 rpm) for 72 h. After incubation, a 100 µL of broth culture was spread on MS agar plates supplemented with 100 g/L glycerol, incubated at 30 °C. From the 24 h culture plates, different colonies of bacteria were selected based on their morphological features like size, color, and colony morphology. All the isolated bacterial strains were identified and screened for their capability of utilizing glycerol under aerobic condition.

Identification of Bacterial Strains

The bacterial isolates were identified using 16S rRNA gene sequencing. However, for identification, the isolated bacterial strains were inoculated in LB broth medium, and incubated at 30 °C in shaker incubator at 200 rpm for 24 h. The genomic DNA of the strains were isolated using bacterial DNA extraction kit (Presto™ mini genomic DNA Bacteria kit, Geneaid) following the manufacturer's protocol. To amplify the genes from bacterial samples, the PCR products for 16 s rDNA sequences were prepared using HDA-1: 5′-GAC TCC TAC GGG AGG CAG CAG T-3′ (forward) and E1115R: 5′-AGG GTT GCG CTC GTT GCG GG-3′ (reverse) primers (Eurofins Scientific, Toronto, Canada). The mixture of PCR reaction composed

of 25 µL Taq mix (2×) (FroggaBio, Toronto, Canada), 1 µL HDA-1 primer, 1 µL E1115R primer, 1 µL genomic DNA template, and 22 µL nuclease-free water. The PCR thermal cycling conditions was followed: primary denaturation for 3 min at 94 °C followed by 35 amplification cycles consisting of denaturation at 94 °C for 30 s, annealing for 30 s at 65 °C, and extension at 72 °C for 1 min, and then concluding with a final extension step at 72 °C for 10 min. The PCR products were visualized using a 1% agarose gel electrophoresis to confirm the quantity and purity of size. The DNA bands from the gel were cut, purified using Geneaid PCR/Gel Purification Kit following the manufacturer's protocol [26]. The nucleic acid DNA concentration was measured using Nanodrop spectrophotometer system. Finally, the purified bacterial DNA samples were sent to the Centre for Applied Genomics (Eurofins Genomics, Toronto, Canada) for sequencing. The sequencing results were input into the National Center of Biotechnology Information (NCBI) database (http://blast.ncbi.nlm.nih.gov) for the possible identification of the strains. The phylogenetic relationship for isolates was constructed using the ClustalX Omega software (Fig. 2).

Preparation of Seed Culture

One set of bacterial strains were stored at − 80 °C with 20% (w/v) glycerol added as a frozen stock. The other set was working culture which was maintained at 4 °C by sub-culturing after each 25–30 days [27]. All experiments including GDH and biotransformation assays were performed using seed culture. Briefly, a loopful of 24 h bacterial culture from LB agar plate was transferred to a fresh 5 mL of LB broth medium, incubated at 30 °C for 24 h. Then 1 mL of the 24 h seed culture from LB broth was inoculated into 200 mL Erlenmeyer flask containing 50 mL of culture medium used for enzyme assay or biotransformation process.

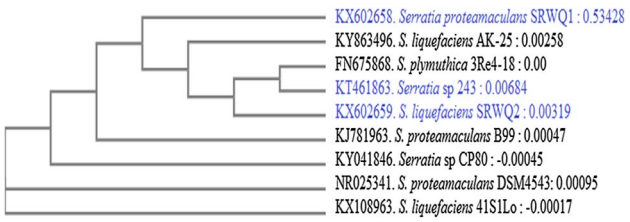

Fig. 2 Evolutionary relationships of the strains: phylogenetic tree drawn from sequence alignment program using ClustalX Omega software. 16S rRNA gene sequences were retrieved by nucleotide BLAST searches in NCBI. The numbers that follow the names of the strains are accession numbers and bootstraps of published sequences

Cell Extraction and Enzyme Activity Assay

For GDH enzyme activity, cells from 1.5 mL aerobic broth culture were harvested by centrifugation at 12,000×*g* for 3 min, and washed twice with 100 mM potassium phosphate buffer (pH 8.0). Cells were resuspended in the same buffer containing 50 mM potassium chloride (KCl) at 4 °C, disrupted using sonicator. Briefly, the mixture was vortexed for 30–60 s, and then sonicated at 4 °C for 2–3 min (10 s at a time until 2–3 min). The lysate was discarded after centrifuged (2–3 min, 15,000×*g*). The supernatant was kept at low temperature using ice, and 50 µL were taken for GDH enzyme assay [28].

However, to determine the GDH enzyme activity, a 300 µL of reaction mixture was contained 30 mM ammonium sulfate, 0.2 M glycerol, 1.2 mM NAD^+ and 50 µL of cells supernatant [29]. By following the method described by Tian et al. [30] and Raj et al. [31], the GDH activity was determined using reduction of NAD^+ to the substrate-dependent absorbance change of NAD(H) at 340 nm ($\varepsilon 340 = 6.22\ mM^{-1}\ cm^{-1}$). In the GDH activity assay, the initial reduction rate of NADH was measured using spectrophotometer (Epoch BioTeck, Gen 5 software) by increasing of absorbance per minute of time. The GDH activity is expressed as µmoles of substrate reduction per minute per milligram of cell protein. As such, one unit of activity is the amount of enzyme required to reduce 1 mmole of substrate per minute under the condition specified [32].

The concentration of the protein (mg/mL) in crude extract was determined by using the standard curve of BSA protein. Bradford Protein Assay kit purchased from Bio Basic Inc., Canada was used to determine protein concentration following the manufacturer's protocol. The activity of the GDH enzyme was measured as units of enzyme per milligram of protein (U/mg protein) [33].

Effect of Different Concentrations of Glycerol and Yeast Extract

For maximum enzyme activity of highly efficient bacterial strains, the concentrations of sole substrate glycerol and a nitrogen source yeast extract in MS medium were optimized in a batch culture at different incubation (24, 48 and 72 h) times. The experiment was performed in a shake flask culture contained 50 mL MS medium supplemented with different concentrations (2, 5 and 7%) of glycerol as a sole carbon source and various concentrations (0.1, 0.25 and 0.5%) of yeast extract. The initial pH of the medium was 7.0. All experiments were repeated at least three times.

Effect of Different Temperature and pH

This study was conducted with the consideration of the effects of temperature on the enzyme production process. The culture medium used for optimization of incubation temperature and medium initial pH was MS medium supplemented with 5% glycerol as a sole carbon source and 0.5% yeast extract. Flasks were incubated at different temperatures (20, 25, 30 and 37 °C). However, for optimization of pH, culture medium of different initial pH values (4, 5, 6, 7, 8 and 9) were used.

Effect of Different Nitrogen Resources

The study was conducted for inducing the GDH activity as well as bacterial growth using different nitrogen sources. However, in this study, the differences in growth and enzyme production were determined when various nitrogen sources were present in the culture medium. The major nitrogen sources viz., peptone, malt extract, ammonium chloride, and ammonium nitrate were used in MS medium where 5% glycerol and 0.5% yeast extract were also added.

Fermentation Process and Sample Preparation for Bioproduct Quantification

The batch fermentation experiment was performed using optimized culture conditions for production of value-added products from glycerol biotransformation process. MS-2 medium was used for biotransformation of glycerol to 2,3-BDO and acetoin in aerobic process. One milliliter of seed culture was transferred to 50 mL of sterilized growth medium (MS-2), incubated at 25 °C for 144 h in a rotary shaker at 200 rpm. After 24 h of incubation, 1.0 mL of the culture broth sample was collected in 1.5 mL centrifuge tube, centrifuged at 12,000×*g* for 5 min, and supernatant was collected. In this way samples from 48, 72, 96, 120 and 144 h incubation also collected. The supernatant was further purified through filtration using a membrane filter of 0.45 nm pore sizer. The purified sample was diluted up to five times using distilled water (%, v/v) and 1 µL was injected into GC-FID for product quantification [34].

Analytical

Bacterial growth was measured spectrophotometrically from absorbance at 600 nm (OD_{600}) using Epoch™ microplate spectrophotometer (BioTek instrument, USA). The pH values of culture broth medium were determined using AB15 pH meter (Fisher Scientific, Canada). The products from fermented broth were identified by GC-MS (Varian 1200 Quadrupole GC/MS) using helium as the carrier gas. Acetoin and 2,3-BDO concentrations in the fermentation broth were quantified periodically using gas chromatograph equipped with a flame ionization detector (GC-FID, Shimadzu/GC-14A) where nitrogen gas was a carrier. The concentration of glycerol in terms of g/L was also determined by GC-FID. The DB-WAXETR column's temperature of GC was set at a range from 45 °C (2 min) to 240 °C at an increasing rate of 10 °C/min. The injector and detector temperatures were set to 250 °C.

Results

Isolation, Identification and Phylogenetic Relationship of Bacterial Strains

Several bacterial strains were isolated from paper mill waste, forest soil and rotting wood samples for screening their glycerol utilizing activity under aerobic condition. Under the experimental conditions described in this research article, the strains SRWQ1, SRWQ2 and SRWQ3 isolated from paper mill waste exhibited significant GDH activities, and were identified using 16S rRNA gene sequencing. Genomic DNA were successfully isolated from all three GDH producing isolates using DNA isolation kit, and amplified 16S rRNA genes using PCR (Polymerase chain reaction). Sequencing and sequence analysis results of all 16S rRNA genes from three strains were successfully obtained. The sequences of 16S rRNA of the three strains SRWQ1, SRWQ2 and SRWQ3 were submitted to the GenBank for their accession numbers. Nevertheless, the sequence of the isolated strain SRWQ3 reported in this paper was found 100% similarity to the strain *Serratia* sp. 243 (accession no. KT461863). Moreover, sequence alignments of other two strains SRWQ1 and SRWQ2 in NCBI revealed 99% similarity to the sequence of *S. proteamaculans* and *Serratia liquefaciens* respectively. Therefore, the potential two strains SRWQ1 and SRWQ2 have been nominated as the new strains *S. proteamaculans* SRWQ1 and *S. liquefaciens* SRWQ2 respectively. The GenBank accession numbers of two newly isolated novel bacterial strains *S. proteamaculans* SRWQ1 and *S. liquefaciens* SRWQ2 released by NCBI are KX602658 and KX602659 respectively (Fig. 2).

Nevertheless, a phylogenetic tree was constructed to analyze the evolutionary relationship among the newly isolated strains as well as other *Serratia* strains retrieved from GeneBank using their 16S rRNA sequences. As shown in Fig. 2, the phylogenetic tree was constructed using ClustalX Omega software. Thus, the identities of our new isolated strains were confirmed by the result of phylogenetic relationship through the distance between all the newly isolated and other strains (Fig. 2).

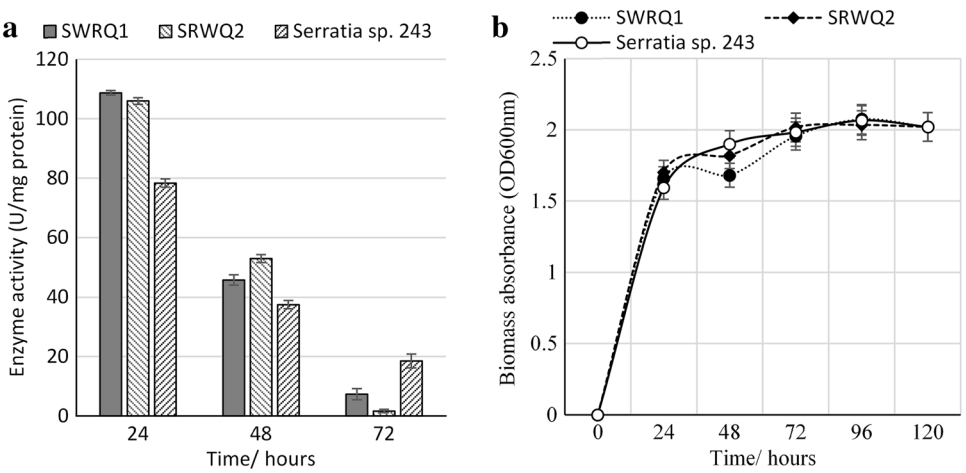

Fig. 3 **a** Specific activity of GDH and **b** bacterial growth curve by SRWQ1, SRWQ2, and *Serratia* sp. 243

Primary Screening for GDH Activity

Three bacterial strains *Serratia* sp. 243, SRWQ1 and SRWQ2 were screened for their GDH enzyme activity and biomass production using MS medium supplemented with 20 g/L (2%) glycerol as the sole carbon source (Fig. 3). All three isolates (SRWQ1, SRWQ2, and *Serratia* sp. 243) were able to utilize glycerol as a sole substrate to produce significant amount (78.31–108.72 units/mg protein) of GDH enzyme in 24 h incubation at 30 °C (Fig. 3a). The use of these bacterial strains can increase attention for the bioconversion of glycerol to produce value-added products. The maximum enzyme activities of all the strains were attained after 24 h of incubation; however, strain SRWQ1 exhibited the greatest enzyme activity (108.72 ± 0.773 U/mg protein) after 24 h incubation compared with the rest of strains (Fig. 3a). However, as shown in Fig. 3b, the biomass productions of all three strains were increased dramatically until 24 h of incubation, and then increased slowly up to 96 h. The maximum biomass productions obtained after 96 h of incubation were almost same (OD_{600} 2.03–2.07) with all the three bacterial strains (Fig. 3b). Overall strain SRWQ1 was found as an efficient strain for capable of utilizing glycerol to produce GDH enzyme. Therefore, the strain SRWQ1 was selected as the best candidate for further study of glycerol biotransformation process. Also, the culture conditions of SRWQ1 strain were optimized for maximum yields of bioproducts from glycerol bioconversion process.

Effect of Glycerol and Yeast Extract Concentrations on GDH Activity

In this study, glycerol was only the substrate, and the yeast extract was the nitrogen source for GDH enzyme production. However, for optimization of biotransformation process, MS medium supplemented with three different concentrations of

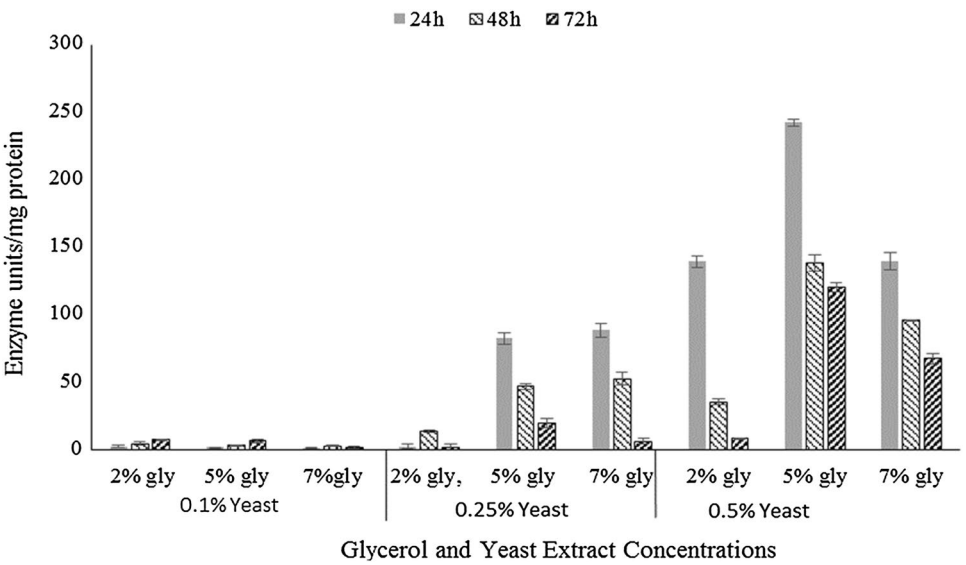

Fig. 4 Effect of different glycerol and yeast extract concentrations on enzyme activity of SRWQ1 strain

both glycerol (20, 50 and 75 g/L) and yeast extract (1.0, 2.5 and 5.0 g/L) were used. The experiment results showed that 50 g/L of glycerol and 5 g/L of yeast extract were the favorable carbon and nitrogen sources respectively for maximum enzyme activity at 30 °C after 24, 48 and 72 h of incubations (Fig. 4). The maximum enzyme activity 242.24 ± 2.6 U/mg protein was attained in 24 h of incubation with 5% (50 g/L) of glycerol and 0.5% (5 g/L) of yeast extract respectively.

Effect of Initial pH and Incubation Temperature on Enzyme Activity and Biomass Production

To determine the effects of different levels of incubation temperature and time for maximum enzyme activity, the experiments were performed in batch fermentation processes under the incubator temperatures 20, 25, 30, and 37 °C respectively. Results of the effect of incubation temperature and initial pH of culture on GDH activity using SRWQ1 strain are presented in Fig. 5. The MS medium containing glycerol (50.0 g/L) and yeast extract (5.0 g/L) was used for growth of the bacterium. The strain SRWQ1 displayed the maximum enzyme activity 343.12 ± 0.52 U/mg protein at 25 °C and pH 7.0 in 24 h of incubation (Fig. 5a). The influence of pH on GDH enzyme and cell growth in the batch bioprocess without pH control was significant. To study the impact of pH on the growth medium, the initial pH levels were adjusted from 4.0 to 9.0. The initial (starting) pH values of the culture medium were adjusted to set a point by adding 1 M KOH/HCl before autoclaving. However, the results of enzyme activity in shake-flask fermentation with various pH ranging from 4.0 to 9.0 are presented in Fig. 5b. As shown in Fig. 5b, the maximum enzyme activity 370.94 ± 0.88 U/mg protein was obtained at pH 6.0.

Moreover, the time profiles of pH changes and the production of cell biomass with different starting pH are shown in Table 1. Low and higher pH inhibited cell growth and GDH enzyme production (Table 1; Fig. 5b). However, a pH higher than 6.0, a similar phenomenon was observed. As shown in Table 1, the maximum cell growths were obtained at initial pH 6.00 and 7.0. Interestingly, the pH values of the culture medium were sharply dropped from starting pH 6.0 and above after 24 h of incubation. On the other hand, the medium pH was increased from pH 4.0 to 4.92 after 24 h (Table 1).

Effect of Different Nitrogen Resources

The effects of five nitrogen sources other than yeast extract viz., peptone, malt extract, $(NH_4)_2SO_4$, NH_4Cl and NH_4NO_3 on GDH enzyme activity were also investigated. MS medium supplemented with 50 g/L glycerol, 5.0 g/L yeast extract and 5.0 g/L of different nitrogen sources was used

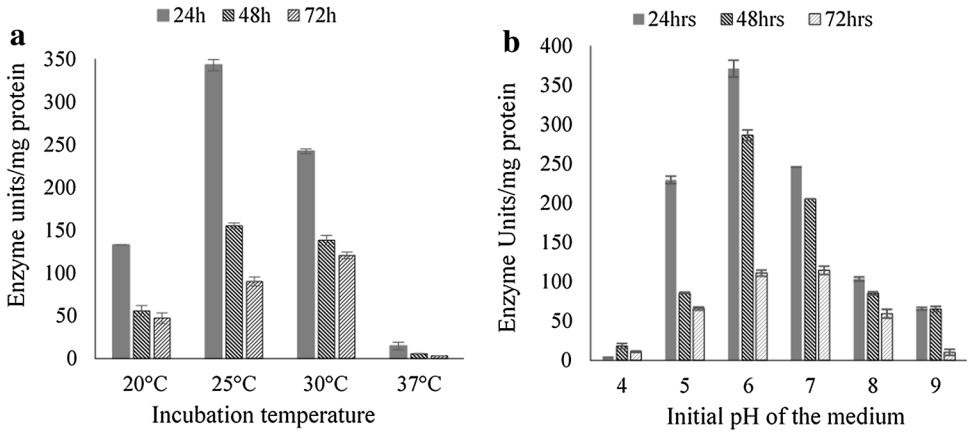

Fig. 5 **a** Effect of different temperatures and incubation times on enzyme activity by SRWQ1strain. **b** Effect of different pH levels on enzyme activity by SRWQ1

Table 1 Effect of different pH levels on cell growth after 24, 48, 72 and 96 h of incubation at 25 °C of the bacterial strain SRWQ1

Initial (pH)	Biomass (OD600nm) of the culture broth[a]				Final pH of the fermented broth[a]			
	24 h	48 h	72 h	96 h	24 h	48 h	72 h	96 h
4.00	0.3423	0.6953	0.6656	0.649	4.52	4.73	4.85	4.92
5.00	1.2903	1.3736	1.43233	1.48633	5.16	5.39	5.84	5.77
6.00	1.3653	1.42466	1.524	1.61766	5.17	5.12	4.89	4.91
7.00	1.404	1.454	1.4906	1.59733	5.15	5.13	5.06	5.03
8.00	1.392	1.445	1.551	1.59733	5.21	5.16	5.06	5.08
9.00	1.35	1.382	1.46533	1.58366	5.34	5.31	5.11	5.09

[a]Mean values are presented of three replications

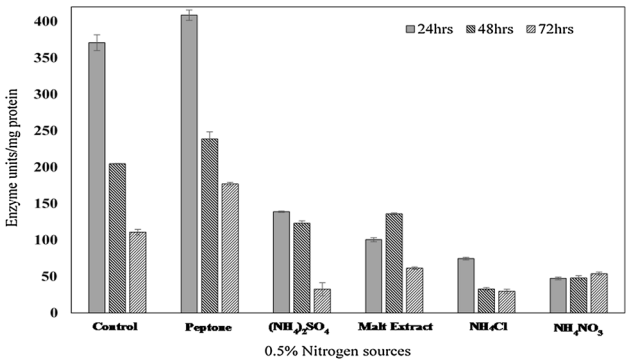

Fig. 6 Effect of different nitrogen sources on enzyme activity of the strain SRWQ1 after 24 h incubation. Minimal salt (MS) medium supplemented with 5.0% of glycerol and 0.5% yeast extract was used. Initial (stating) pH and incubation temperature were 6.0 and 25 °C respectively

throughout the experiments. As shown in Fig. 6, peptone showed the highest enzyme activity compared to that of other nitrogen sources. In the case of peptone, the maximum enzyme activity was obtained 408.69 ± 0.069 U/mg protein at a concentration of 5 g/L peptone after 24 h (Fig. 6).

Production Kinetics of Major Products of the Strain SRWQ1

Combining the results from above optimization experiments, an optimum system for maximum enzyme activity was developed using glycerol as the sole substrate. However, to evaluate glycerol utilization, biomass and value-added bioproduct productions using the efficient strain *S. proteamaculans* SRWQ1, experiment was carried out under aerobic biotransformation process with glycerol as the sole substrate. Biotransformation of glycerol to bacterial metabolic products was conducted at 25 °C in 200 mL flasks containing 50 mL MS-2 medium at initial pH 6.0. Shake flask batch culture was carried out under optimized conditions, and two major bio-products 2,3-BDO and acetoin were revealed. The concentrations of the substrate (glycerol) as well as metabolic products (2,3-BDO and acetoin) as a function of incubation time in a batch culture process of pure glycerol biotransformation by the SRWQ1 are presented in the Fig. 7a. Moreover, cell biomass production and GDH activity were also determined, and results are presented in Fig. 7b. The GDH enzyme activity were gradually decreased after 24 h incubation by increasing incubation time. After 144 h of the incubation, glycerol was totally consumed by SRWQ1 strain with yielding 7.01 g/L of 2,3-BDO and 8.11 g/L of acetoin (Fig. 7a). The maximum product yields of 2,3-BDO obtained from the broth culture in 96 and 120 h incubations were 13.913 ± 1.24 g/L and 18.43 ± 1.55 g/L respectively. Acetoin is another major metabolic product that was detected from the fermentation medium. However, the maximum concentration of acetoin obtained after 144 h of the incubation was 8.38 ± 0.76 g/L using SRQW1 strain.

In addition to our novel strain SRWQ1, the other two strains *S. liquefaciens* SRWQ2 and *Serratia* sp. 243 were also screened for their glycerol assimilation and metabolic products accumulation. These strains SRWQ2 and *Serratia* sp. 243 were grown under the same optimized condition of strain SRWQ1. Comparison the final values of products,

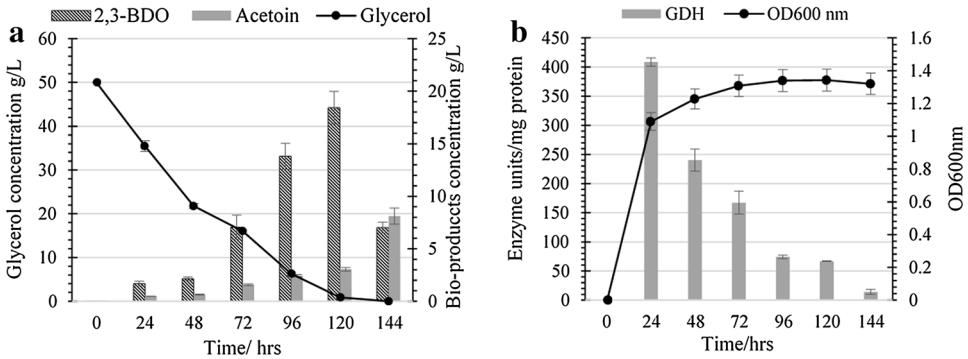

Fig. 7 **a** Different concentration of bioproducts produced by *Serratia proteamaculans* SRWQ1. **b** biomass production and GDH enzyme activity of SRWQ1. The MS-2 medium was used for growth of bacterium, and culture pH and incubation temperature were maintained at 6.0 and 25 °C respectively

Table 2 Comparison the final values of metabolic products, GDH and biomass obtained from metabolized of glycerol by three strains at 25 °C incubation temperature

Strain	Glycerol utilized (%)		Biomass (OD600)		GDH (U/mg protein)		2,3-BDO (g/L)		Acetoin (g/L)	
	96 h	120 h	96 h	120 h	96 h	120 h	96 h	120 h	96 h	120 h
S. *proteamaculans* SRWQ1	87.4	98.14	1.34	1.4	74.3	66.89	13.91	18.43	2.4	3.04
S. liquefaciens SRWQ2	82.32	100.0	1.7	1.8	196.4	140.5	15.4	12.3	3.1	9.0
Serratia sp. 243	74	100.0	1.6	1.8	106.0	88.8	13.8	11.4	2.9	6.6

GDH and biomass obtained from metabolized of glycerol by three strains is presented in Table 2. In case of strain SRWQ2, the maximum product concentrations of 2,3-BDO after 96 and 120 h incubations were 15.4 ± 0.6 g/L and 12.3 ± 0.83 g/L respectively (Table 2). However, for strain *Serratia* sp. 243, the maximum yields of 2,3-BDO obtained from the culture medium after 96 and 120 h incubation were 13.8 ± 0.22 g/L and 11.41 ± 0.5 g/L respectively.

Discussion

Biodiesel is being adopted to replace petroleum products in order to promote an eco-friendly way of harnessing energy [35]. The use of fossil fuel has had a few drawbacks such as having adverse effects on the environment, created a global ecological disturbance and harmful effect on environment in the long run [36]. Nonetheless, biodiesel is an environmental friendly renewable source of energy. With the flourishing of biodiesel industry, global surplus of glycerol is increasing [37]. Consequently, biotransformation of glycerol to value-added products including platform chemicals and liquid fuel or fuel additive from different perspectives through cost-effective processes with a main focus on novel bacterial strain would lead to both environmental and economic dividends of biodiesel plant [38]. Microbial conversion of glycerol to valuable chemicals is a subject of interest in the last few years [35, 38]. There were several reasons of this research, namely bacteria can be easily cultured, can be found inhabiting unlimited environments and can survive in extreme environmental stresses. These attributes lend to the potential exploitation of hardier bacterial strain for the industrial biotransformation of a large volume of glycerol to biofuel and other high-value products.

First of all, our focus was given on isolation and characterization of novel glycerol utilizing microbes, as well as the aerobic conversion of glycerol (a core by-product of biodiesel production). In this research work, our three newly isolated strains SRWQ1, SRWQ2, and *Serratia* sp. 243 isolated from soil were found to have the greatest GDH activity. However, until now, in biotechnological application like production of high-value products from glycerol looking for efficient GDH enzymes producing bacteria, traditional microbiological isolation techniques are still important [38, 39]. The conversion of glycerol to 2,3-BDO is an oxidation process reported in many previous studies [40].

2,3-BDO is a valuable building blocks chemical, and it can be used for the synthesis of 2-3-butanone [41]. The aerobic condition is often employed for 2,3-BDO production in which oxygen can be used as an electron acceptor. In anaerobic condition, the lake of external electron acceptor causes the hampering in microbial growth [37, 38]. Therefore, very little works have been done on the aerobic process [35, 38]. In our study under aerobic condition, both of the high enzyme activity and low pH of the growth culture boosted 2,3-BDO product yield, while low enzyme reaction and higher pH can enhance the accumulate of 1,3-propanediol (1,3-PDO) [42]. There is no 1,3-PDO as we expected to get it from the culture broth based on the literature review [37]. In our study, strain *S. proteamaculans* SRWQ1 with optimal pH 6.0 of the growth culture was enhanced GDH activity, in which the SRWQ1 strain blocked the synthesis of 1,3-PDO and produced a high concentration of 2,3-BDO using glycerol as a sole carbon source under the optimized fermentation condition. The maximum 2,3-BDO product yield 0.4 g/g was achieved by SRWQ1, where 98.2% (49.1 g/L) of glycerol was utilized after 120 h of incubation. This is the first study that showed 2,3-BDO and acetoin productions by *S. proteamaculans* SRWQ1 and until today there is no any report on bioconversion of glycerol by *S. proteamaculans*.

Glycerol has been converted to value-added products by several microorganisms only in cultures controlled at pH 7.00 [43]. Similarly, glycerol bioconversion has been reported by *Serratia* sp. only in a culture controlled by pH 8.00 and incubation temperature 40 °C [44]; however, our new isolate *S. proteamaculans* SRWQ1 able to convert glycerol and produce 2,3-BDO and acetoin at the optimal pH 6.0 and below, and optimum temperature 25 °C. Some microorganisms have developed a strategy to escape progressive of pH decrease by switching over to the production of less toxic products such as alcohol or glycols [45].

In glycerol fermentation, 1,3-PDO is the characteristic bio-product formed through the reduction step of pyruvate pathway [46, 47]. In our research, 1,3 PDO was expected to produce as reported earlier using different microorganisms through glycerol bioconversion [48, 49]. However, our new isolate SRWQ1 was not able to produce 1,3-PDO. The GDH enzyme can produce 1,3-PDO, 2,3-BDO and acetoin, but this does not mean all the bacteria which can produce GDH enzyme are able to produce all these three bio-products [50]. Some of the bacteria could produce a high amount of 2,3-BDO, little 1,3-PDO and quite amount of acetoin. Also, sometimes there is no cell wall pathway to produce 1,3-PDO [51]. According to the preliminary experiment results at slightly acidic pH 6.0, 1,3-PDO formation is reduced in the presence of oxygen, probably because of the oxygen sensitivity of the two enzymes of the propanediol route [52]. Those, it seems possible that, under aerobic conditions and low pH, glycerol is converted to 2,3-butanediol and acetoin only. Moreover, Kim et al. [53] stated the highest yield 0.4 g/g of 2,3-BDO without production of 1,3-PDO by *Raoultella ornithinolytica* B6 strain using a co-substrate casamino acid in addition to glycerol where fed-batch fermentation parameters including pH were controlled by bioreactor. However, we reported 0.4 g/g of 2,3-BDO using a different organism S.

proteamaculans SRWQ1 in a batch flask culture without control of fermentation parameters including pH where glycerol was only the substrate (carbon source). Furthermore, in batch culture, considering that the 2,3-BDO product yield (18.43 g/L and 0.4 g/g glycerol) obtained from glycerol fermentation by S. *proteamaculans* SRWQ1 was much higher than that reported (8.9 g/L and 0.12 g/g glycerol) by Cho et al. [42] using crude glycerol. Also, Ripoll et al. [54] demonstrated 0.3 g/g of 2,3-BDO production in batch culture using pure glycerol as the substrate.

Separation and purification of 2,3-BDO and acetoin from fermented broth may represent one of the major technological challenges for large scale production. Acetoin and 2,3-BDO can be recovered from fermentation broth using aqueous two-phase system (ATPS) proposed by Sun et al. [55] However, for ATPS method, the recovery coefficients of 2,3-BDO and acetoin are 96.4 and 95.5% respectively. Moreover, the precipitation and vacuum distillation process can be used for recovered of 2,3-BDO from the fermentation broth using alcohol [35, 40]. Briefly, the cell-free extract is collected from the culture broth using centrifugation and filtration, concentrated up to 500 g/L of 2,3-BDO through vacuum evaporation at 50 °C and 50 mbar vacuum pressure. The concentrated solution is further treated with alcohols like ethanol, methanol and isopropanol to precipitate organic acids and inorganic salts. At the final step, about 76.2% of 2,3-BD, with 96.1% purity can be recovered from the supernatant by vacuum distillation process.

Conclusion

In this study, we have demonstrated that a novel bacterial strain *S. proteamaculans* SRWQ1 represents a foundation for the exploration of GDH producing microorganisms which might be more efficient in the industrial environment. It showed that *S. proteamaculans* SRWQ1 has a great ability to produce high concentrations of 2,3-BDO and acetoin without producing any co-product at higher glycerol concentrations. Therefore, the newly isolated strain SRWQ1 could be a better organism for aerobic conversion of glycerol to 2,3-BD. Our work would lead to contribute in the development of biodiesel industries, and reduce industrial waste disposal. Moreover, this work can evaluate the efficiency of bioconversion of glycerol to bioproducts by novel bacterial strains under the aerobic condition with potential in future studies for advancement in bioconversion processes.

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