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Bioconversion of crude glycerol to biofuels and value-added bioproducts

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Bioconversion of Crude Glycerol to Biofuels and Value-added Bioproducts

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Summary

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. Biodiesel, a renewable fuel produced by transesterification of animal fats and vegetable oils, generates about 10% (v/v) of crude glycerol as a core by-product. Consequently, the recent booming of biodiesel industry all over the world has generated a large amount of crude glycerol, creating an oversupply problem. The economic feasibility of the biodiesel industry has been crucially affected due to a high volume (by worldwide surplus) of crude glycerol generated from the biodiesel production process. Consequently, with the increasing number of biodiesel production plants, a large number of glycerol production plants can be expected to be shut down within a few years due to the price drop that will result from the oversupply of glycerol. Therefore, this abundance of glycerol provides an opportunity for the development of new commercial uses.

Glycerol, a core by-product of biodiesel production has become an inexpensive and easily obtainable product for which new applications have to be discovered. At present, there is a lack of microorganisms which can efficiently convert crude glycerol to value-added bio-products. The new isolate of bacteria that would permit screening, isolation and over-expression of enzyme would help overcome these challenges. Thus, 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. In Chapter 2 and 3, this study shows a number of bacterial strains isolated from environmental consortia were screened for their capability of converting low or negative-value biodiesel-derived crude glycerol to value-added products. Primarily, an aerobic batch biotransformation process was carried out to observe the kinetics of glycerol dehydrogenase (GDH) activity, bio-product formation and glycerol utilization. Therefore,

the major bio-product obtained from this biotransformation of glycerol was 2,3-butanediol (2,3-BD) with minor co-products including acetoin, 1,3-propanediol (1,3-PDO) and acetate. In this study, three bacterial species *Klebsiella pneumoniae*, *K. variicola* and *Serratia liquefaciens* newly isolated from soil and paper mill waste were the highest producers of 2,3-BD. The novel strains *K. pneumoniae* SRP2 and, *K. variicola* SRP3 were used to construct a co-culture, capable of simultaneously converting crude glycerol to concurrently produce up to 27.87 g/L of 2,3-BD, yielding 0.73g 2,3-BD per gram glycerol (0.73g/g) using 37.0 g/L glycerol under aerobic conditions in batch culture, showing great potential for biotransformation bioprocess.

Therefore, an attempt has been made to produce a major product 2,3-butanediol (2,3-BD) from glycerol as a sole carbon source using newly isolated novel bacterial strains *Klebsiella variicola* SRP3 and *K. pneumoniae* SRP2 in a series of batch and fed-batch processes under aerobic process. These studies also compare the bacterial cell biomass, bio-products and a key enzyme glycerol dehydrogenase (GDH) production of *K. variicola* SRP3 and *K. pneumoniae* SRP2 isolated from paper mill waste when grown in aerobic condition. The incubation temperature, pH, glycerol concentration and nitrogen sources were the most important factors ruling the GDH. This study also revealed that an increased GDH activity led to a substantially enhanced production of 2,3-BD as a principal product with 1,3-propanediol (1,3-PDO), acetoin and acetate as minor.

In Chapter 4-6, the studies of high production of 2,3-butanediol (2,3-BD) from pure and biodiesel derived crude glycerol using an ethyl methanesulfonate (EMS) mutant *K. pneumoniae* SRM2, and two adapted mutants *K. variicola* SRM3 and *K. variicola* SW3 developed from the newly isolated wild type strains *K. pneumoniae* SRP2 and *K. variicola* SRM2 respectively are reported. However, as stated in Chapter 4, an adapted mutant strain *K. variicola* SRM3 withstanding 200 g/L glycerol could efficiently convert glycerol to 29.87 g/L 2,3-BD and 7.08 g/L

acetoin from 50.0 g/L glycerol in a batch culture, and an acidic initial pH (pH 5.0) led to enhanced 1.3-fold increased GDH activity of 721.5 units/mg protein from 558.2 units/mg protein. The optimal conditions for maximal GDH enzyme activity were defined, and total 0.79 g/g product yield was attained by the muted strain *K. variicola* SRM3, which is the highest amount obtained from glycerol as a sole carbon source until now. Furthermore, another mutant *K. variicola* SW3 developed from wild type strain *K. variicola* SRP3 withstanding 200 g/L biodiesel-derived raw glycerol able to tolerate growth inhibitory agents like methanol, soap, catalyst and other non-glycerol organic matters present in crude glycerol, capable of efficiently converting biodiesel-derived crude glycerol to 2,3-BD under aerobic process where crude glycerol was only the substrate or sole carbon source. As shown in Chapter 5, the SW3 strain displayed a high yield of 2,3-BD production up to 82.5 g/L, yielding 0.62 g/g using glycerol as the sole carbon source in a fed-batch culture. Moreover, in a batch culture, a final 33.5 g/L (0.67 g/g) of 2,3-BD was accumulated within 96h from 50 g/L glycerol. This evolutionary adapted mutant strain SW3 exhibited the minimum inhibitory concentration of glycerol as high as 325.0 g/L. Real-time qPCR and glycerol dehydrogenase (GDH) enzyme activity assay revealed that the overexpression of GDH gene resulted in an increased GDH enzyme activity, led to a markedly boosted 2,3-butanediol (2,3-BD) production. However, for metabolic study of *K. variicola* SW3, eight important genes (dhaD, gldA, dhaB, dhaK, glpK, budB, budC and pdhC) which are involved in glycerol metabolisms were identified and studied for their gene expression levels. For this expression study, both wild type (SRP3) and mutant (SW3) strains were used. The 16S rRNA gene, obtained based on the primers 5′-GCGGTTGTTACAGTCAGATG-3′ and 5′-GCCTCAGCGTCAGTATCG-3′ was used as an internal standard. Glycerol boosted the expressions significantly of the genes dhaD (glycerol dehydrogenase), budB (acetolactate

synthatase and budC (acetoin reductase) over control. The mutant strain (SW3) exhibited higher expression levels compared to that of wild type strain. Until now, a high production (82.5 g/L) of 2,3-BD reported in this study using glycerol as the sole carbon source is the highest amount obtained from biotransformation process. Therefore, the research has for the first time proved that this *K. variicola* species can efficiently convert glycerol to produce 2,3-BD with minor co-products, and it is also the first report on biosynthesis gene expression study of glycerol metabolisms for metabolic engineering. These expression studies could be used for metabolic engineering to improve the metabolic product yield. However, to the best of my knowledge, the product yield of 0.63 g/g of 2,3-BD obtained from crude glycerol is the highest point on biotransformation of biodiesel-derived crude glycerol until now.

However, as shown in Chapter 6, *K. pneumoniae* SRM2, an EMS mutant strain developed from newly isolated *K. pneumoniae* SRP2 withstanding a high glycerol concentration (220 g/L of medium) could rapidly convert glycerol aerobically to 2,3-BD. The mutated strain SRM2 exhibited higher GDH activity compared to that of its wild type strain (500.08 vs. 638.6 μmol/min/ mg protein), yielding 32.3 g/L and 77.5 g/ L of 2,3-BD in batch and fed-batch process respectively, where glycerol was the sole carbon source or only the substrate. Also, 0.59 g/g and 0.66 g/g product yields of 2,3-BD were obtained using pure and crude glycerol respectively by the mutated strain *K. pneumoniae* SRM2. Thus, this research paper reported a role of GDH involved in oxidative pathway of glycerol metabolism of 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.

In chapter 7, a novel expression system was developed in order to improve 2,3-butanediol (2,3-BD) production by metabolic engineering of *Escherichia coli*. The dhaD gene encoding

glycerol dehydrogenase (GDH) enzyme from *Klebsiella pneumoniae* SRP2 was expressed in *E. coli* BL21(DE3)pLys under the control of T7lac promoter. Over-expression of the gene dhaD confirmed that the 2,3-BD synthesis pathway gene was expressed on the protein level. The enzyme GDH was over-expressed (38.9-fold), and the recombinant *E. coli* showed a nearly 84.0-fold increased expression of dhaD gene, and an increase of 2.4-fold in yield of 2,3-BD with respect to the non-recombinant strain. From these results, the first reported expression system of dhaD gene has paved the way for improvement of 2,3-BD production and will be available and efficient for other heterologous gene expression in *E. coli* as well as *K. pneumoniae* or other bacterial strain.

Therefore, this bioconversion of crude glycerol to 2,3-BD –a value-added green product with potential industrial applications as a liquid fuel or fuel additive would represent a remarkable alternative to add value to the biodiesel production and thus help in the development of biodiesel industries.

Keywords

Glycerol, Crude glycerol, 2,3-Butanediol, Acetoin, Bioconversion, Biotransformation, Co-culture, Bacterial consortium, Klebsiella pneumoniae, K. variicola, Biosynthesis genes, E. coli, Glycerol dehydrogenase, Mutagenesis, EMS mutant, Value-added products.

List of Abbreviations

2,3-BD – 2,3-butanediol

BLAST - Basic Local Alignment Search Tool

DHA - Dihydroxyacetone

DNA – Deoxyribonucleic acid

DHAP – Dihydroxyacetone phosphate

GDH – Glycerol dehydrogenase

GC-MS – Gas chromatography linked to mass spectroscopy

HPLC – High performance liquid chromatography

LB – Luria Bertani

MONG – Matter organic nonglycerol

MS – Minimal salt

NCBI - National Center for Biotechnology Information

NAD – Nicotinamide adenine dinucleotide

NADH - nicotinamide adenine dinucleotide-Hydrogen

rDNA – Ribosomal deoxyribonucleic acid

PCR - Polymerase Chain Reaction

RPM/rpm – Revolutions per minute

rRNA – Ribosomal ribonucleic acid

16S - Small subunit of a prokaryotic ribosome

1,3-PDO – 1,3-propanediol

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Finally, it is with much thankfulness and sincerity I thank my wife Leema Farha and my son Iram Rahman for encouraging me throughout the many adventure of my PhD. I also wish to thank my brothers, sisters, grand parents, friends and all well wishers for encouraging me.

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Chapter 1: Background Information and Literature Review

1.1. Background Information

In 1779, Carl W. Scheele, a Swedish chemist discovered a new transparent, syrupy liquid by heating olive oil and litharge. Now it is known as glycerol (propane-1, 2, 3-triol). Studies later showed glycerol to be a principal component of all fats and oils, in the form of esters called glycerides. However, the discovery of glycerol had no further impact up to 1866, till the production of dynamite. At the end of the nineteenth century due to the booming of the processing of natural oils and fats, the production of glycerol increased fast [1]. Today glycerol is found in market in two forms, synthetic glycerol and natural glycerol. Synthetic glycerol is produced by the chemical conversion of propylene and constitutes around 10 % of the total glycerol market.

In recent years, there has been a rapid increase in demand for fuels and chemicals, and at the same time there are concerns over global warming and limited availability of fossil resources. These have put scientist in tremendous pressure to explore alternative sources of energy and chemicals. Biofuels such as biodiesel are promising and environmentally safe alternatives to fossil fuels, which have attracted increasing interest from academia and industry.

Biodiesel has become one of the vital renewable fuels with potential to replace fossil fuels. It is produced from animal fats and vegetable oils by reacting with a primary alcohol in the presence of a catalyst [2]. During the last few years, the production of biodiesel has increased dramatically with EU as the biggest biodiesel producer in the world. In 2005, EU countries produced about 3.2 million tons of biodiesel, followed by the USA producing approximately 0.25 million tons of biodiesel [3]. The global biodiesel productions are presented in Figure 1.1. However, a typical biodiesel production process produces a significant amount of crude glycerol as the core by-product, approximately 10% of the biodiesel produced. Due to its low price, glycerol is now

considered as a waste instead of a useful product. The high volume of glycerol generated from biodiesel industries has become an environmental problem since it cannot be safely disposed in the environment.

Specifically, crude glycerol contains impurities which can negatively influence the bioconversion processes. Thus, the crude glycerol is considered a hazardous waste to the environment. Therefore, conversion (chemical or biological) of crude glycerol into value added products would lead to both environmental and economic dividends. Presently, increasing efforts around the globe have been focused on the development of novel and efficient bioconversion processes of glycerol into value-added products. Glycerol can be used as carbon and energy sources for microbial growth to produce valuable chemicals. Therefore, my research work attempts to produce valuable chemicals from the bioconversion of glycerol.

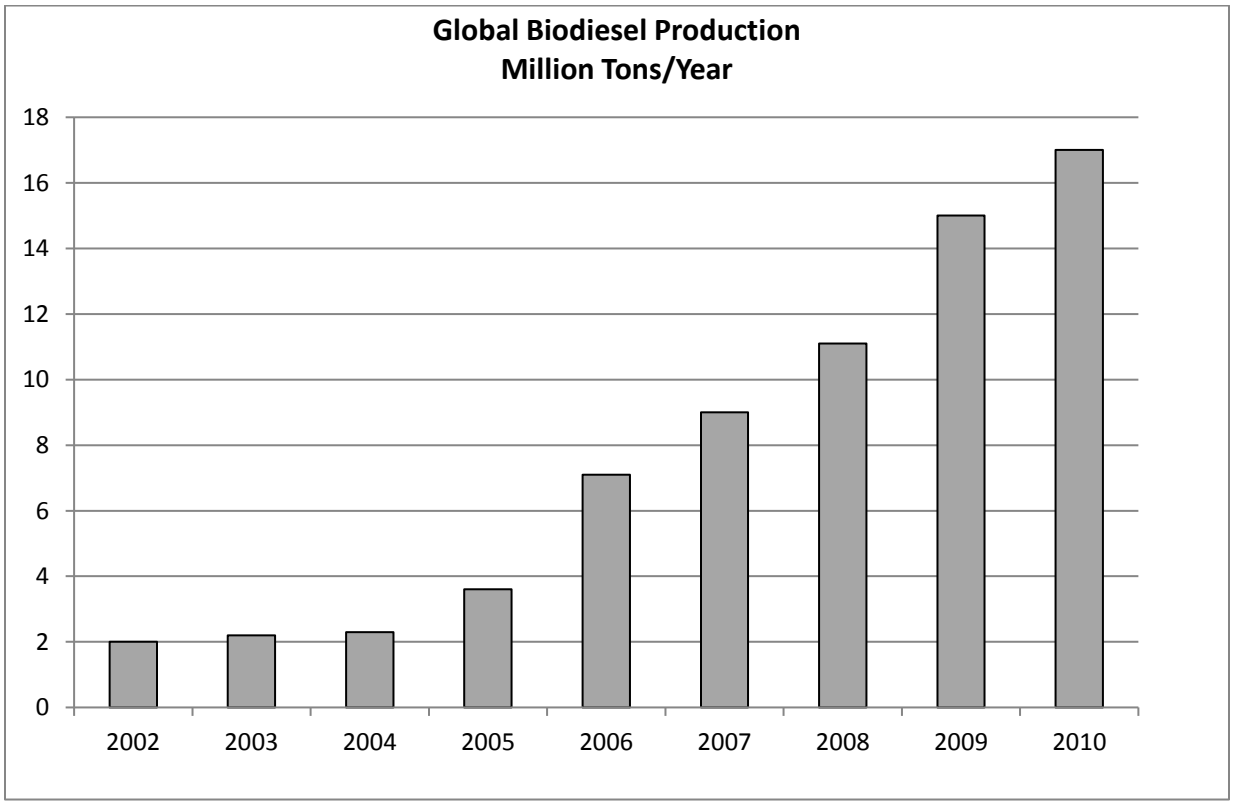

Fig 1.1. Worldwide biodiesel production [4].

1.1.1. Sources of Glycerol

Natural glycerol is produced as a by-product in oleo chemical industry mainly for biodiesel production. Animal fats and vegetable oils are natural sources of glycerol esters (glycerides). Synthetically, glycerol is derived using petroleum as a feedstock. The mainstream of the glycerol available in the market is from purification of crude glycerol. The crude glycerol is produced from fats and oils by three different processes. However, naturally it is derived by two methods such as hydrolysis in soap and fatty acid production process, and transesterification in biodiesel production process. In transesterification process about 10% crude glycerol is obtained as a co-product of biodiesel. The world scenario of crude glycerol (in million tons) is presented in Figure 1.2 [4].

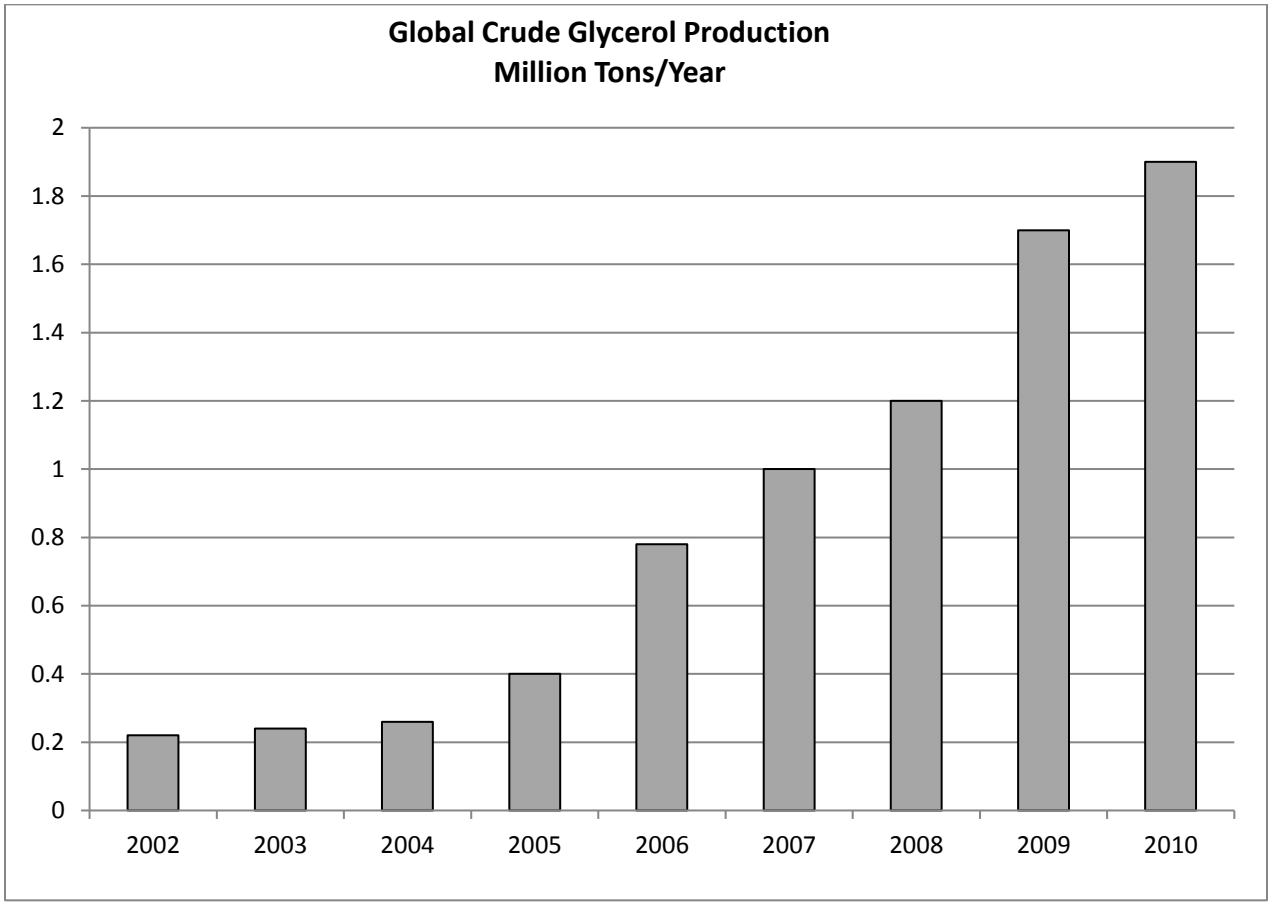

Fig 1. 2. Worldwide biodiesel-derived crude glycerol production [4].

1.1.2. Crude Glycerol

The component of crude glycerol varies widely, but it is generally 85% glycerin (range from 40%-90%), 10% water (range from 8% to 50%), 4% salt (range 0% to 10%), less than 0.5% methanol, and around 0.5% free fatty acids [9]. Glycerin is generally sold as 99.5% and 99.7% pure. Crude glycerol is purified to make three different grades. The refined grades of glycerin are:

● Technical grade – used as a building block in chemicals, not used for food or drug formulation

● United States Pharmacopeia (USP) – glycerin from animal fat or plant oil sources, suitable for food products, pharmaceuticals

● Kosher – glycerin from plant oil sources, suitable for use in kosher foods

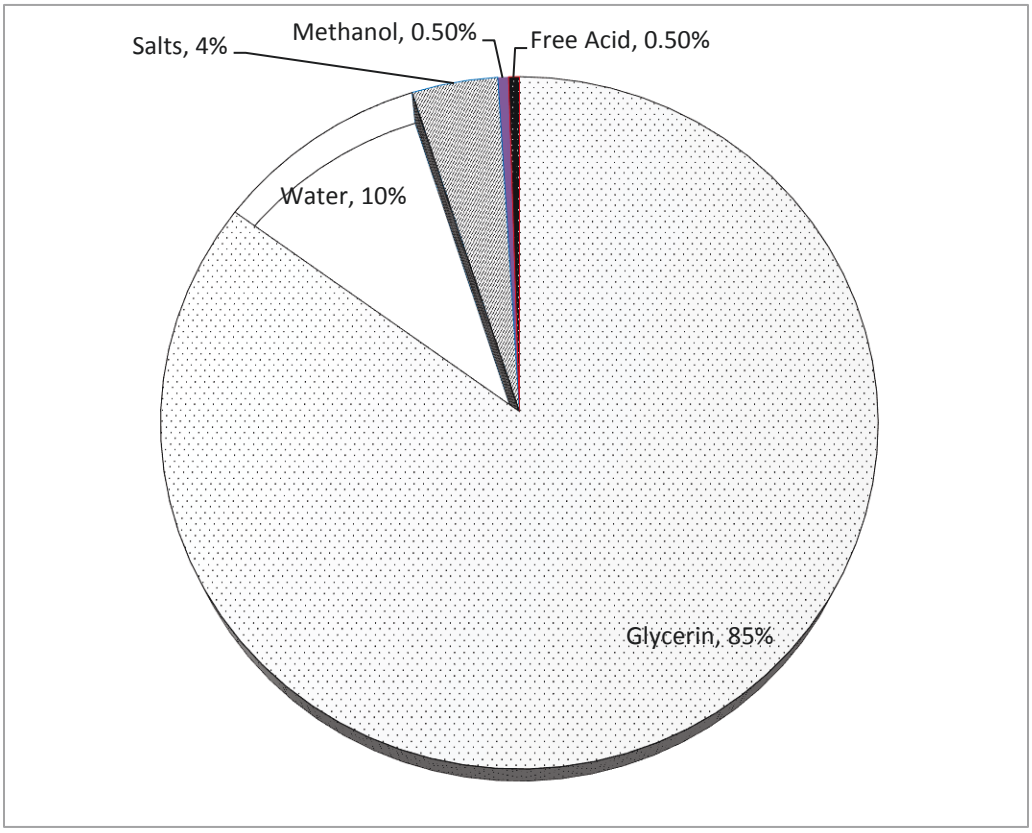

Fig 1.3. Contents of Crude Glycerol (Source: US Soybean Export Council Inc.: Glycerin Market Analysis) [5].

1.1.3. Glycerol Properties

Glycerol, aka, glycerine or glycerin, is a three-carbon sugar alcohol with a molecular formula of $C_3H_8O_3$ and an IUPAC name of propane-1,2,3-triol. It is an odorless, colorless, nontoxic and sweet tasting hygroscopic liquid. It is completely soluble in water, alcohols and hydrocarbons, and only slightly soluble in many common solvents like ether and dioxane. The common properties of glycerol are presented in Table 1.1[6]:

Table 1.1. Properties of Glycerol

Parameters	**Property**
Chemical formula	$C_3H_8O_3$
Molar mass	92.09 g mol^{-1}
Relative density	1.261 g/cm^3
Freezing point	- 46.5°C
Melting point	17.8 °C (64 °F)
Boiling point	290 °C (554 °F)
Surface tension	63.4 mN/m
Auto-flammability	393°C
Flash point	160^0C (177^0C in open cup)
Viscosity	1410 mpas at 20^0C
Electrical Conductivity (20 °C)	0.1 μS·cm -1
Thermal conductivity (0 °C)	0.000691 Cal cm deg /sec
Auto ignition point	429 °C

1.1.4. Uses of Glycerol

Glycerol plays an important role in nearly every industry. Pure glycerol is widely used in medical, pharmaceuticals, cosmetics, paint, pulp and paper, leather, textile, food, tobacco and automotive industries [8] It is also used as a personal health care product for improving smoothness, providing lubrication and moisturizing the skin. Moreover, glycerol is an important ingredient of cough syrups, expectorants, toothpaste, mouthwashes, shaving cream and soaps [4]. One of the important uses of glycerol is suppository into the rectum as a laxative. As well, it is an important moistening agent; glycerol is widely used in the food industry for baked goods. It is also used in candies and icings to prevent crystallization. Glycerol is a major starting material for nitroglycerine, which is used in the manufacture of pain-relieving drugs for heart patients. For humectant properties, glycerol is sprayed on pre-processed tobacco to prevent crumbling. However, glycerol can causes of side effects including headaches, dizziness, bloating, nausea, vomiting, thirst, and diarrhoea.

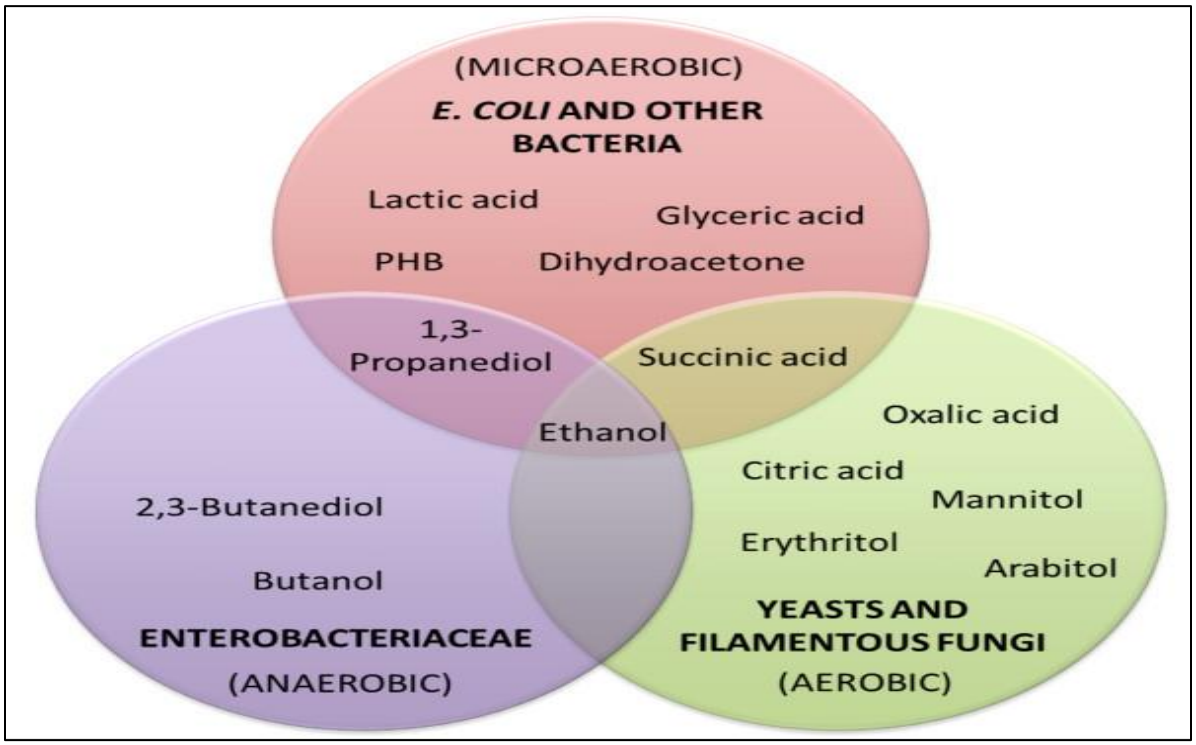

Fig 1.4. Products from aerobic, microaerophilic and anaerobic fermentation of glycerol [4].

1.1.5. Significant of this project

Biodiesel, one of the promising alternative and renewable fuels, has been observed with increasing interest and its production capacity has been well developed in recent years. Biodiesel production will generate about 10% crude glycerol as the main by-product. For every 9 kg of biodiesel produced, about 1 kg of a crude glycerol by-product is formed [7]. It was projected that the world biodiesel market would reach 37 billion gallons by 2016, which implied that approximately 4 billion gallons of crude glycerol would be produced [8]. The usage of low-grade quality of glycerol obtained from biodiesel production is a big challenge as this glycerol cannot be used for direct food and cosmetic uses. Thus, too much glycerol waste produced from biorefineries is a global oversupply crisis due to lack of refining capacity. An effective usage or conversion of crude glycerol to specific products will cut down the biodiesel production costs. Therefore, development of sustainable process for utilizing this organic raw material is imperative. Bioconversion of a large amount of biodiesel glycerol waste would directly benefit the environment by obtaining renewable products, encouraging the use of biodiesel and reducing fossil fuel use. Bioconversion processes of crude glycerol waste by microorganisms represent a remarkable alternative to add value to the biodiesel production helping biorefineries development. However, to establish more biodiesel industries and to make biodiesel production more economical, expedient bioconversion processes and more efficient microbial strains should be developed. Glycerol could be used as a useful carbon and energy source in industrial fermentation process with many possible applications. Most of the research work has been done on the production of small alcohols from glycerol feed stock and no systematic work has been done. Under this backdrop my research project aims to cover possible conversion of glycerol into useful bioproducts.

To address the aforesaid shortcoming of the conversion of glycerol to different value-added products, the research work of this PhD thesis project divided in to five parts:

i. **Screening of novel bacterial and fungal strains for their glycerol utilization.**
ii. **Characterization (identification) of novel strains which showed promising activity**
iii. **Screening and optimization of their enzyme activity**
iv. **Development of the active strain by mutagenesis/metabolic engineered to improve product yields of value-added products.**
v. **High production of value-added bio-products**

1.1.6. Significance of the Project

The effective utilization of glycerol by-product obtained from biodiesel production is globally important for commercialization and improvement of biodiesel production. The renewable value-added products derived from glycerol will not only shrink our dependence on non-renewable products, but will also enforce the development of biorefinery. Thus, bioconversion processes of crude glycerol waste by microorganisms have been imposed on a new dimension for biorefinery. The conversion of abundant and low-priced glycerol co-product of biodiesel biorefinery into higher priced products denotes a promising way to attain economic sustainability in biofuel industries. This project mainly focused on bioconversion of low value glycerol or glycerol waste to high value bioproducts. The successful completion of this project may result in the development of an economical and energy efficient technology for continuous production of biofuels; fine chemicals as an important building block of other valuable chemicals, and, high-value bioproducts. The successful completion of the project would greatly benefit the biodiesel industry for sustainability and strengthen the bio-economy of Canada.

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1.2. Literature Review

Value-added Utilization of Crude Glycerol from Biodiesel Production

Published Book Chapter: Advances in Enzymatic and microbial Conversion of Biomass to Biofuels, Future Science Group, Chapter-9, 2015.

Authors: M. Shafiqur Rahman, Chunbao (Charles) Xu, Zi-Hua Jiang, Wensheng Qin

1. Introduction:

1.1. Background Information

In recent years, there has been a rapid increase in demand for fuels and chemicals, and at the same time there are concerns over global warming and limited availability of fossil resources. These have put scientists in tremendous pressure to explore alternative sources for energy and chemicals. Biofuels such as biodiesel are promising and environmentally safe alternative to fossil fuels, which attracted increasing interest from academia and industry.

Biodiesel has become one of the vital renewable fuels with potential to replace fossil fuels. It is produced from animal fats and vegetable oils by reacting with a primary alcohol in the presence of a catalyst [1]. During the last few years, the production of biodiesel has increased dramatically, and the European Union (EU) is the biggest biodiesel producer in the world. In 2011, EU countries produced 178.15 thousand barrels/day of biodiesel, followed by the North America which produced 65.91 thousand barrels/day of biodiesel [2]. A typical biodiesel production process, however, produces a significant amount of crude glycerol as a core by-product, approximately 10% of the biodiesel produced. Due to its low price, glycerol is now considered as a waste instead of a useful product. The high volume of glycerol generated from biodiesel industry has become an environmental problem since it cannot be safely disposed in the environment. Crude glycerol contains methanol, salts, soaps, non-glycerol organic matter, and catalysts as the main

impurities which can negatively influence the bioconversion processes. Thus, the crude glycerol is considered as an organic waste that might create eutrophication problems to the environment. Due to oversupply of crude glycerol from biodiesel refinery and high cost of purification (where as pure glycerol being very low in cost), negative influence is being impacted on purification of crude glycerol. If all biodiesel producers were to invest in making pharma-grade, the present high price of pharma-grade glycerin would collapse. Therefore, conversion (chemical or biological) of crude glycerol into value added products would lead to both environmental and economic dividends. Presently, increasing efforts around the globe have been focused on the development of novel and efficient bioconversion processes of glycerol into value-added products. Glycerol can be used as carbon and energy sources for microbial growth to produce valuable chemicals. This chapter attempts to summarize the current state of the research on various aspects of glycerol metabolism.

1.2. Glycerol Chemistry and Uses

Glycerol, aka, glycerine or glycerin, is a three-carbon sugar alcohol with a molecular formula of $C_3H_8O_3$ and an IUPAC name of propane-1,2,3-triol. It is an odorless, colorless, nontoxic and sweet tasting hygroscopic liquid readily soluble in water. Its molar mass is 92.09 g mol^{-1}, relative density 1.261 g/cm^3, melting point 17.8 °C (64 °F), boiling point 290°C (554 °F), surface tension 63.4 mN/m, auto-flammability 393°C, flash point 177^0C (open cup) and viscosity 1410 mpas at 20^0C [3]. Animal and vegetable fats and vegetable oils are the natural sources of glycerol esters (glycerides). Pure glycerol is widely used in medical, pharmaceuticals, cosmetics, paint, pulp and paper, leather, textile, food, tobacco and automotive industries. It is also used as personal health care product for improving smoothness, providing lubrication and moisturizing the skin. Glycerol is an important ingredient of cough syrups, expectorants, toothpaste, mouthwashes,

shaving cream and soaps. One of the important uses of glycerol is suppository into the rectum as a laxative. Glycerol can cause side effects including headaches, dizziness, bloating, nausea, vomiting, thirst, and diarrhoea.

2. Biological Methods for Conversion

Bioconversion of glycerol into biofuel or other value added bioproducts can play a significant role for the development of biodiesel biorefinery industry. Glycerol can be used as a promising and abundant carbon source by the biotechnological production process. Anaerobically many microorganisms are able to utilize glycerol as a sole carbon and energy source, and the use of these microorganisms has increased attention for the bioconversion of glycerol [1]. Although anaerobic condition lacks external electron acceptor hampering microbial growth, some engineered strains of bacteria and fungi can utilize glycerol to produce 1,2-propanediol (1,2-PDO), 1,3-propanediol (1,3-PDO), H_2 and other higher value molecules without external electron acceptor [4,5]. In 2005, Gonzale-Pajuelo *et al*. [6] explored that some biotechnologically important organisms could not naturally ferment glycerol. However, these engineered strains constructed by importing genes from natural glycerol fermenting organisms could ferment; for example, the 1,3-PDO producing gene from the pathway of *Clostridium butyricum* was introduced into *Clostridium acetobutylicum* resulting in efficient glycerol fermentation and 1,3-PDO synthesis [6]. To increase productivity of high value chemicals by the microbial conversion of glycerol, extensive genetic manipulation and metabolic engineering should be imposed on these biotechnologically important organisms.

3. Glycerol Uptake and Catabolism

An earlier report indicated that the glycerol transport facilitated its diffusion across the *Escherichia coli* cytoplasmic membrane [7]. In this pathway, glycerol facilitator and glycerol

kinase are the two proteins which are principally involved in the entry of external glycerol into cellular metabolism of bacterial cell. The glycerol facilitator acts as a carrier, whereas the kinase traps the glycerol inside the cell as glycerol-3-phosphate. Therefore, the kinase activity is inspired by an interaction between the glycerol facilitator protein and glycerol kinase. In the first step, glycerol passes the biological membrane through energy independent passive diffusion process which is catalysed by glycerol facilitator, a membrane protein [7]. After entering into a bacterial cell, intracellular glycerol is then converted to glycerol-3-phosphate by glycerol kinase enzyme. Glycerol facilitator operon is the inducer in many bacteria. Nevertheless, glycerol-3-phosphate is not a substrate for the glycerol facilitator, and it remains trapped in the cell until it is further metabolized. However, glycerol uptake in most fungi occurs by active transport which requires energy to operate. Several yeasts and other fungi can take up glycerol by proton symporters. In *saccharomyces cerevisiae*, active transport is mediated by permeases, which are highly specific membrane proteins [8].

Glycerol conversion to 1,3-PDO under anaerobic conditions by Enterobacteriaceae takes two pathways. These are oxidative pathway and reductive pathway. In the oxidative pathway glycerol is dehydrogenated by an NAD^+-dependent glycerol dehydrogenase to dihydroxyacetone (DHA), which is then phosphorylated by phosphoenolpyruvate and ATP-dependent DHA kinase. On the other hand, glycerol is dehydrated by the coenzyme B_{12}-dependent glycerol dehydratase to 3-hydroxypropionaldehyde in the parallel reductive pathway. With the help of the NADH-dependent 1,3-PDO dehydrogenase, this is then reduced to the major product 1,3-PDO, which leads to regenerating NAD^+ [9].

4. Value-added Products from Glycerol

Bioconversion of glycerol waste is initiated by microorganisms such as bacteria and fungi which are able to utilize glycerol as a carbon source. In biotechnological process, biodiesel waste

could be used to produce high value chemicals to avoid waste disposal and increase the profit of biorefineries. Throughout this section, biotechnological production processes as well as the economic value of microbial metabolites from glycerol are evaluated.

4.1. Alcohols

4.1.1. 1,3-Propanediol

A simple organic chemical 1,3-propanediol (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-propanediol, 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 [10]. The precursor 1,3-PDO of PTT is produced through chemical synthesis and fermentatively from glucose by microbes. Nowadays, many researchers are trying to develop more competent technology for the production of 1,3-PDO by more cost-effective glycerol fermentation technique. Thus, the anaerobic fermentation is the most promising option for bioconversion of glycerol by *Klebsiella*, *Citrobacter*, *Clostridium, Lactobacillus* and *Bacillus* [11]. The organisms best known to produce 1,3-PDO are *Clostridium butyricum, C. pasteurianum* and *Klebsiella pneumonia.* Some approaches have achieved 1,3-PDO levels close to 100g/L by adopting advantage of both genetic and bioprocess strategies [12]. In the recent report on pilot scale production of 1,3-PDO, crude glycerol from Jatropha biodiesel was converted by using *K. pneumonia* ATCC 15380 to yield 56 g/L of 1,3-PDO, with a purity and recovery of 99.7% and 34%, respectively [13]. Also, several strains of *C. butyricum* and an engineered strain of *E. coli* could convert glycerol to 1,3-PDO [14].

4.1.2. Butanol

Only the bacterial species *Clostridium pasteurianum* has the natural ability to use glycerol as a sole carbon and energy source for bioconversion into butanol. Butanol is a high value chemical which is widely used in industrial applications. Many valuable chemicals like acrylates, glycol ethers and butyl acetate can be obtained from the industrially converted butanol, which are utilized in the formulation of paints, lacquers and resin. As an alternative biofuel, butanol could be mixed with standard oil based fuels directly. Butanol has greater advantage over the commonly used ethanol, because it has lower enthalpy of vaporization, lower solubility in water, less corrosiveness, and a much higher energy density. As a result, without making any kind of structural modification of the engine, it is possible to replace 100% fossil fuels. Likewise, using existing infrastructure, butanol can also be blended directly at the refinery for delivery [15]. *Clostridium pasteurianum* is the only microorganism that can convert glycerol directly into butanol through anaerobic fermentation process, which is the current attraction to produce high-energy and high value biofuel. Recently, it was observed that a mutant strain *C. pasteurianum* MNO6 developed by chemical mutagenesis using ethane methyl sulfonate (EMS) is capable of utilizing an average of 2.49 g/L/h to a maximum of 4.08 g/L/h glycerol and a total of 111 g/L of crude glycerol, and the maximum production of butanol was 12.6 g/L with productivity 1.8 g/L/h at the maximum glycerol utilization rate [16]. Due to slow growth rate of this organism and requiring long time for fermentation, immediate attention should be given to construct super strain or to look for new microorganism for increasing the production rate. It remains an interesting and challenging research topic to engineer new microorganism for the production of high value butanol from the low value and fermentable glycerol.

4.1.3. 2,3-Butanediol

The 2,3-butanediol (BDO) is widely used as an antifreeze chemical and lubricant. 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 [17]. It has been observed that *K. pneumoniae* G31 strain is able to produce 2,3-BDO as a major product of glycerol fermentation under certain cultivation conditions. The maximum 2,3-BDO concentration obtained was 70 g/L, with a maximum yield and productivity of 0.39 g/g glycerol and 0.47 g/L/h, respectively, in a batch culture under aerobic condition [17].

4.1.4. Ethanol

Ethanol is a biofuel and widely used as a solvent and chemical intermediate. It is mainly produced from sugarcane sucrose, corn starch and lignocellulosic feedstock through the fermentation of yeast. Recently, more attention has been paid to the production of ethanol from bioconversion of glycerol. Many bacterial species of the Enterobacteriaceae, anaerobic *Clostridium* and yeasts are able to produce bioethanol by the fermentation of glycerol [5,6]. However, *Escherichia coli* can utilize glycerol both aerobically and anaerobically. It was demonstrated that *E. coli* SY4 can convert glycerol to ethanol in a batch culture, and the total yield was 85%, productivity 0.15 g/L/h and product concentration 7.8 g/L [18]. The bacterium *Enterobacter aerogenes* is also capable of bioconversion of glycerol to ethanol. It has been investigated that the strain *E. aerogenes* HU-101 exhibited high yields and high production rate of ethanol. The maximum production yield of ethanol was 0.85 mol/mol glycerol [6]. Moreover, a mutant strain *K. pneumoniae* GEM167 greatly enhanced ethanol production by the utilization of glycerol to a maximum level of 25.0 g/L [19].

4.2. Hydrogen

Hydrogen (H_2) is a potential next generation renewable fuel with low molecular weight. It has exceptional environmental capability. H_2 is highly flammable and the cleanest fuel that turns into water when it is burned. In chemical processes and reactions, H_2 is most commonly used and it is an essential part. Moreover, H_2 can be used to fuel cells to produce electricity and heat without emission of CO_2 [5]. Therefore, more and more attention has been paid to the production of H_2 from glycerol bioconversion [4,5]. It has been proved that many bacterial strains like *Enterobacter aerogenes* HU-101 and *Clostridium pasteurianum* DSMZ 525 are capable of biological production of H_2 from glycerol [4,5]. Recent report displayed that the strain *E. aerogenes* HU-101 exhibited high yield and high production rate of H_2. The maximum rate of H_2 production was 80 mmol/L/h in a continuous culture with a packed-bed reactor using self-immobilized cells [5]. The biological production of H_2 can be one of the promising future fuel solutions.

4.3. Dihydroxyacetone (DHA)

A simple carbohydrate dihydroxyacetone (DHA) produced from glycerol fermentation is primarily used as an ingredient in sunless tanning skincare products and works as a valuable building block for the synthesis of many fine chemicals [20]. Chemical synthesis of DHA is very expensive due to laborious safety requirements. Thus, recent attention has been set for the production of DHA economically by using the microbial process. Very few studies have been done on microbial production of DHA through oxidative fermentation by *Gluconobacter oxydans* via a membrane-bound glycerol dehydrogenase [20]. However, both substrate and product have an inhibitory effect on microbial DHA synthesis. To overcome this problem, the strain *G. oxydans* has been developed by mutagenesis, and at the same time the medium was optimized for increasing production rate of DHA even at a higher concentration of glycerol. As a result, the developed *G.*

oxydans ZJB09112 strain significantly enhanced glycerol conversion to DHA by optimization of medium and fermentation condition, and the maximum rate of DHA production was 165.5 g/L with a corresponding productivity of 2.3 g/L/h in a feed batch process [21].

4.4. Biogas

Biogas is a clean and renewable energy, because the renewable carbon comes from plant sources. Anaerobic digestion of animal waste is a biological process that converts organic matter into biogas like methane (CH_4) by microorganisms. Biogas production from agricultural biomass offers environmental benefits and is an additional revenue source for farmers. The utilization of large quantities of crude glycerol produced from biodiesel industries could lead as a boost for biogas production. Biogas plants have been consuming crude glycerol for increasing CH_4 production. In last few years, much attention has been attained on the improvement of digester biogas production and for improving methane yields in co-digestion system, especially in Denmark [22]. It has been proved that glycerol addition can boost biogas yields [22]. Recent report explored that the reactor treating the sewage sludge produced 1106 ± 36 ml CH_4/d before the addition of glycerol and 2353 ± 94 ml CH_4/d after the addition of glycerol 1% (v/v) in the feed [22]. They also showed that the extra glycerol added to the feed did not have a negative effect on reactor performance, but seemed to increase the active biomass (volatile solids) concentration in the system.

4.5. Other chemicals

Most of the work on the productivity of chemicals and biofuels from glycerol fermentation by microorganisms have been focused on the production of 1,3-PDO, DHA, ethanol and butanol. There are many other valuable chemicals such as citric acid, succinic acid, lactic acid, oxalic acid, propionic acid, glyceric acid, mannitol, arabitol, biosurfactants, phytase, erythritol and 3-

Hydroxypropanoic acid that can be achieved by the glycerol fermentation. The production of these chemicals through bioconversion of glycerol has also been reported briefly. Succinic acid is a dicarboxylic organic acid that is largely used in the production of various pharmaceutical products including vitamins, amino acids and antibiotics. It is an important intermediate chemical used to produce tetrahydrofuran, γ-butyrolactone, 1,4-diaminobutane, 1,4-butanediol, and many other important chemicals [23]. In 2010, Yuzbashev *et al.* [23] reported that the recombinant yeast strain *Yarrowia lipolytica* Y-3314 exhibited maximum production of succinic acid at 45.5 g/L and a yield of 0.45 g per gram of glycerol. Citric acid is a weak organic acid used as an additive and preservative of foods and candies. It is commercially produced through fermentation of molasses by the fungus *Aspergillus niger*. Because of the increase of global demand for citric acid, its production from glycerol as a feed stock is also of great interest. The strain *Yarrowia lipolytica* A-101-1.22 showed high level of citric acid production (124.2 g/L) with a yield of 0.77 g/g and a productivity of 0.85 g/L/h during batch cultivation in the medium with glycerol-containing waste of biodiesel industry [24]. Lactic acid is widely used in food, cosmetic and pharmaceutical industries. It is produced from glycerol through chemical synthesis.

Therefore, more attention has recently been paid to the fermentation of glycerol to lactic acid since the process is more cost effective and the yield is higher. Several microorganisms including *Klebsiella, Clostridia*, and *Lactobacillus* enable to produce lactic acid by bioconversion of glycerol. Hong *et. al.* (2009) [25] reported that *E. coli* AC-521 could produce lactic acid in high amounts up to 85.8 g/L, and the total yield and productivity were 0.9 mol/mol and 0.49 g/L/h, respectively. Oxalic acid is another important organic acid used in dying process, bleaching, baking powder, paper and ceramic manufacturing. A recent report explored that *Aspergillus niger* could produce oxalic acid by using glycerol as a feed stock, and the maximum production was

recorded as 48.9 g/L with yield of 0.88 g/g [26]. An industrially important chemical, 3-Hydroxypropanoic acid (3-HPA) is a natural biodegradable platform chemical used as a precursor for the preparation of many valuable chemicals such as 1,3-propanediol, acrylic acid, methyl acrylate, malonic acid and its esters, hydroxy amide and acrylamide [27]. Many bacteria are able to produce 3-HPA by anaerobic fermentation of glycerol. An engineered strain of *Klebsiella pneumoniae* has been reported for the maximum production of 3-HPA 142.63 mmol/L with yield 26.7% [28]. It was observed that mannitol, erythritol and arabitol could be produced with biodegradation of glycerol (Table 1.1) [29-31].

5. Problems of Glycerol Bioconversion

Although a lot of work has been done at different routes to establish a glycerol fermentation process by using wild type natural microbes and engineered strains, there are many obstacles to increase the productivity. Impurities present in glycerol by-product obtained from biofuel refineries inhibit microbial growth and result in decrease in production yield and production rate. Another problem for bioconversion is the concentration of impurities varies in crude glycerol which is dependent on the biodiesel production process and nature of feed stock. Purification and partial purification of the crude glycerol is an alternative solution to reduce inhibition problem of microbial growth. The wild type microbial strains are employed to improve productivity of a desired high value product by the optimization of fermentation conditions. But end product toxicity, need of anaerobic conditions or lack of genetic tools hindered their industrial application. However, due to absence of external electron accepter in anaerobic fermentation process the redox potential is dropped and microbial growth is inhibited. During anaerobic growth, drop of redox potential might be a result of redox active metabolites produced by bacteria into the culture medium that leads to decrease of external pH, or might be connected with the process on the

bacterial membranes. The other problem is that the high concentration of glycerol as well as fermentation products inhibits cell growth during fermentation. To overcome these problems, a better understanding of metabolic pathways and development of new technologies are needed, such as optimizing reaction parameters and fermentation conditions, developing mutant and engineered strains. Moreover, metabolic engineering approaches have to be used to optimize metabolite synthesis through homologous and heterologous pathways. If the metabolic pathways and enzymes are identified, they can be used for the development of recombinant strains.

6. Conclusion

Renewable products from biodiesel co-product waste glycerol could add great advantage to the reduction of waste treatment cost and increase the economy value of by-products. In addition to the economy value, bioconversion of a large amount of biodiesel glycerol waste would directly benefit the environment by obtaining renewable products, encouraging the use of biodiesel and reducing fossil fuel use. Nowadays, effective utilization of glycerol by-product obtained from biodiesel production is globally important for the commercialization and improvement of biodiesel production. The renewable value-added products derived from glycerol will not only shrink our dependence on non-renewable products, but also will enforce the development of biorefinery. Thus, bioconversion processes of crude glycerol waste by microorganisms have been imposed on a new dimension for biorefinery. The conversion of abundant and low-price glycerol co-product of biodiesel biorefinery into higher priced products denotes a promising way to attain economic sustainability in biofuel industry. Except the production of small alcohols from glycerol, more attention should be taken for the production of other economically important pharmaceutical and industrial products like enzymes, proteins, antibiotics, vitamins, drugs and other fine chemicals by using glycerol as a sole carbon source through microbial fermentation.

Table 1.2. Value-added products produced from bioconversion of glycerol.

Products	Microorganisms/strains	Product	Productivity	References
1,3-	*K. pneumonia* LDH 526	102.1 g/L	2.13 g/L/h	[32]
Propanidiol	*C. pasteurianum* MNO6	-	1.21 g/L/h	[4]
	Engineered *E. coli K12*	104.4 g/L	2.16 g/L/h	[14]
Butanol	*C. pasteurianum* MNO6	12.6 g/L	1.80 g/L/h	[16]
2,3-Butanidol	*K. pneumoniae* G31	70.0 g/L	0.47 g/L/h	[17]
Ethanol	*K. pneumoniae* GEM167	25.0 g/L	-	[19].
	E. coli SY4	7.8 g/L	0.15 g/L/h	[18]
H_2	*E. aerogenes* HU-101	-	80 mmol/L/h	[5]
1,3-DHA	*G. oxydans* ZJB09112	165.5 g/L	2.3 g/L/h	[21]
3-HPA	*K. pneumonia*	142.63 mmol/L	-	[28]
Succinic acid	*Y. lipolytica* Y-3314	45.5 g/L	0.27 g/L/h	[23]
Citric acid	*Y. lipolytica* 101-1.22	124.2 g/L	0.85 g/L/h	[24]
Lactic acid	*E. coli* AC521	85.8 g/L	0.49 g/L/h	[25]
Oxalic acid	*A. niger* XP	48.9 g/L	0.29 g/L/h	[26]
Mannitol	*Candida magnolia*	51.0 g/L	0.53 g/L/h	[29]
Erythritol	*Y. lipolytica* Wratislavia K_1	80.0 g/L	1.00 g/L/h	[30]
Arabitol	*Debaryomyces hansenii* SBP1	15 g/L	0.13 g/L/h	[31]

Key Terms:

1) Key Term 1: Biodiesel: a biodegradable, nontoxic, and clean combustible fuel which is derived from transesterification of fat and vegetable oils in the presence of a catalyst leading to fatty acid methyl ester.

2) Key Term 2: Bioconversion of glycerol: the conversion of glycerol into valuable products or energy sources by biological process using microorganisms.

3) Key Term 3: Engineered strain: a microorganism constructed by introducing a gene or genes from other organisms.

4) Key Term 4: Anaerobic fermentation: a process in which microorganisms convert organic compounds into alcohol or acids under oxygen free conditions.

Summary

● Glycerol waste produced from biorefineries is a global oversupply crisis due to lack of refining capacity.

● Mmicroorganisms are able to utilize glycerol as a sole carbon and energy source.

● Engineered and mutated strains of bacteria and fungi have utilized glycerol to produce biofuels and other higher value molecules.

● Different strategies employed to produce biofuels and industrially important chemicals by microbial fermentation of glycerol.

● Bioconversion processes of crude glycerol waste by microorganisms represent a remarkable alternative to add value to the biodiesel production helping biorefineries development.

● Bioconversion of a large amount of biodiesel glycerol waste would directly benefit the environment by obtaining renewable products, encouraging the use of biodiesel and reducing fossil fuel use.

● The bright future of biofuels (alcohols) and many other valuable chemicals that can be achieved by the glycerol bio-fermentation.

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Chapter 2: Isolation and characterization of some efficient glycerol utilizing bacteria from soil and paper mill waste

Abstract

Biodiesel production process generates 10% crude glycerol as a core by-product, and this high volume of crude glycerol is a serious concern for development of biodiesel industries. A wide variety of microorganisms in environment permits screening for more efficient glycerol dehydrogenase (GDH) to help overcome current challenges in biodiesel industry. This study focuses on the isolation of efficient GDH producing bacteria in soil and paper mill waste which can be considered for use in large scale biotransformation of biodiesel-derived raw glycerol. More than one hundred bacterial strains were isolated from different sources including soil and paper Mill waste at Thunder Bay area, Canada based on their capability of utilizing glycerol at an initial concentration of 100 g/L pure glycerol. Minimal Salt (MS) medium was used where glycerol was the sole carbon source. Thirteen bacterial strains were selected as the best candidates for utilization of glycerol based on their biomass production and glycerol dehydrogenase (GDH) enzyme activity. The 16S rRNA genes of the high GDH enzyme producing seven isolates (P1, P2, P3, P4, P5, S1, S2, and S3) were amplified, sequenced, and then BLASTed to identified likely genera or species. All isolates were tested for their GDH activity at a concentration of 25 g/L glycerol in aerobic batch flask process. The isolates P3 and P3 exhibited superior GDH activity and represent excellent candidates for further GDH analysis and bioconversion of glycerol studies.

2.1. Introduction

Biodiesel become an important renewable source of energy due to increasing of fossil fuels price, depletion of its sources, environmental concern as well as increasing energy demand [1-3]. The major advantages of biodiesel comprise low pollutant emissions and renewability, high flashpoint, high biodegradability and excellent lubricity. It is estimated that 3.2 kg of CO2 could

be reduced by 1.0 kg of biodiesel that is used instead of the fossil fuel [4,5]. Therefore, biodiesel fuel is an environmental friendly alternative to fossil fuel. Generally, biodiesel is producing by transesterification of vegetable oil and animal fats with methanol in the presence of either alkaline or strong acid catalysts [1–3], generates 10% crude glycerol as the core by-product [6,7]. Biodiesel production has increased noticeably across the world in the last decade [8] and the large volume of this non-biodegradable by-product crude glycerol is creating a serious issue for development of biodiesel industry due to its high purification cost. Also, the availability of crude glycerol has resulted in a collapse in pure glycerol prices [9]. Chemical or biological conversion of glycerol into various products has been reported [10]. Bioconversion of the cheaper feedstock crude glycerol to value-added products can bring down the overall cost of production and make it competitive to fossil based diesel. Until now, only a few numbers of bacterial and fungal strains are capable of utilizing glycerol to accumulate high-value bioproducts [10]. Therefore, the use of microorganisms for converting glycerol could be the best choice for development of biodiesel industry. Moreover, glycerol dehydrogenase (GDH) is a key enzyme for biotransformation of glycerol to produce biofuels and other value-added bioproducts under aerobic process. Thus, crude glycerol from biodiesel industries is the focus of considerable attention as it is readily available and cheapest carbon source for microbial bioconversion process. The proposed use of glycerol to produce microbial metabolic high-value products is a highly beneficial and attractive option. In this study, our primary focus is to isolate highly efficient GDH producing bacterial strains form environmental samples.

2.2. Materials and Methods

2.2.1. Isolation of Glycerol Utilizing Bacteria

Soil samples were collected from different locations at Thunder Bay. About 10 g of soil sample inoculated (10g) into 250-mL Erlenmeyer flasks containing 50 ml Minimal Salt (MS) medium (0.1g/L $NaNO_3$, 0.1g/L K_2PO_4, 0.1 g/L KCl, 0.05 g/L $MgSO_4.7H_2O$) and incubated at 30°C with shaking (200 rpm) for 3-5 days. MS medium contained pure glycerol as a sole carbon source and comprised of 100 ml/L commercial glycerol (99.0% analytical grade, Sigma). The pH of the medium was adjusted with 1 M NaOH or 1 M HCl. Batch fermentations were carried out at pH 7.0. Following incubation, inoculum from flasks showing growth was plated onto MS agar plates containing 10% analytical glycerol. Positive isolates were considered as glycerol utilizer. The pure isolates were then preserved or maintained at 4°C on MS agar medium supplemented with glycerol 50 g/L, peptone 2.5 g/L and yeast extract 2.5 g/L. These isolated strains were considered for confirmation further by their biomass production. Primarily, the biomass production was measured as the absorbance (optical Density/OD_{600}) by spectrophotometer at 600 nm wave length in the above broth medium after 0, 24 and 48h of incubation. If the OD is found higher than control (MS medium without glycerol) after incubation was considered as a glycerol utilizer.

2.2.2. Glycerol Degrading Enzyme Assay

The oxidation of glycerol is catalyzed by glycerol dehydrogenase (GDH), which leads to the formation of dihydroxyacetone (DHA) under the generation of reducing equivalent (NADH2). Dihydroxyacetone formed by GDH is further metabolized to various products such as 2,3-BD, acetoin, ethanol and acetic acid through pyruvate.

For determination of GDH enzyme activity, strains were grown in MS medium supplemented with 25.0g/L glycerol and 2.5 g/L yeast extract. To measure the enzyme activity of each strain, cells were washed twice with 100 mM potassium phosphate buffer (pH 8.0) and then re-suspended in the same buffer containing 50 mM KCl. The re-suspended cells were disrupted using a sonicator. The lysate was centrifuged at 15,000-25,000 *g* for 5 min, and the enzyme activity in the resulting supernatant was determined using the method described by Ahrens et at [11] with some modification. Briefly, one mL reaction mixture contained 30 mM ammonium sulfate, 0.2 M glycerol, 1.2 mM NAD (adjusted to pH 7.0 with 1 M NaOH), and the elute with enzymes in 0.1 M potassium phosphate buffer solution (pH 8.0). The assay was initiated by adding the cell extract (50 µl), and the increase of absorbance (NADH2 formation) was followed with a spectrophotometer reading at 340 nm and 30^{o} C for 1-5 minutes. One unit of activity is the amount of enzyme required to reduce 1 mmol of substrate per minute under the conditions specified.

i. Units enzyme = µmol of substrate reduced (µmole of NADH) per minute

The concentration c (µmol) of NADH in the reaction mixture was measured by Beer-Lambert Law: $A = \varepsilon c l$; where absorbance A, extinction coefficient, or molar absorptivity ε (ε_{340nm} =6.22 $mM^{-1}cm^{-1}$ for NADH), path length ℓ, and the concentration c.

ii. Specific activity = Units/mg protein

Or, µmole of substrate/minute/mg of cell protein

Concentration of protein (mg) in crude extract was determined by using standard curve of BSA protein [12].

2.2.3. Identification of isolates (strains)

16S rDNA sequencing and genetic analysis of selected bacterial isolates:

Bacterial genomic DNA was extracted using Norgen Biotek Genomic DNA Isolation kit (Canada) following the protocol provided by the manufacturers. The genomic DNA of bacterial isolates was subjected to amplify using the polymerase chain-reaction (PCR). The 16S rRNA gene will be targeted for amplification by universal primers HDA-1 (forward: GACTCCTACGGGAGGCAGCAGT) and E-1115R (reverse: AGGGTTGCGCTCGTTGC GGG). These primers code for a portion of the sequence of the 16S rRNA gene, which is 769 bp in length [13]. PCR reactions for bacteria were performed under the following conditions: initialization of 5 minutes at 95°C for 1 cycle; 35 cycles of denaturation (1min at 95°C), annealing (1 min at 60°C), and extension (1 min at 72°C); 1 cycle of a final extension for 10 minutes at 72°C. PCR products will be reviewed on a 1% agarose gel and sent to Eurofins MWG Operon Canada for sequencing.

Characterization by morphological, cultural and biochemical analysis:

For identification, highly active isolates (strains) were further characterized by their morphological, cultural and biochemical characteristics.

2.3. Results and Discussion

In this study, a lot of bacterial strains were screed for their glycerol utilizing activity. Among them, only thirteen bacterial strains showed positive activity towards 2.5% glycerol. Results of these screening are presented in Fig. 2.1, and Fig. 2.2. Optical density (OD_{600}) and pH of the broth culture medium was determined after 48h incubation. Results of the OD_{600} and pH are presented in Fig. 2.1. Among the 13 bacterial strains, strain no P1, P2, P3, P4, P5, H8, H10, S3, N1 and E2 showed higher biomass production (OD_{600}) ranging from 0.94 to 1.53 after 48h incubation at 25.0 g/L glycerol. Moreover, strain P3 exhibited highest biomass production (OD_{600}) compared to that of all other strains (Fig. 2.1). The OD_{600} value of control was about 0.096 (0.094-0.098) for each

bacterial strain (Fig. 2.1). Furthermore, the isolated strains P1, P2, P3, P4, P5 and S1 displayed slightly acidic pH, ranging from 5.17 to 5.51 after 48h incubation. In this 48h incubation period, bacterial growth and biomass production were increased rapidly, and probably organic acid was produced by those strains. Rest of the other strains (H10, H8, S2, S3, N1, E1 and E2) showed the pH value about 6.0 or higher. The initial pH of the media was set at 7.0 by adding NaOH or HCl.

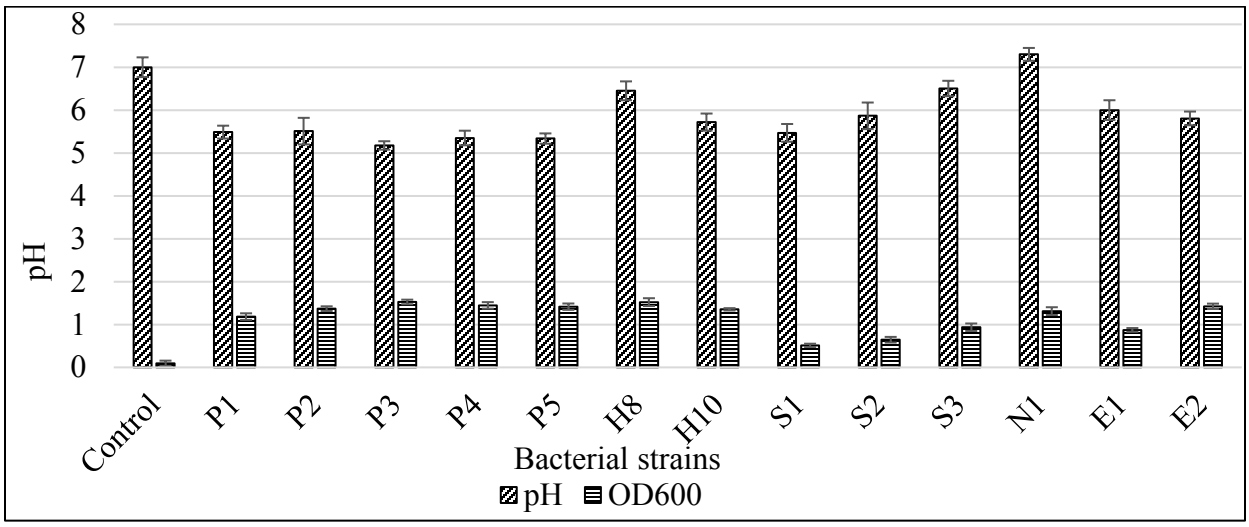

Fig 2.1. Biomass production and medium pH after 48h incubation at 30^{o}C. Minimal Salt (MS) medium supplemented with 25.0 g/L pure glycerol.

An attempt was made to determine glycerol dehydrogenase (GDH) enzyme activities of all the selected 13 bacterial strains after 24h and 48h incubations respectively. MS medium supplemented with 25.0 g/L glycerol and 2.5 g/L yeast extract was used for the growth of bacteria. Results of the GDH activities of thirteen bacterial strains are presented in Figure 2.2. As shown in Figure 2.2, the best candidates (strains) for GDH production were P1, P2, P3, P4 and P5 after 24h incubation at a 25.5 g/L initial concentration of glycerol. Moreover, after 48h incubation, the strains P3 and E2 exhibited comparatively the better GDH activities. Strain No. P3 displayed the highest activity 132.0 and 122.0 units/mg protein of GDH after 24h and 48h incubation respectively. Besides this, Strain No. P2 also showed comparatively the better activity of GDH. Similar GDH activities were

reported by other researchers [11, 14, 15]. Therefore, P2 and P3 strains could be the best candidate for biotransformation of glycerol.

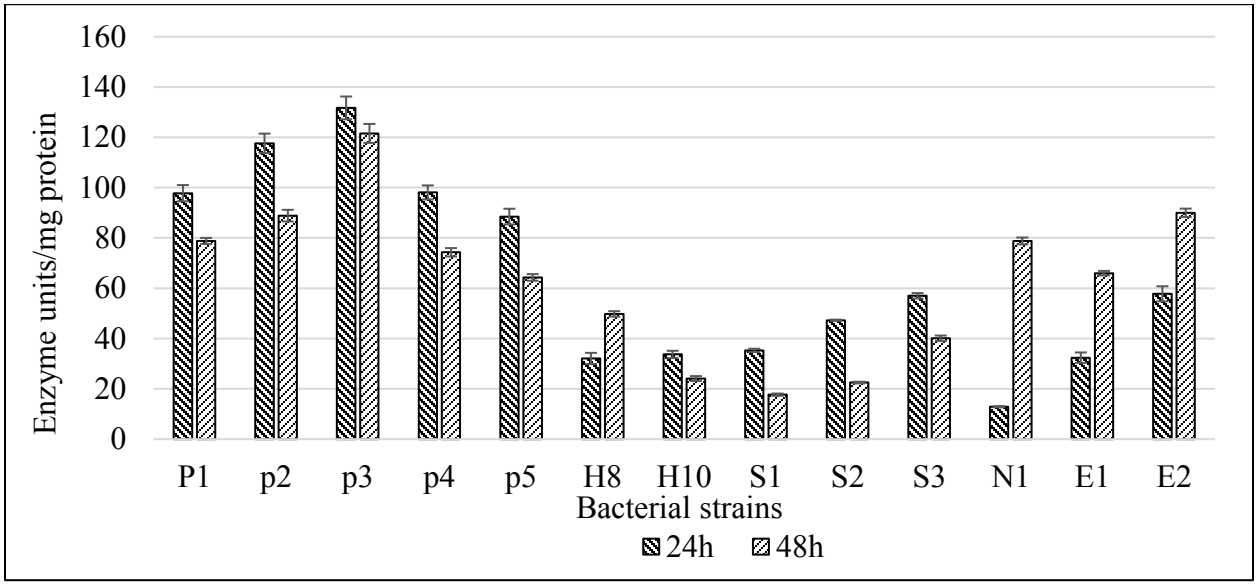

Fig 2.2. Glycerol dehydrogenase (GDH) enzyme activities of thirteen selected strains.

2.4. Significance of the Project

The effective utilization of glycerol by-product obtained from biodiesel production is globally important for commercialization and improvement of biodiesel production. The renewable value-added products derived from glycerol will not only shrink our dependence on non-renewable products, but will also enforce the development of biorefinery. Thus, bioconversion processes of crude glycerol by microorganisms have been imposed on a new dimension for biorefinery. The conversion of abundant and low-priced or negative-priced crude glycerol of biodiesel biorefinery into higher priced products denotes a promising way to attain economic sustainability in biofuel industries. This project mainly focuses on bioconversion of low value glycerol or biodiesel-derived crude glycerol to high-value bioproducts. The successful completion of this project may result in the development of an economical and energy efficient technology for continuous production of biofuels; fine chemicals as an important building block of other valuable

chemicals, and, high-value bioproducts. The successful completion of the project would greatly benefit the biodiesel industry for sustainability and strengthen the bio-economy of Canada.

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Chapter 3: Biotransformation of biodiesel-derived crude glycerol using newly isolated bacteria from environmental consortia

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Abstract

Biodiesel-derived crude glycerol, a core by-product of biodiesel production process is becoming of great environmental and economical concern for growth of biodiesel industries due to its ever-growing over supply problem. Biotransformation of this large volume of crude glycerol to value-added bio-products may directly benefit the environment and economic feasibility, encouraging the development of biodiesel plants. In our study, numerous bacterial strains isolated from environmental consortia were screened for their capability of converting crude glycerol to 2,3-butanediol (2,3-BD), a high worth green product uses as a liquid fuel or fuel additive. An aerobic batch flask cultivation was carried out to access the kinetics of bio-product formation and glycerol utilization. The greatest producers of 2,3-BD identified by 16S rRNA gene sequences were *Klebsiella pneumoniae*, *K. variicola* and *Serratia liquefaciens*. The co-culture constructed by *K. pneumoniae* SRP2 and, *K. variicola* SRP3 was capable of simultaneously converting crude glycerol to concurrently produce up to 27.87 g/L of 2,3-BD, yielding 0.73 gram 2,3-BD per gram glycerol (0.73 g/g)) using 37.0 g/L glycerol under aerobic conditions in batch culture, showing great potential for biotransformation bioprocess.

Keywords: Glycerol dehydrogenase, Crude glycerol, 2,3-Butanediol, Bioconversion, *Klebsiella*.

3.1. Introduction

Biodiesel has become one of the vibrant renewable fuels produced from animal fats and vegetable oils by reacting with a primary alcohol in the presence of catalyst, generating a significant amount of crude glycerol (10% v/v) as a core by-product. Thus, the crude glycerol produced from biodiesel production process is a global oversupply problem due to a lack of refining capacity, and this high volume of crude glycerol has become an environmental problem since it can't be safely disposed in the environment. However, due to its low price, crude glycerol is now considered as a waste instead of a useful product. A high volume of crude glycerol is the crucial problem for the development of biodiesel industries, which is greatly affected on economic viability [1]. In addition to the economic value, conversion of this large amount of biodiesel waste (crude glycerol) would directly benefit the environment by obtaining renewable value-added products, encouraging the development of biodiesel industry and reducing non-renewable fossil fuel use. Glycerol, a simple sugar alcohol, can be used as a carbon and energy source for microbial growth to produce biofuels and other valuable products [2-4]. It has been proved that bioconversion of pure glycerol to fuels or reduced products may result in yields higher than those obtained with the use of common sugars due to the highly-reduced state of carbon present in glycerol [5]. The ability to conduct fermentative metabolism of pure glycerol in the *Enterobacteriaceae* family is shared by only a few members such as *Enterobacter aerogenes*, *K. oxytoca* and *Klebsiella pneumoniae* [6-8]. There is no effective microorganism which can convert biodiesel waste (crude glycerol) efficiently to produce high value products 2,3-butanediol (2,3-BD) until today. Moreover, due to lack of biocatalysts, the biodiesel bio-refining process remains economically unfeasible that can overwhelm the problems of glycerol conversion. Furthermore, crude glycerol contains methanol, salts, soaps as matter organic non-glycerol

(MONG), and catalysts as the main impurities which can negatively influence the bioconversion process. Almost all the works have been carried out on bioconversion of pure glycerol to biofuels and value-added bio-products through microbial fermentation process [9,10], but very few works have been done on bioconversion of biodiesel waste crude glycerol under anaerobic process [11-12]. Our primary goal is to isolate and develop new strains of bacteria capable of efficiently converting crude glycerol to value-added biotechnological products under completely aerobic process. In oxidative pathway of glycerol metabolisms, *K. pneumoniae* metabolizes glycerol for synthesis of major products including dihydroxyacetone (DHA), 2,3-BD, acetoin, acetate and 1,3-propanediol (1,3-PDO) [2,13]. In this oxidative pathway, NAD^+-dependent glycerol dehydrogenase (GDH) enzyme dehydrogenates glycerol to DHA by generating reducing equivalent NADH [1,14]. Subsequently, DHA is then phosphorylated by ATP or PER-depended DHA kinase to generate dihydroxyacetone phosphate (DHAP), which is then further metabolized to various products including 2,3-butanediol (2,3-BD), acetoin, lactate, acetate, succinate and ethanol through pyruvate [14-16].

2,3-BD and acetoin is a significant platform chemical which can be attained from oxidative pathways of glycerol metabolisms of many bacteria [7,17]. 2,3-BD is a reduced form of acetoin which is widely used as an antifreeze agent, lubricant, liquid fuel or fuel additive, and a precursor of many synthetic materials including polymers and resins [18]. 2,3-BD is using for the manufacturing of printing ink, perfumes and fumigants, polymer, pharmaceutical carrier, moistening and softening agents, and reagent in different asymmetric chemical synthesis [19]. However, an important metabolic product acetoin is widely used in food, flavor, cosmetics, and chemical synthesis [19]. In the recent years, biotechnological production of 2,3-BD from glycerol has been demonstrated for only few bacterial strains including *K. pneumoniae, K. variicola, K*

oxytoca and *Bacillus amyloliquefaciens* [2,12,13,21]. Now-a-days, the anaerobic fermentation is the most promising option for bioconversion of crude glycerol by *Klebsiella* [22,23]. Several bacterial strains including *Lactobacillus lycopersici* and *Bacillus subtilis* are capable of fermenting sugars producing glycerol, but are unable to further convert glycerol to other product [24]. However, several bacteria including *K. pneumoniae, Enterobacter, Citrobacter*, and few species of *Clostridium* have already been isolated which are able to ferment glycerol, residual glycerol or mixture of glycerol and sugars, and the main product was
1,3-PDO, while 2,3-BD was not reported along with other products [25-27].

Consequently, more work must be needed without any delay if the enormous amounts of surplus glycerol are to be cost effectively converted into value-added commercial products. In this context, the aim of our present work is to make a co-culture from bacterial strains isolated from environmental consortia to increase metabolic product yield of 2,3-BD to make a process relevant for industrial application. The biotransformation kinetics of batch culture processes was studied in detail, and the best co-culture of bacterial strains providing the gain of increased 2,3-BD product yield were evaluated.

3.2. Materials and methods

3.2.1. Crude glycerol

Biodiesel-derived crude glycerol was kindly supplied by Dr. Chunbao Xu of Western University (Canada) which was obtained from a small biodiesel plant Centre for Agricultural Renewable Energy and Sustainability (CARES), Guelph, Canada. This crude glycerol composition was (in weight based) 50.0±4.6 % glycerol, 4.3±0.3% ash, 6.7±1.05% moisture and 36.2±3.3% MONG (Matter organic non-glycerol). The pH of the crude glycerol was 10.6±0.4.

3.2.2. Isolation of bacterial strains from a microbial consortium

The paper mill sludge, an environmental bacterial consortium was collected from Resolute Forest Products, Thunder Bay, Ontario, Canada. This sample was immediately refrigerated to inhibit the growth of microbes, transported to the research laboratory for glycerol utilization study. For isolation of glycerol degrading strains, 5.0 g of paper mill waste sample was inoculated into in a 250 ml Erlenmeyer flasks containing 100 ml minimal salt (MS) broth medium supplemented with 100 g/L analytical grade glycerol (Sigma Aldrich), incubated at 30°C with shaking (200 rpm) for 48 h. MS medium containing glycerol which is a sole carbon source, and contained the following components: K_2HPO_4 (0.1 g/L), $MgSO_4.7H_2O$ (0.05g/L), KCl (0.1 g/L), $NaNO_3$ (0.1 g/L) and an alytical grade glycerol (100 g/L. Subsequent incubation, culture from flasks displaying growth was streaked onto MS agar plates containing 100.0 g/L glycerol, transferred pure culture to LB agar plate, and stored at 4°C. These isolated strains were further screened for their glycerol assimilation, GDH activity and 2,3-BD production.

3.2.3. Identification and construction of phylogenetic tree using 16S rRNA partial sequencing

The molecular identification of glycerol degrading bacterial isolates was conducted using 16S rRNA gene sequencing. Genomic DNA of the selected bacterial isolates was extracted using bacterial DNA Genomic Mini Kit (FroggaBio, Canada). The extracted DNA was further amplified using universal primers designed within conserved regions of the 16S rDNA for Eubacteria, which were HDA-1 (5′-GACTCCTACGGGAGGCAGCAGT) and E1115R (5′-AGGGTTGCGCTCGTTGCGGG). The 50 µl PCR mixture contained 0.5 µg template DNA, 0.5 µM forward and reverse primers, 25 µl double strength Tag Mix (0.4 mM dNTPs, 3.2 mM $MgCl_2$, 2X PCR buffer, 0.25 U/ µl Tag DNA polymerase and 0.02% bromophenol blue) and nuclease-free water. The PCR reactions were performed in an automated thermal cycler (Bio-Rad, My Cycler^{Tm} thermal cycle) using following thermal cycling conditions: initial denaturation at 94^{o}C for 3 min

followed by 30 cycles at 94^0C for 30 s, 52^0C for 30 s and 72^0C for 1 min, and a final extension at 72^0C for 10 min. The PCR products were purified using Geneaid Clean-up kit (FroggaBio, Canada) following the manufacturer's protocol. Confirmation of amplified 16S rRNA gene fragments was validated by a band on a 1% agarose gel. The purified samples were sent to Euroffins Genomics (USA) for sequencing. Sequencing result was inputted in the nucleotide blast tool through the NCBI database (http://blast.ncbi.nlm.nih.gov/) for possible identification of bacterial genera. The phylogenetic relationship was analyzed using sequence alignment program ClustalX Omega software. For confirming the species identification, morphological and physiological characteristics were determined [28].

3.2.4. Biotransformation kinetics

The stock culture of bacterial isolates was maintained at 4°C by sub-cultured every month, and one set stored at −80°C with 20% (w/w) glycerol added. For batch culture, the seed culture was prepared from stock culture (1 loopful culture from LB agar plate) by inoculating into a Luria-Bertani (LB) broth (yeast extract 5.0 g/L; peptone 10.0 g/L; NaCl 5 g/L and pH 7.0) medium. The seeds were grown in 125 ml shake flask containing 50 ml medium at 30°C and 200 rpm under aerobic conditions for 18-20h. Following incubation, the seed culture was inoculated into appropriate medium for aerobic biotransformation of glycerol. Batch culture was carried out in 250 ml Erlenmeyer flasks with a working volume of 100 ml including 100 µl of 18 h culture. Incubation temperature was maintained at 30° when *Serratia* (S1 – S3) strains were used and 37°C when only *Klebsiella* (P1 – P5) strains were used. However, agitation was maintained at 200 rpm using a rotary shaker incubator (New Brunswick Scientific, C25 incubator shaker, NJ, USA). The batch culture medium contained glycerol as a sole carbon source. The MS-2 medium supplemented with different concentrations of pure and crude glycerol was used for batch biotransformation

comprised (in per L): $NaNO_3$ (0.1 g), K_2HPO_4 (0.1 g), KCl (0.1 g), and $MgSO_4$.$7H_2O$ (0.05g), yeast extract (2.5 g) and peptone (5.0 g). The initial pH (7.0) of the medium was adjusted by using 1 M HCl. All experiments were conducted in triplicates.

3.2.5. Enzyme activity assay

The membrane-bounded GDH enzyme activity was determined at room temperature by measuring the initial reduction rate of NAD at 340 nm absorbance according to the method described by Ahrens et al [29] with some modification. Momentarily, 1.0 ml of culture (OD_{600} of ~0.8) broth was centrifuged at 12,000-15,000 x *g* for 5 min, and cells were collected. Cells were washed twice using 100 mM potassium phosphate buffer (pH 8.0), re-suspended in 100 mM potassium phosphate buffer containing 50 mM KCl. Using a sonicator (Model CL-18) cells were disrupted at 2-4°C for 2 minutes (6 secs at a time, and until 2 min). The supernatant was then collected by centrifugation at 12,000-15,000 x *g for 4-5 minutes,* kept at low temperature (2-4°C) or on ice for GDH enzyme activity. Enzyme activity was measured by microplate spectrophotometer (EPOCH, BioTek Gen5^{TM}, USA). The GDH activity was determined by the change of substrate-dependent absorbance per minute at 340 nm. Reaction mixture of 0.3 ml for each microplate well contained 0.2 M glycerol, 30 mM ammonium sulfate, 1.2 mM NAD, 50.0 mM potassium phosphate buffer (pH 8.0), and 50 µl cell extract. The absorbance increase (NADH) was followed with a spectrophotometer for 5 minutes. One unit of GDH activity was defined as the amount of enzyme required to reduce 1 µmole of NAD^+ to NADH per minute. The specific activity of GDH is defined as enzyme units / mg of cell protein. Protein concentration in the cell free extract was determined according to Bradford method [30] using a microtiter plate reader and bovine serum albumin as the standard.

3.2.6. Analytical methods

Cell concentration of the culture medium was measured using microplate spectrophotometer (EPOCH, BioTek). The biomass production was expressed as optical density (OD_{600}) at 600 nm absorbance. GC-MS (Varian 1200 Quadrupole GC/MS using helium as the carrier gas) was used to identify the end products. To determine concentrations of glycerol and major metabolic products including 2,3-BD, acetoin and 1,3-PDO, gas chromatograph GC 14A (Shimatzu Corp., Kyoto, Japan) equipped with a flame ionization detector (FID) was used. Briefly, the supernatant of culture broth obtained after centrifugation (accu Spin Micro 17, Fisher Scientific) at 12,000 – 15,000 ×g for 5 min was filtered through a syringe filter (pore size 0.22µm; Progene, UltiDent Scientific, Canada). DB-WAXetr column was used under the following conditions: sample volume 1 µl; column temperature range from 45°C (2 min) to 240°C at the rate of 10°C/min; the injector and detector temperature 250°C; carrier gas nitrogen.

3.3. Results and discussion

3.3.1. Glycerol utilizing bacterial strains from environmental consortium

Total eight strains (P1, P2, P3, P4, P5, S1, S2 and S3) were isolated from the sample, a microbial consortium based on their ability to tolerance (100 g/L) and utilize glycerol as the sole carbon source to exhibit GDH enzyme activity under aerobic condition. These highly active eight strains were identified using 16S rRNA gene sequencing, confirmed by their morphological, physiological and biochemical properties. Genomic DNA for 16S rRNA Gene amplication was successfully isolated from all eight GDH producing isolates using DNA isolation kit. Sequencing and sequence analysis results of all eight different 16S rRNA genes were successfully obtained. The partial sequences of 16S rRNA of the isolates/strains P1, P2, P3, P4, P5, S2, and S3 were submitted to the GenBank for their accession numbers. However, the strain S1 reported in this paper is not a new strain which has 100% similarity to the strain Serratia sp. 243 (accession No.

KT461863). The potential seven isolates reported in our research paper has been nominated as the new strains of *Klebsiella* and *Serratia* sp, and their GenBank accession numbers are released in the NCBI website (Table 3.1).

Table 3.1. List of strains which were identified by 16S rRNA sequencing as well as morphological and biochemical characters.

Isolate No.	Strain identified as	GenBank Accession No.
P1	*Klebsiella pneumoniae* SRP1	KU550763
P2	*K. pneumoniae* SRP2	KR092085
P3	*K. variicola* SRP3	KR092086
P4	*K. pneumoniae* SRP4	KU550764
P5	*K. pneumoniae* SRP5	KU550765
S1	*Serratia* sp. 243.	KT461863
S2	*S. liquefaciens* SRWQ2	KX602659
S3	*S. proteamaculans* SRWQ1	KX602658

However, for the analysis of evolutionary relationship among the newly isolated strains, phylogenetic tree was constructed using 16S rDNA sequences of strains *Klebsiella pneumoniae* SRP1, *K. pneumoniae* SRP2, *K. pneumoniae* SRP4, *K. pneumoniae* SRP5, *K. variicola* SRP3, *Serratia liquefaciens* SRWQ2, *Serratia proteamaculans* SRWQ1, *Serratia* sp., and other *Klebsiella* as well as *Serratia* strains retrieved from GeneBank. The phylogenetic tree constructed using the ClastalX Omega software is presented in Fig. 3.1. The evolutionary history was inferred using the same software (ClastalX Omega). The result of phylogenetic relationship confirmed the identity of our new strains through the distance between all the newly isolated and other strains.

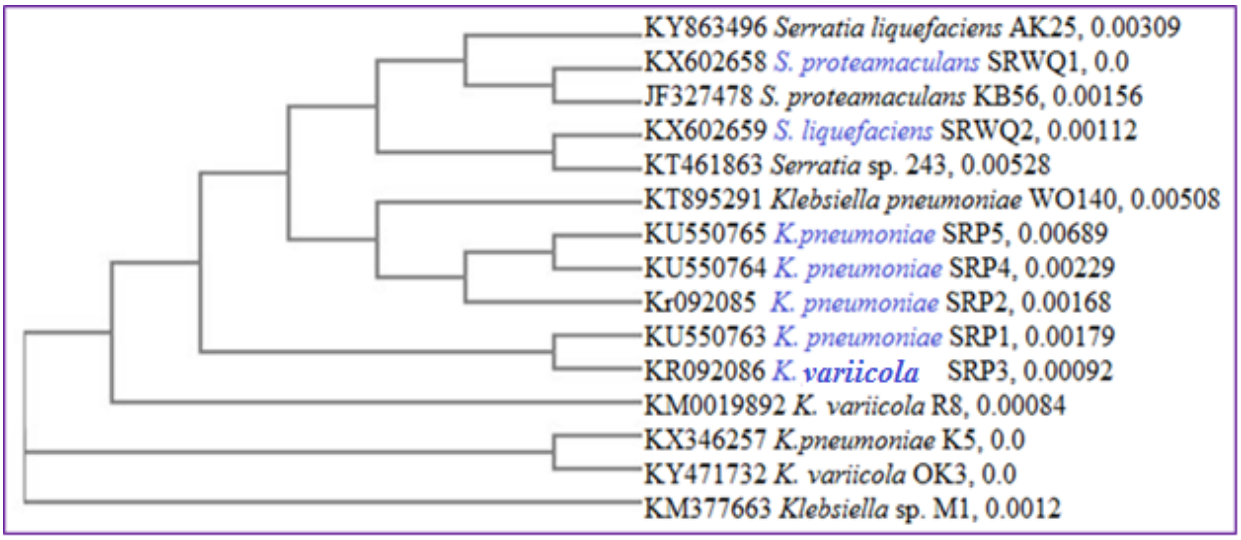

Fig. 3.1. Evolutionary relationships of the strains: Phylogenetic tree drawn from sequence alignment program using ClustalX Omega software. 16S rRNA gene sequences were retrieved by nucleotide BLAST searches in NCBI. The numbers that follow the names of the strains are accession numbers and bootstraps of published sequences.

3.3.2. Glycerol degrading capability of the consortium

All the identified bacterial strains were used to perform the ability of metabolizing crude glycerol from biodiesel production process by cultivating environmental consortia in a batch flask culture at 30^{o}C. In this aerobic batch culture process, glycerol was only the substrate for GDH and 2,3-BD production. Comparison the capabilities of bacterial consortium to grow in either pure or crude glycerol is shown in Figure 3.2. As shown in Figure 3.2a, 25.0 g/L of pure glycerol was consumed in 60h with a production of 14.07 g/L 2,3-BD, yielding 0.56 g/g and productivity of 0.24 g/L/h. The highest GDH activity was 177.21 units/mg protein when the bacterial consortium utilized 11.5 g/L glycerol after 24h incubation. However, for crude glycerol, the highest GDH activity was 157.31 units/mg protein in 24h, and utilization of 97.4% (24.3 g/L) glycerol was completed within 60h of incubation with nearly the same amount (13.44 g/L) of 2,3-BD being produced (Fig. 3.2b). However, when the culture temperature was maintained at 37^{o}C the 2,3-BD product yield and glycerol utilization rate was increased using the consortium containing P1, P2,

P3, P4 and P5 strains. Isolated strains under *Serratia* (S1, S2 and S3) were not capable of growing as high as 37°C in batch culture. In case of incubation temperature at 37°C in a batch culture as shown in Figure 3.3, almost 100% (25.0 g/L) glycerol was consumed within 60h with a product concentration of 15.03 g/L 2,3-BD and a productivity of 0.25 g/L/h, and a product concentration of 14.67 g/L 2,3-BD and a productivity of 0.245 g/L/h in case of pure and crude glycerol respectively. The GDH enzyme activities were also increased at 37°C of culture temperature compared to that of 30°C with the consortium contains P1, P2, P3, P4 and P5. The highest GDH activities 195.97 and 187.75 units/mg protein were recorded after 24h using pure and crude glycerol respectively (Fig 3.3a and 3.3b). Only one research work has been conducted on the production of 1,3-PDO by environmental consortia using crude glycerol as a sole carbon source until now [22]. There is no any report on the production of 2,3-BD using environmental consortia or bacterial co-culture until today. Our findings displayed that some of these strains were present in the bacterial consortia, and were capable of competently converting glycerol or biodiesel derived crude glycerol into 2,3-BD.

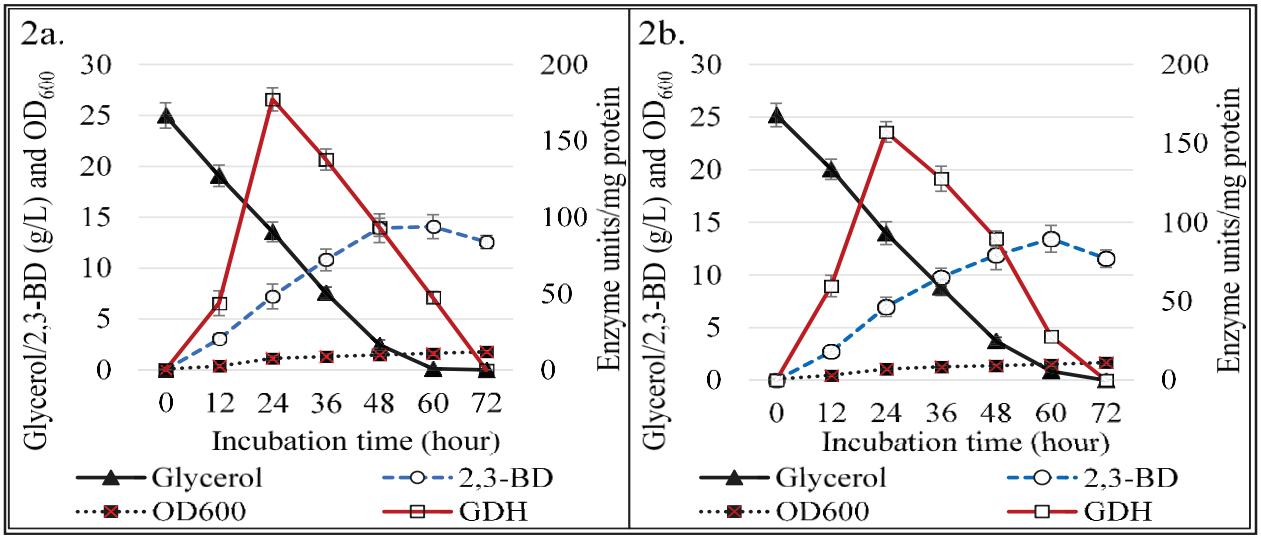

Fig 3.2. Profile of glycerol utilization, GDH enzyme activity, 2,3-BD and biomass production of bacterial consortium containing P1+P2+P3+P4+P5+S1+S2+S3 strains; (a) pure glycerol 25.0 g/L,

(b) biodiesel derived crude glycerol (50% glycerol) 50.0 g/L. MS medium supplemented with pure/crude glycerol, yeast extract 2.5 g/L and peptone 5.0 g/L. Incubation at 30^0C.

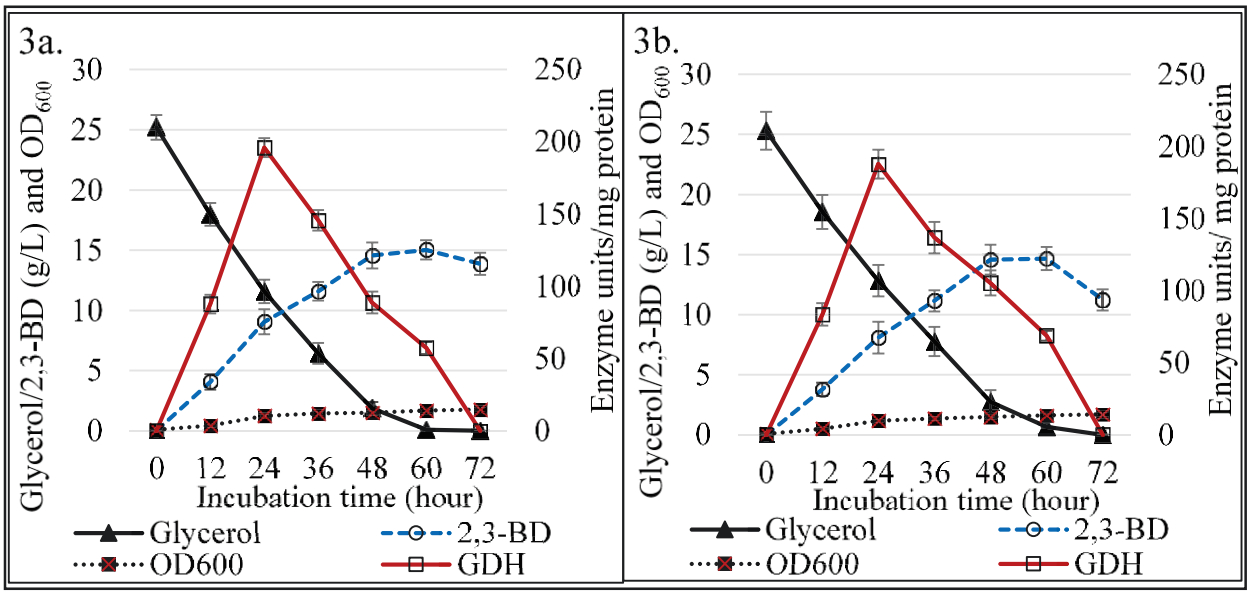

Fig 3.3. kinetics of glycerol consumption, GDH enzyme activity, 2,3-BD and biomass production of bacterial consortium containing P1+P2+P3+P4+P5 isolates (S1+S2+S3 strains do not grow at 37^0C); (a) pure glycerol, (b) biodiesel derived crude glycerol. MS medium supplemented with pure glycerol 25 g/L or biodiesel derived crude glycerol (50% glycerol) 50 g/L, yeast extract 2.5 g/L and peptone 5.0 g/L, and incubation at 37^0C.

3.3.3. Utilization of glycerol by single cultures

The GDH activity, biomass production and glycerol consumption were observed for 8 bacterial isolates (Table 3.1) nominated based on previously characterized glycerol utilizing capabilities. As shown in figure 3.4, none of the bacterial isolates produced significantly demonstrable GDH enzyme in 24h at 30^oC, nor did they consume significant amount of glycerol after 24h of incubation under aerobic condition in a batch culture. Moreover, there was an extensive disparity in glycerol utilization, GDH activity and biomass production between individual bacterial strains with both pure and crude glycerol as the sole carbon source. Under aerobic conditions, strains P2, P3 and P4 were the best producers of GDH. As shown in Figure

3.4, the best three isolates P2, P3 and P4 displayed the maximum GDH activities of 117.6, 131.7 and 102.1 units/mg protein respectively using pure glycerol as the sole carbon source (Fig. 3.4a). However, as the sole carbon source crude glycerol, the maximum GDH activities were 102.74, 110.43 and 98.07 units/mg protein with the same isolates P2, P3 and P4 respectively after 24h at 30ºC (Figure 3.4b). The highest GDH activity (131.7 units/mg protein) under aerobic conditions was achieved using isolate P3 (*K. variicola*). Furthermore, the isolate P3 exhibited the better glycerol consumption capability of 9.27 g/L in 24h as well as biomass production (OD_{600} 1.53) with pure glycerol, and almost same results were obtained using crude glycerol. Finally, S1, S2 and S3 isolates displayed a significantly less enzyme activity and glycerol consumption capability, and also these three isolates do not grow at 37ºC. The remaining isolates P1 and P5 did not display significant activity of GDH when compared with the other isolates. Recently, Homann et al. [31] isolated bacterial strains from an environmental consortium, characterized as *K. pneumoniae, K. oxytoca and Citrobacter freundii*, and these bacterial strains produced between 9.3 and 13.1 g/L of 1,3-PDO in shake flask cultures using 20.0 g/L of pure glycerol. It is notable to point that these researches worked with pure glycerol at low concentrations to produce 1,3-PDO, but there is no report on GDH as well as 2,3-BD production.

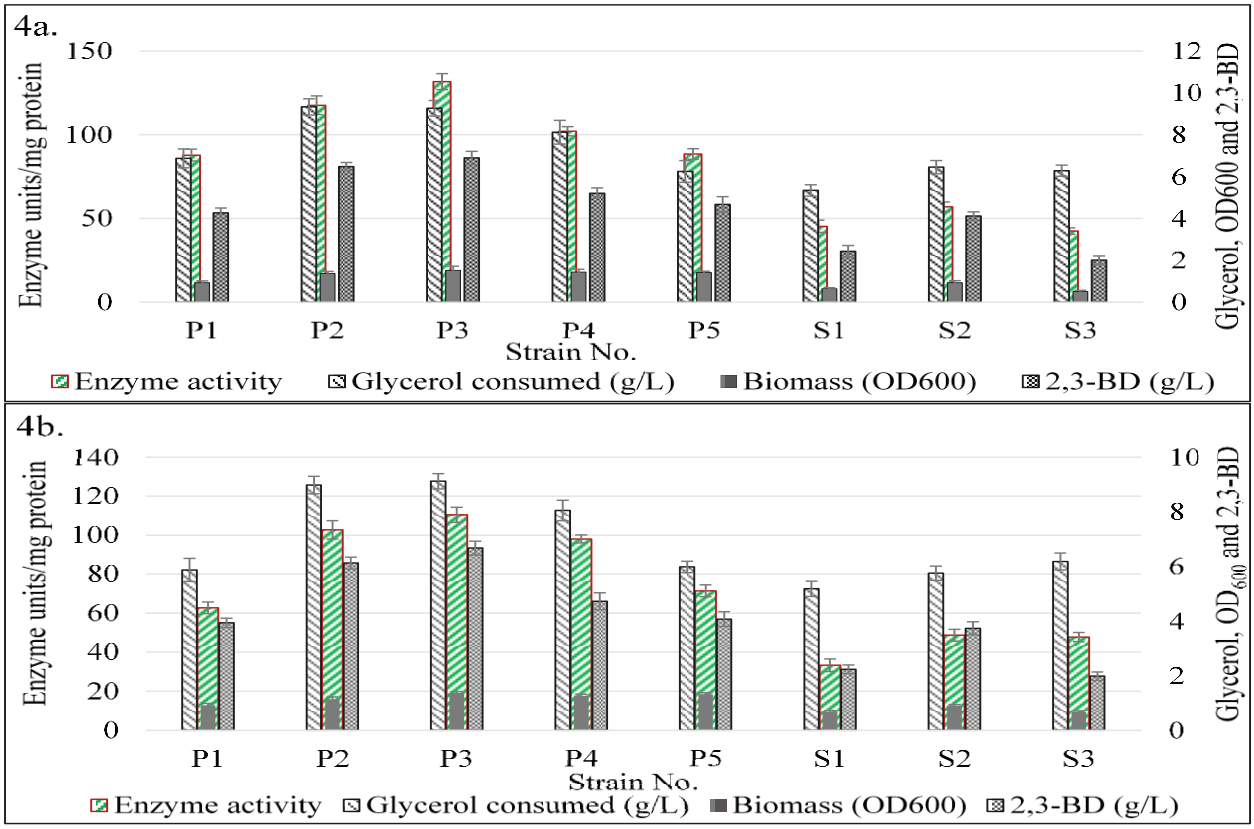

Fig 3.4. Profile of glycerol utilization, GDH enzyme activity, 2,3-BD and biomass production of bacterial strains isolated from bacterial consortium; (a) pure glycerol 25.0 g/L, (b) biodiesel derived crude glycerol (50% glycerol) 50.0 g/L. MS medium supplemented with pure/crude glycerol, yeast extract 2.5 g/L and peptone 5.0 g/L. Incubation at 30^0C for 24h.

3.3.4. Utilization of glycerol by co-cultures

Likewise, glycerol consumption, GDH activity and production of 2,3-BD were evaluated for bacterial co-cultures constructed by pairing each of the five nominated isolates P1, P2, P3, P4 and P5 with each other once. Like this, a total of ten co-cultures were prepared (Fig. 3.5). For determination of the best co-culture, the experiment was carried out in shake flask batch culture under aerobic condition at a concentration of 50.0 g/L biodiesel-derived crude glycerol which was contained 25.0 g/L glycerol, incubated at 37°C for 48h. Consequently, all co-cultures produced significant amount of 2,3-BD, ranging from 6.89 to 15.73 g/L after consumed 15.27 – 25.0 g/L glycerol from biodiesel-derived crude glycerol in 48h at 37°C in batch culture (Fig. 3.5). As shown

in Figure 3.5, the best productions of 2,3-BD were 15.73, 14.12 and 13.01 g/L obtained from three co-cultures of P2+P3, P2+P4 and P3+P4 respectively. Meanwhile, from these results four co-cultures (P1+P5, P4+P5, P1+P4 and P1+P2) exhibited less than 10.0 g/L 2,3-BD. In the meantime, the remaining co-cultures, P1+P3, P2+P5 and P3+P5 were showed 10.27 – 11.3 g/L 2,3-BD using 19.94 -22.73 g/L glycerol. Furthermore, co-culture P2+P3 with the greatest potential towards 2,3-BD production was selected from this eight co-culture groups for the construction of the best bacterial consortia (co-culture), based on its ability to utilized 100.0% (25.0 g/L) of glycerol in 48h, yielding 0.63 g/g as well as 0.33g/L/h. In regards, to the group of eight co-cultures, when looking at their efficacy of glycerol utilization and 2,3-BD production the isolates P2 and P3 containing co-cultures were demonstrated as the best candidate, it is readily apparent that the co-culture P2+P3 was capable of producing noticeable 2,3-BD which is the highest amount (15.73 g/L) among eight co-cultures reported in Figure 3.5. Specifically, it is clearly seeming that isolates P2 and P3 made the best combination for construction of effectual co-culture (P2+P3) for a high production of metabolic product 2,3-BD (Fig. 3.5).

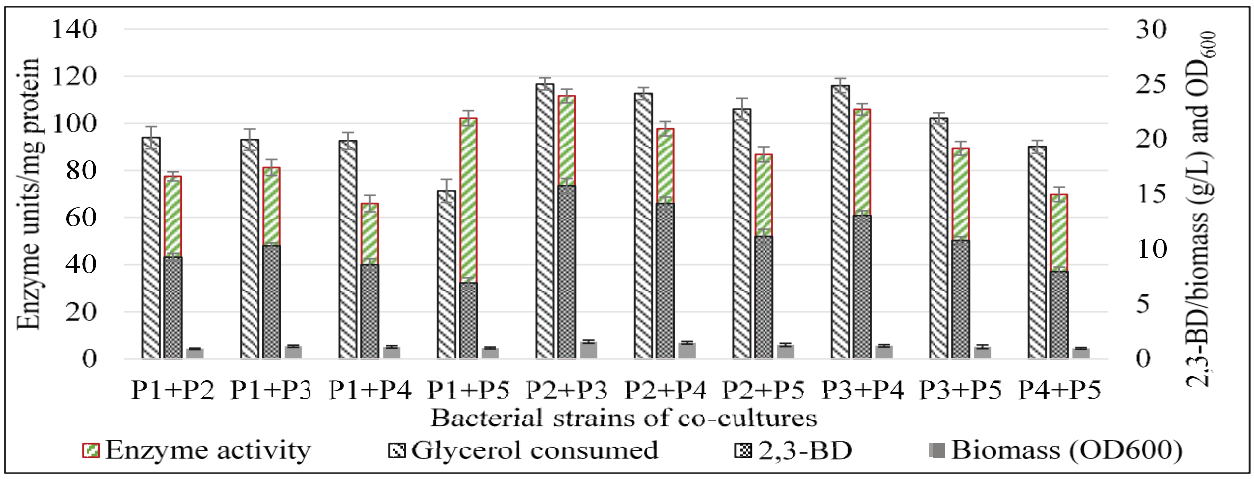

Fig 3.5. Profile of glycerol utilization, GDH enzyme activity, 2,3-BD and biomass production of bacterial co-cultures after 48h at 37^0C on biodiesel waste glycerol. MS-2 medium supplemented with biodiesel derived crude glycerol 50.0 g/L (glycerol 25 g/L).

3.3.5. Utilization of glycerol by a newly constructed bacterial consortium

Based on the outcomes from glycerol utilization in eight co-cultures using biodiesel-derived crude glycerol as only the sole carbon source, the co-culture P2+P3 produced the greatest yield of 2,3-BD, selected as the best bacterial consortium for this study. Aerobic batch biotransformation process was used for a high production of 2,3-BD using 75.0 g/L crude glycerol as a sole carbon source. The kinetics of batch cultivation of co-culture P2 (*K. pneumoniae* SRP2)+P3 (*K. variicola* SRP3) under aerobic conditions are presented in Figure 3.6. Specifically, the biotransformation under aerobic condition (Fig 3.6b) displayed a very lower production of acetate (0.14 – 0.37 g/L) and 1,3-PDO (0.34 -0.77 g/L) in 120h when the glycerol from biodiesel-derived crude glycerol was completely consumed. The maximum production of 2,3-BD was 27.87 g/L, yielding 0.73 g/g (0.29 g/L/h) in 96h by utilized 97.37% or 37.0 g/L glycerol using the consortium P2+P3 (Fig 3.6a). Additionally, co-culture P2+P3 exhibited the highest concentration 3.7 g/L of acetoin obtained in 144h after completely consumed glycerol (carbon source) from the culture medium. The biomass production was increased dramatically until 48h of incubation, and OD_{600} value reached up to 1.12 (Fig. 3.6b). After 48h of incubation, biomass production was increased very slowly, and OD_{600} value reached from 1.12 to 1.38 after 120h incubation (Fig. 3.6b). However, during the stationary phase (OD_{600} 1.12-1.38) of bacterial co-culture (P2+P3) under aerobic biotransformation process, the 2,3-BD product yield was increased until 96h of incubation (Fig 3.6a). After 96h when glycerol was completely consumed by two isolates P2 and P3, the concentration of acetoin was increased and 2,3-BD production was decreased. Once more, when glycerol is absence in the medium, acetoin is produced instead of 2,3-BD, and 2,3-BD is converting to acetoin. Our results revealed that the co-culture constructed using *K. pneumoniae* SRP2 and *K. variicole* SRP3 was capable of converting biodiesel-derived crude glycerol to 2,3-BD in alike way

to results stated in other researchers where the culture was environmental consortium for the production of 1,3-propanediol and monoculture for 1,3-PDO or 2,3-BD [22,32,33], but there is no report on co-culture for production of 2,3-BD using glycerol as a carbon source.

An enteric bacteria *K. pneumoniae* has been known to produce 1,3-PDO through anaerobic fermentation of glycerol [11,34,35]. Moreover, in the recent years, a very few works have been reported on aerobic biotransformation of glycerol to 2,3-BD production [2]. Therefore, Table 3.2 compares the product yields of 2,3-BD reported earlier and this study in batch fermentation using biodiesel-derived crude glycerol as the only carbon source. However, we have confirmed that our co-culture (consortium) constructed from newly isolated strain *K. pneumoniae* SRP2 and *K. variicola* SRP3 could be fermentatively metabolized glycerol to produce notable amount of important liquid fuel or fuel additive product 2,3-BD in a GDH-dependent oxidative pathway.

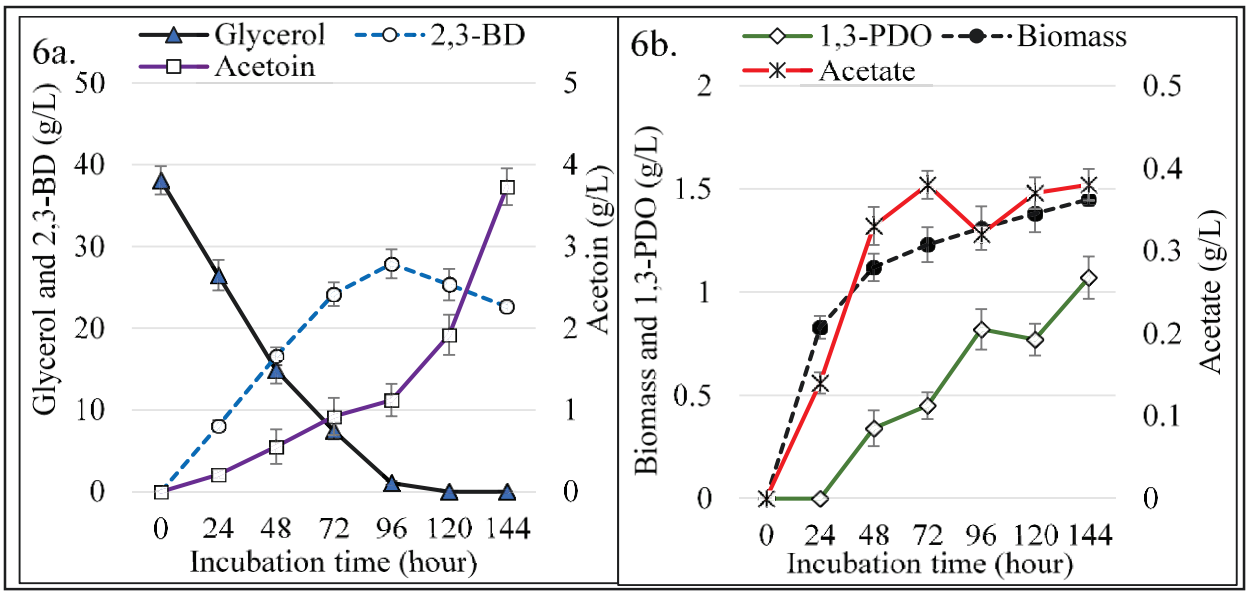

Fig 3.6. Kinetics of biotransformation of biodiesel derived crude glycerol to metabolic bioproducts in aerobic batch culture at 37^0C by the co-culture P2+P3 (*K. pneumoniae* SRP2+*K. variicola* SRP3); a) glycerol consumption, 2,3-BD and acetoin production, (b) 1,3-PDO, acetate and biomass production. MS-2 medium supplemented with crude glycerol 75.0 g/L.

Table 3.2
Comparison of 2,3-BD production using crude glycerol as the sole carbon source in batch culture process.

Name of bacterial strains	Carbon source	Product (g/L)	Yield (g/g)	References
Klebsiella oxytoca M1	Crude glycerol	8.9	0.12	36
K. oxytoca	Crude glycerol	4.3	0.24	37
Enterobacter aerogenes	Crude glycerol	22.0	0.4	37
Bacillus amyloliquefaciens	Crude glycerol	18.2	0.35	39
K. oxytoca FMCC-197	Crude glycerol	4.8	0.18	38
Raoultella terrigena CECT 4519	Crude glycerol	33.6	0.38	40
K. pneumoniae SRP2 and *K. variicola* SW3 (co-culture)	Crude glycerol	27.87	0.73	This study

3.4. Conclusions

Biotransformation of a core by-product crude glycerol generated from biodiesel production process offers a substantial advantage to produce 2,3-BD in relation to usage of glycerol, almost all the works have been conducted on pure glycerol as a substrate. Nonetheless, only a few research works have been reported on the possible use of this core by-product crude glycerol generates from biodiesel synthesis process to produce 2,3-BD. Our results consistently revealed that it is possible to isolate novel bacterial strains capable of producing a high yield of 2,3-BD from environmental consortia of microorganisms. We have demonstrated that the co-culture developed by using two strains *K. pneumoniae* SRP2 and *K. variicola* SRP3 isolated newly from natural bacterial consortium could be a potential system for efficiently utilized glycerol to produce a high product yield of 2,3-BD using low-value or negative -value biodiesel-derived raw glycerol as a feed-stock in industrial bioconversion process. Consequently, further studies with these newly isolated novel

strains are granted to increase 2,3-BD production as well as the utilization rate of crude glycerol. Moreover, as finding alternatives to chemical methods of crude glycerol conversion remains an important goal, biotransformation involving microbes as biocatalysts is an expressively promising and advanced green method.

Acknowledgements

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Chapter 4: 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 1.3fold 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*

4.1. 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 4.1) [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 1^{st} 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 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, 20-21]. 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.

4.2. Materials and methods

4.2.1. 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 flask, 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].

4.2.2. 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 $MgSO_4$.$7H_2O$ (0.05g), glycerol (50.0g), yeast extract (2.5 g) and peptone (5.0 g).

4.2.3. 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.

4.2.4. 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, 15,000x*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 µmoles 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.

4.2.5. 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.

4.2.6. 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.

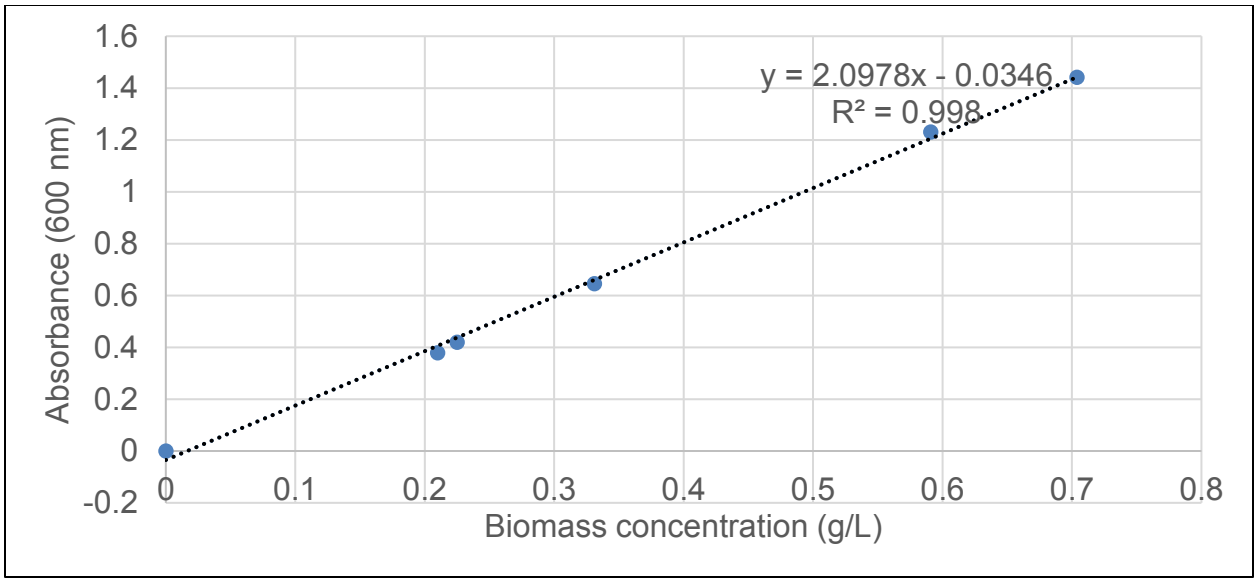

Figure 4.1. Standard curve for conversion of absorbance to dry cell weight (DCW) of bacteria.

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

4.3. Results

4.3.1. 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 4.1). 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.

4.3.2. 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 4.2).

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 4.3).

The influence of pH on GDH and cell growth in the batch bioprocess without pH control was significant (Table 4.2 and Figure 4.4). 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 4.2. However, the results of enzyme activity under shake-flask fermentation with various pHs ranging from 4.0 to 9.0 are presented in Figure 4.4. 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 4.4 and Table 4.2, 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 (5.0 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 4.5, 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 4.5).

4.3.3. 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 4.7 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 4.8 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 4.3). 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 4.3). 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 4.3. The adapted mutant strain *K. variicola* SRM3 utilized 97.5% glycerol within 96h incubation.

4.4. 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 4.2). 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 4.6, 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.

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 by-products like acetate, lactate and succinate inhibit of 2,3-BD production [31]. To overcome these 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 50^0C 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.

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Table 4.1. Morphological and physiological characters of strain *K. variicola* SRP3

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

+, positive; -, negative

Table 4.2. Effect of pH on cell growth after 24, 48, 72 and 96 hours of incubation at 35ºC of the bacterial strain *K. variicola* SRP3.

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

*pH after incubation

Table 4.3. 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 37^0C.

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±0.9	0.58	7.6±0.26	0.17	2.2±0.12	0.05
SRP3 (MC)	7.0	5.46	0.64	84.0	23.1±1.0	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±0.9	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±1.5	0.60	7.08±1.2	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

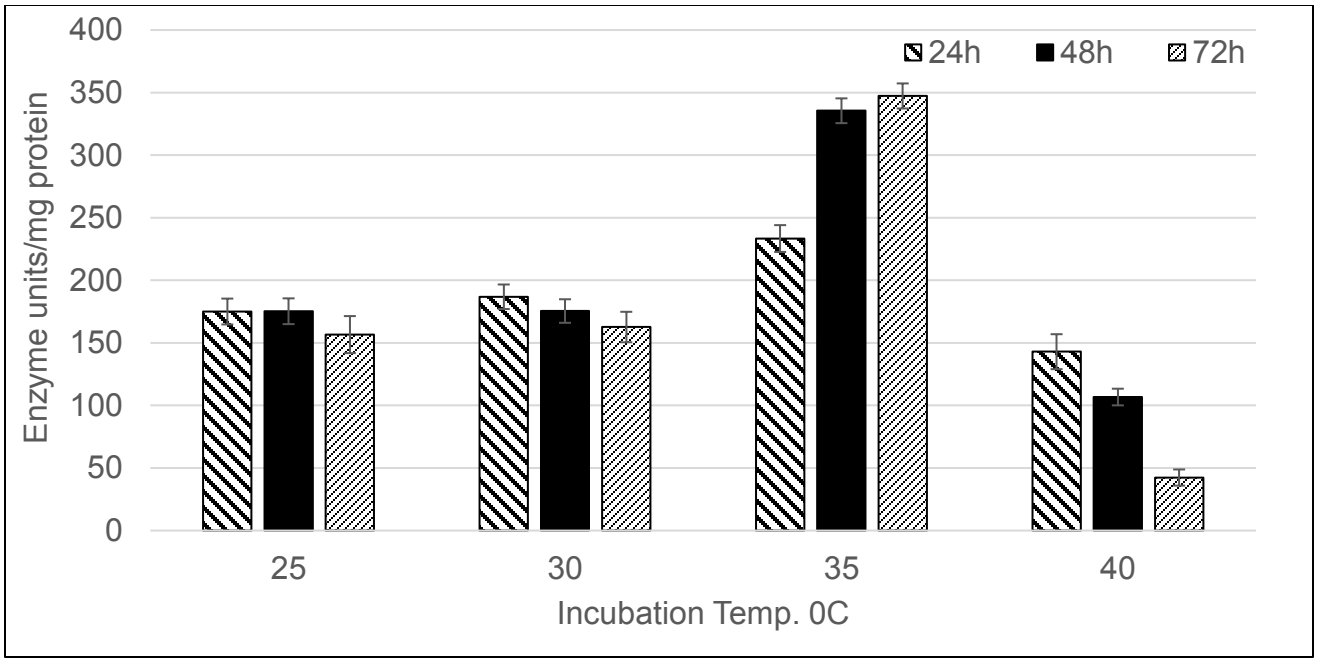

Figure 4.2. Effect of incubation temperature on specific activity of enzyme of strain SRP3 in batch process, minimal salt (MS) medium supplemented with yeast extract 0.25% and glycerol 5.0%, stating pH 7.0 and incubation temperature 35^0C.

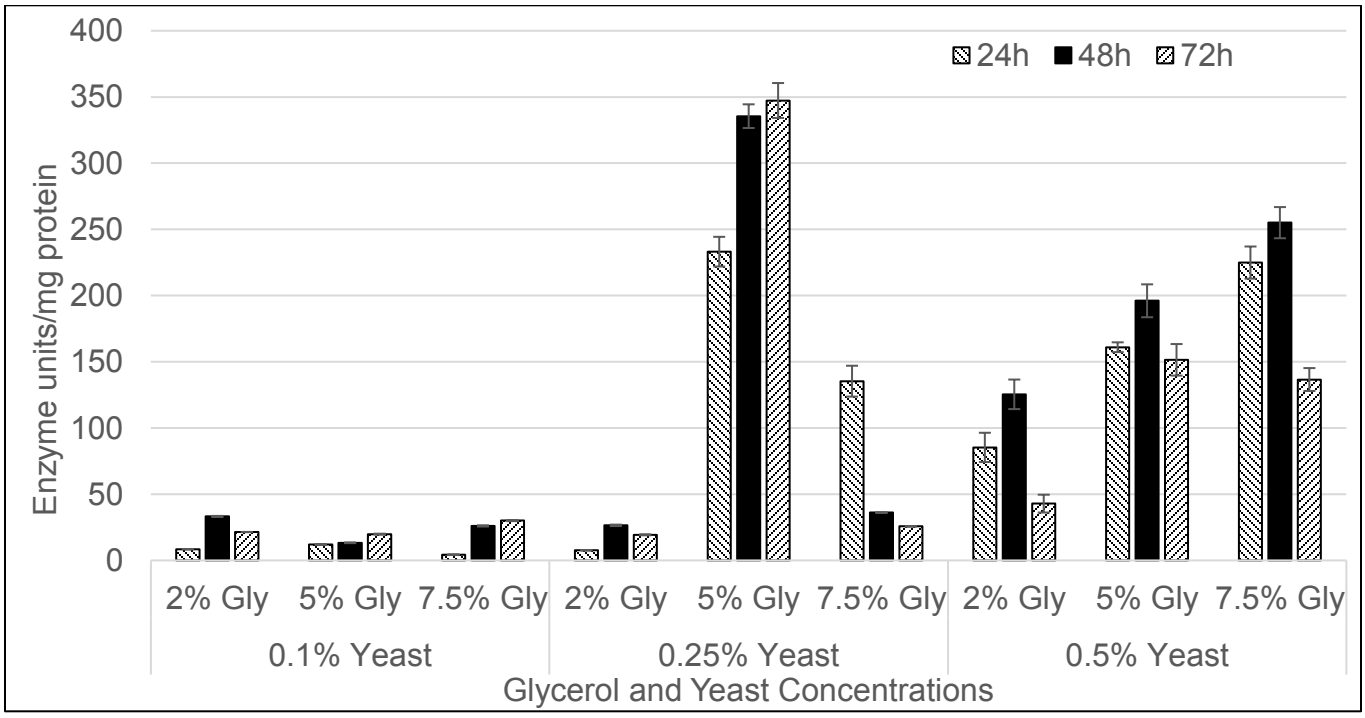

Figure 4.3. Effect of glycerol and yeast extract concentration on enzyme activity of SRP3 strain,

minimal salt (MS) medium supplemented with different concentrations of glycerol and yeast extract, stating pH 7.0 and incubation temperature 35^0C.

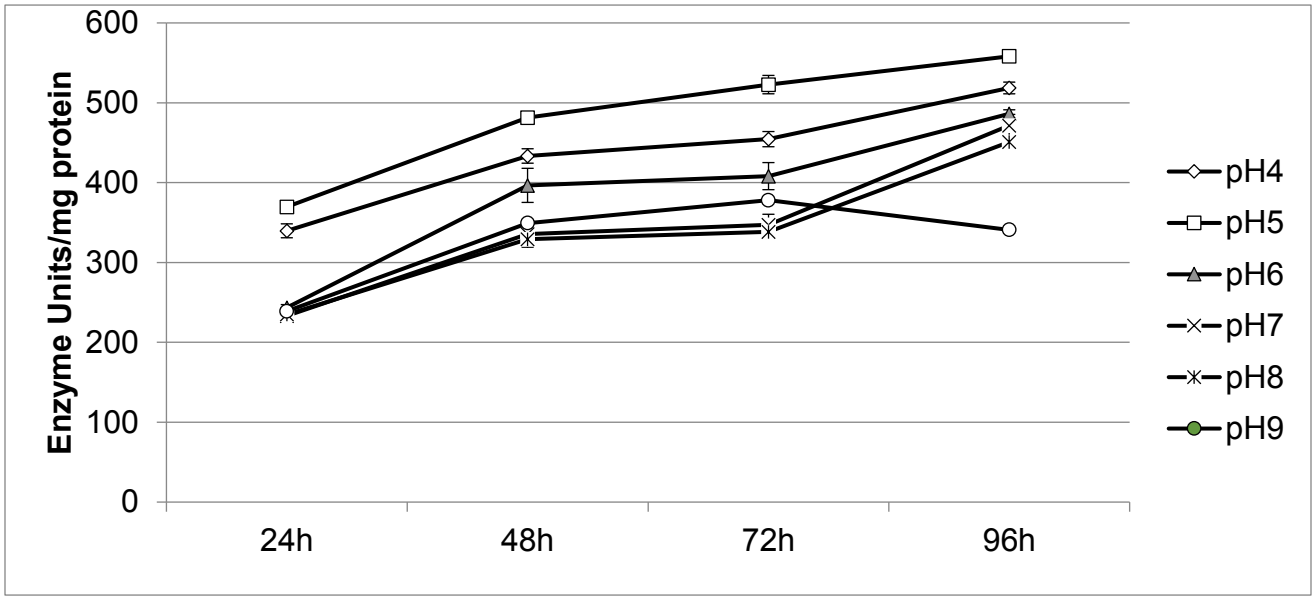

Figure 4.4. Influence of initial pH on enzyme activity of SRP3 without pH control, incubation temperature 35^0C, minimal salt (MS) medium supplemented with glycerol 5.0% and yeast extract 2.5%, and incubation temperature was 35^0C.

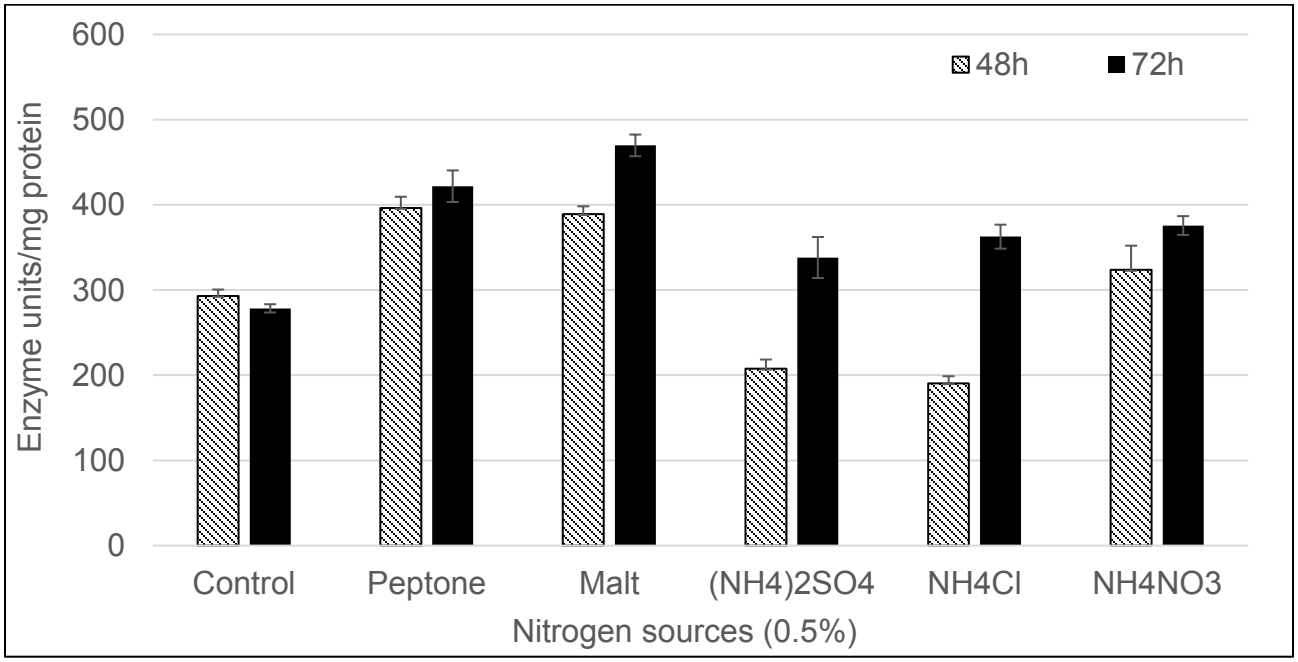

Figure 4.5. Effect of nitrogen sources on enzyme activity of strain SRP3, minimal salt (MS)

medium supplemented with glycerol 5.0% and yeast extract 0.25%, stating pH 7.0 and incubation temperature 35^0C.

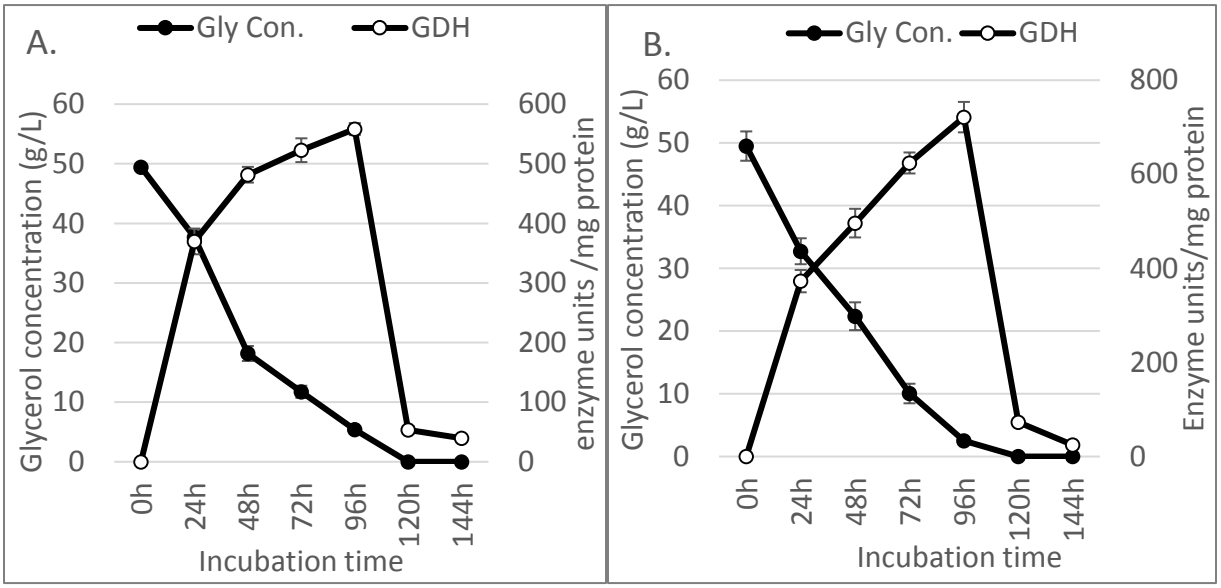

Figure 4.6. Glycerol consumption and specific activity of GDH by SRP3 (A) and SRM3 (B) in fermentation medium at initial pH 5.0 and incubation temperature 35^0C.

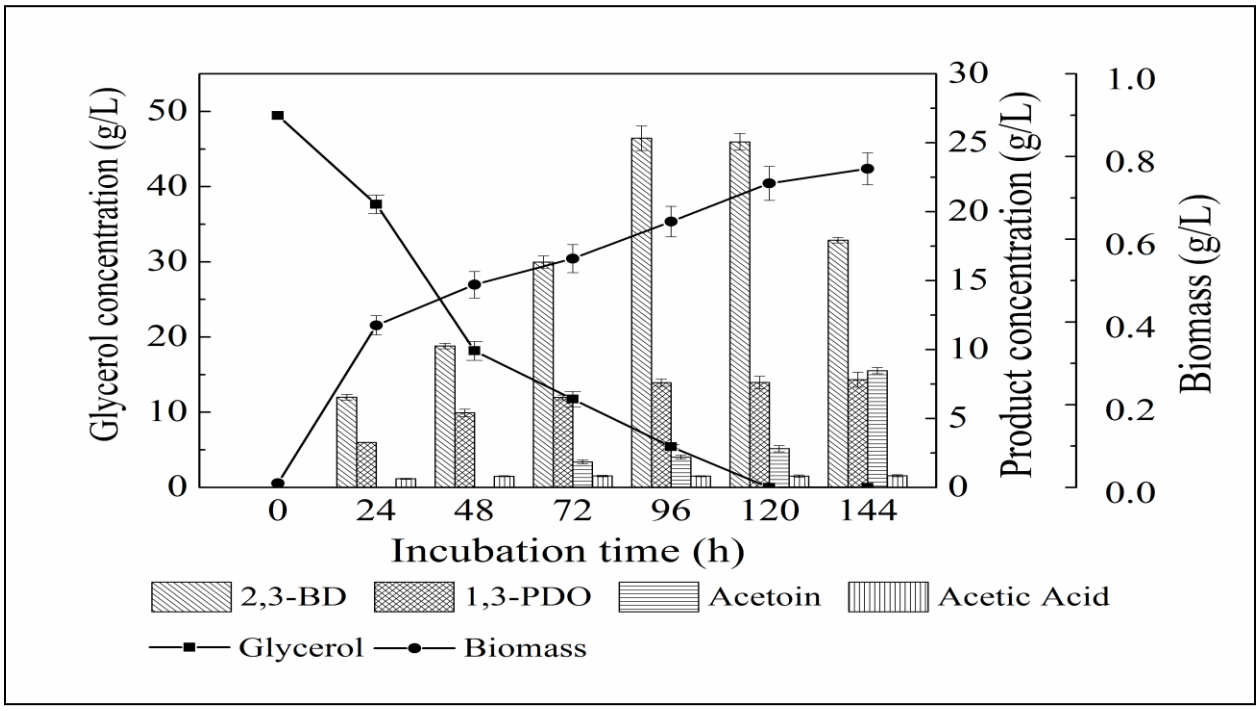

Figure 4.7. Production kinetics of major products obtained by *K. variicola* SRP3 in fermentation medium without pH control, starting pH 5.0 and incubation temperature was 35^0C.

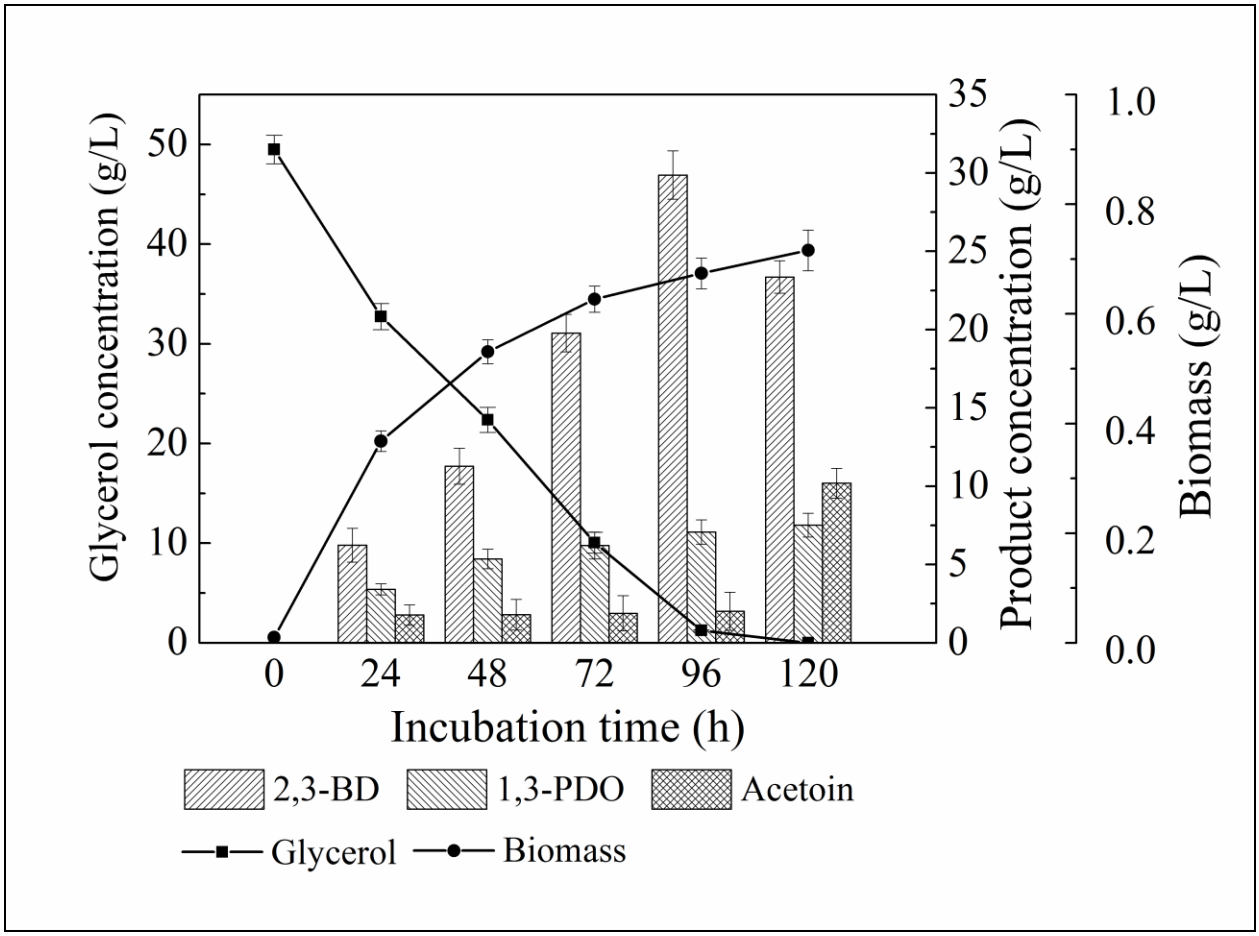

Figure 4.8. Production kinetics of major products obtained by adapted strain *K. variicola* SRM3 in fermentation medium without pH control, started at pH 5.0, incubation temperature 35^0C.

Chapter 5: Utilization of by-product glycerol from bio-diesel plants as feedstock for 2,3-butanediol accumulation and biosynthesis genes response of *Klebsiella variicola* SW3

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Abstract

The booming of biodiesel industry all over the world has led to generate a large amount (10% v/v) of crude glycerol, created an oversupply problem. Herein, we compared the product concentrations of major metabolic products attained from pure and crude glycerol biotransformation process using an adapted mutant strain *Klebsiella variicola* SW3. Real-time qPCR and glycerol dehydrogenase (GDH) enzyme activity assay revealed that the overexpression of GDH gene resulted in an increased GDH enzyme activity, led to a markedly boosted 2,3-butanediol (2,3-BD) production. Based on these results, the SW3 strain obtained from wild type strain *Klebsiella variicola* SRP3 displayed a 1.39-fold increased 2,3-BD production of 82.5 g/L from 59.3 g/L, yielding 0.62 g/g using pure glycerol. However, in a batch culture, a final 33.5 g/L of 2,3-BD of was accumulated within 96h from 50 g/L glycerol. Moreover, the strain SW3 withstanding high concentration (200 g/L) of crude glycerol displayed 64.9 and 29.25 g/L 2,3-BD in fed-batch and batch cultures respectively. Therefore, this bioconversion of crude glycerol to 2,3-BD –a value-added green product with potential industrial applications as a liquid fuel or fuel additive would represent a remarkable alternative to add value to the biodiesel production helping biodiesel industries development.

Keywords: Crude glycerol; 2,3-Butanediol; Biodiesel; Liquide fuel; Fuel additive; *Klebsiella variicola*.

5.1. Introduction

In recent years, the demand of biofuels has increased dramatically. Biodiesel, a renewable vibrant biofuel, is produced from vegetable oils and animal fats by transesterification. A large amount (10% v/v) of crude glycerol is generated as a core by-product during the synthesis of biodiesel. Therefore, crude glycerol produced from biorefineries is a worldwide overflow problem due to a nonexistence of purifying facility. The increasing market for biodiesel has conspicuously altered the cost and obtainability of glycerol generated from biodiesel manufacturing plants. Consequently, a high volume of this biodiesel derived crude glycerol is a crucial problem for development and economic feasibility of biodiesel industry [1]. Moreover, with rising biodiesel plants, a considerable number of glycerol production industries will be shut down within few years due to price drop by overflow of glycerol [2,3] that require new profitable products from glycerol conversion. Besides this, the purification or disposal of non-biodegradable biodiesel by-product is intricate, costly, and it is becoming a noteworthy cost factor for the biodiesel industries [4]. Consequently, due to its low price, biodiesel derived crude glycerol is considered as waste and it is, therefore, important that new and sustainable applications should be explored for glycerol utilization. Therefore, biotechnological approach, a biological process of glycerol conversion could help evade the disadvantages of chemical conversion process, whereas offering the opportunity to produce numerous value-added products [5-6]. Glycerol, a simple three carbon sugar alcohol would be a potential carbon and energy source for microbial growth to synthesize a number of valuable biotechnological products [6]. A large number of microorganisms is capable of utilizing glycerol as a sole carbon source in the synthesis of a lot of value-added products

including 1,3-PDO (1,3-propanediol), ethanol, hydrogen, 2,3-BD, and organic acids [6-8]. There is no metabolically efficient microorganism which can convert glycerol effectually to produce high value products. The major microbial conversion route of glycerol leads to produce of 1,3-PDO through an anaerobic fermentation process [4-5,9]. Biodiesel derived crude glycerol contains methanol, salts, catalyst and organic acids as the main impurities which are negatively influenced on bioconversion process [10]. If the crude glycerol is used as a renewable waste substrate to produce value-added products through bioconversion process would offer a substantial option for the inventive and profitable waste management of biodiesel plants. Bioconversion of glycerol to 2,3-BD is a promising and alternative to chemical synthesis. Our main objective is high production of 2,3-BD from glycerol as well as crude glycerol by developing an adapted mutant strain(s) with increased tolerance toward crude glycerol under completely aerobic process.

2,3-BD, a glycol, is an expensive and important bulk chemical which is used in pharmaceutical, plastic, antifreeze solution and solvent preparation. Furthermore, as a platform chemical, 2,3-BD is widely used as reagent for the synthesis of a number of chemicals, and it could be converted to liquid fuel or fuel additive [13-14]. 2,3-BD is also a reduced from of acetoin. A metabolic product acetoin is an important platform chemical with many applications in cosmetics, food, and synthesis of many chemicals [15]. 2,3-BD and acetoin can be transferred to each other in cells [15].

The metabolic reactions proposed for glycerol assimilation in *K. pneumoniae* basically follow oxidative and reductive pathways (Fig 5.1), which results in the synthesis of major products including dihydroxyacetone (DHA), 2,3-BD, 1,3-PDO, acetoin, ethanol, acetate and other organic acids. In the oxidative branch, an important cell-bounded (intracellular) and NAD^+-dependent enzyme GDH converts glycerol to DHA, producing $NADH_2$ as a reducing equivalent [11-12].

Subsequently, dihydroxyacetone phosphate is generated from DHA by adenosine triphosphate-depended dihydroxyacetone kinase, which is then further undergoes glycolysis to form various products through pyruvate [12]. Meanwhile, $NADH_2$ produced after receiving electrons from oxidation of substrate organic compounds could stimulates 1,3-PDO production in aerobic process [12]. Moreover, glycerol is firstly converted to 3-hydroxypropionaldehyde by the coenzyme B_{12}-dependent glycerol dehydratase through the parallel reductive pathway, and it is then converted by $NADH_2$-linked 1,3-PDO dehydrogenase to the major product 1,3-PDO [12].

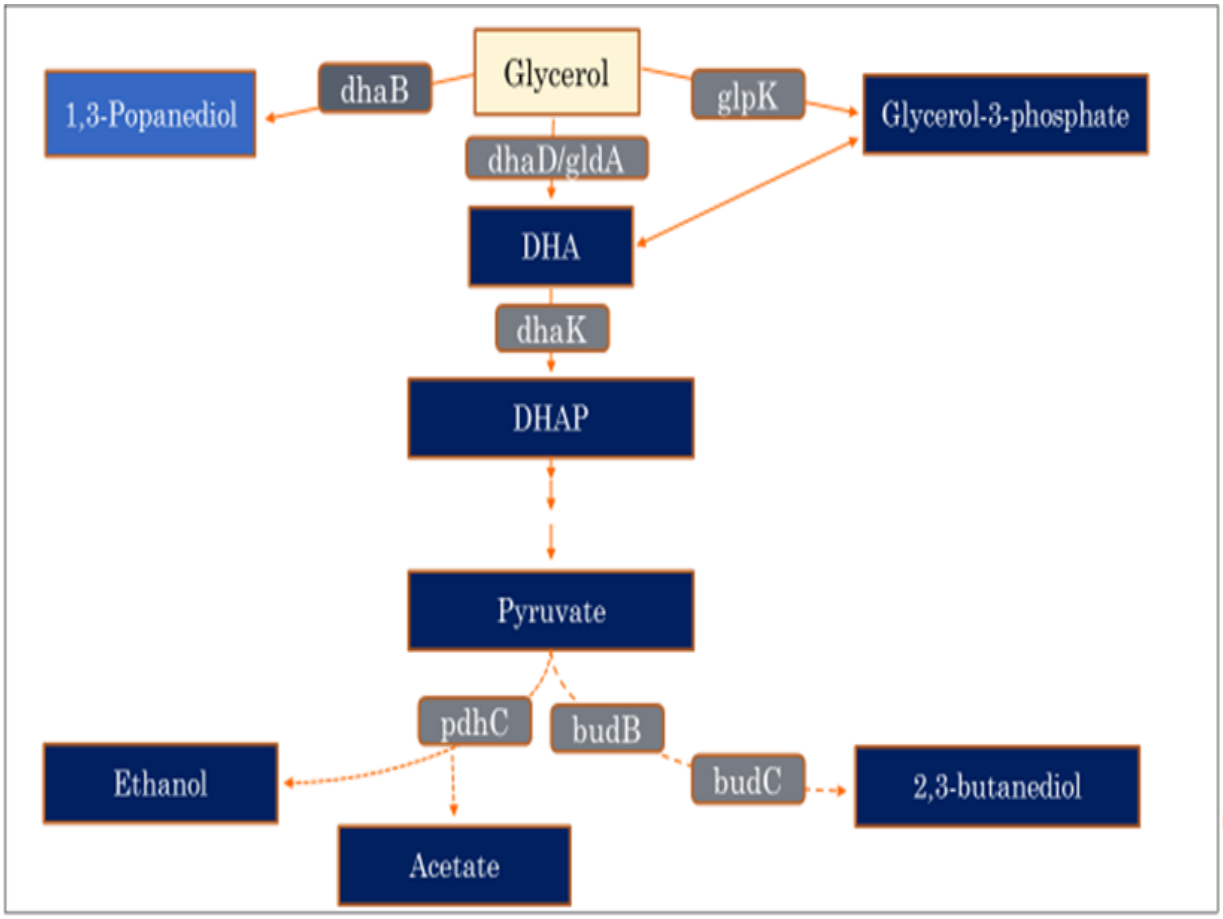

Fig 5.1. Schematic representation of initial steps in glycerol metabolism pathway of *K. pneumoniae*. The key enzymes/representative genes related to glycerol metabolism are glycerol dehydratase (dhaB), glycerol dehydrogenase (dhaD and gldA), dihydroxyacetone kinase (dhaK), glycerol kinase (glpK), acetolactate synthatase (budB), acetoin reductase (budC) and pyruvate dehydrogenase (pdhC).

Several species of *Klebsiella* are known to produce 1,3-PDO as a core metabolic product through glycerol bioconversion process [4-5,9-10]. Our intention of this research is to study the development of a new strain of bacteria capable of competently accumulation and convert by-product glycerol from biodiesel plant into value-added products under completely aerobic condition. Moreover, the aim of this study is to pinpoint transcriptional profiles of key enzyme genes related to the biosynthesis pathways of 2,3-BD. Instead, through investigation of metabolic activity, we developed an adapted mutant strain (*K. variicola* SW3) that is withstanding a high concentration of biodiesel waste (residual glycerol). In the current study, we have, therefore, chosen a newly developed strain of *K. variicola* SRP3 with increased tolerance towards crude glycerol by evolutionary adaptation (mutagenesis). Here, we report a high product concentration of 2,3-BD from glycerol as a sole carbon source by an adapted mutant strain SW3 obtained from our newly isolated strain *K. variicola* SRP3.

5.2. Materials and methods

5.2.1. Biodiesel by-product glycerol/crude glycerol

Crude (raw) glycerol obtained from biodiesel synthesis was supplied by CARES (Centre for Agricultural Renewable Energy and Sustainability) biodiesel plant (Guelph, Canada), which uses vegetable oil as the raw material. In this small plant, about 600,000 L/year biodiesel production is obtained by the transesterification of vegetable oil using a catalyst methanol. This crude glycerol was directly used as a feedstock for microbial growth. The composition of the crude glycerol was determined as mass fractions (glycerol 50.0%, ash 4.2%, MoNG 36.2%, water 6.7%, and pH 10.6).

5.2.2. Bacterial strains and culture conditions

K. variicola SW3 (GenBank Accession No. KY437692), an adapted mutant strain was used throughout the study. *K. variicola* SRP3 (GenBank Accession No. KR092086), a wild type strain isolated from paper mill (Resolute Forest Products, Canada) waste, reported in our earlier paper [13] was used to develop the adapted mutant SW3. Seed culture for batch and fed-batch process was prepared from stock slant culture by inoculating into a Luria-Bertani (LB) broth medium contained (g/L): peptone 10.0, yeast extract 5.0, NaCl 5.0. The seed LB broth culture was incubated at 37°C and 200 rpm under aerobic conditions for 20 h. For batch and fed-batch cultures, the seed culture was inoculated into MS-2 medium contained K_2HPO_4 (0.1 g/L), $NaNO_3$ (0.1 g/L), $MgSO_4.7H_2O$ (0.05 g/L), KCl (0.1 g/L), yeast extract (2.5 g/L) and peptone (5.0 g/L). Different concentrations of pure or crude glycerol were used in the MS-2 medium as a carbon source. All the growth parameters were performed in triplicates. The batch and fed-batch cultures were carried out in 125 ml Erlenmeyer flasks (50 ml medium, 100 µl of 20 h culture) at 37^0C and 200 rpm. In case of fed-batch culture, the glycerol concentrations were varied. In our fed-batch culture, an initial concentration of glycerol was 50 g/L, and then a concentrated solution containing 800 g/L of pure glycerol was fed into the culture as required. However, in fed-batch culture, the initial crude glycerol was 100 g/L, and this crude glycerol was directly fed into the culture as required.

5.2.3. Strain development and identification

The wild type strain *K. variicola* SRP3 was mutagenized by evolutionary adaptation technique using pure glycerol which was described in our earlier work [13]. One of the utmost 2,3-BD producing strains achieved from this evolutionary adaptation process was further gradually developed (adapted) with increased tolerance toward crude glycerol using the same protocol [13]. This evolutionary developed adapted mutant strain nominated as *K. variicola* SW3 could grow at

a concentration of 200 g/L crude glycerol. The partial sequence of 16S ribosomal RNA gene of this adapted mutant SW3 was input into NCBI BLAST tool, compared with the sequences in the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/). The phylogenetic relationship was analyzed using sequence alignment program of the Neighbor-Joining method with MEGA6. The partial sequence of 16S rDNA of the strain SW3 was submitted to the NCBI GenBank for accession

5.2.4. RNA isolation and quantitative real-time PCR

For isolation of RNA, seed cultures of SRP3 and its adapted mutant SW3 were prepared from stock slant culture by inoculating into a LB broth medium, incubated at 37°C and 200 rpm for 20h. Following incubation, 1% (v/v) of the LB broth seed culture was inoculated into 125 ml flash containing 50 ml MS-2 medium supplemented with different concentrations (0 g/L, 25g/L, 50 g/L and 75 g/L) of glycerol, incubated at 37°C and 200 rpm into a shaker incubator for 72h. Subsequent incubation, the cells collected after centrifugation at 5000g and 4°C for 5 min was used for RNA isolation. RNA was isolated according the protocol of PureLink^{Tm} RNA extraction kit (Ambion, Thermo Fisher Scientific, USA). The concentration of each RNA sample was determined using NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). The first-stand cDNA was synthesized using cDNA synthesis kit (Tetro cDNA synthesis Kit, Bioline, UK) and RNA as a template. Quantitative gene expression was carried out using SensiFAST^{TM} SYBR No-ROX Kit (Bioline, UK) on C1000^{TM} thermal cycler quantitative real-time PCR (qRT-PCR) detector system (BioRad, USA). The 16S rRNA, obtained based on the primers 5′-GCGGTTGTTACAGTCAGATG-3′ and 5′-GCCTCAGCGTCAGTATCG-3′ was used as an internal standard. The primer sequences of glycerol metabolism genes used in this study are

presented in Table 5.1. The $2^{-\Delta\Delta CT}$ method was used to analyze the fold change gene expression over control [16].

Table 5.1. List of primers designed for gene expression studies by qRT-PCR.

Genes	Enzymes	Primer sequences
dhaD	Glycerol	F: AGCGGGCGAGTTTGAAGAGTATCTG
	dehydrogenase	R: GCCAGCAGCGTATCATAGCACAGG
gldA	Glycerol	F: CGAACACTCACCGCCAAACGG
	dehydrogenase	R: TCGCATTATTCAATCACCAGGCAAAT
dhaB	Glycerol dehytratase	F: ACCAGTGCCACGTCACCAATCTC
		R: CGGACTGCGAACCGACCAACA
dhaK	Dihydrohyacetone	F: GCTGAGATGGCGATTCTGACCC
	kinase	R: GGTGGATGGCACGACGTTGATAG
glpK	Glycerol kinase	F: CGGGTCCAGACCGCTCAAAT
		R: GGGCTGGGCGAACAGGTAAA
budB	Acetolacteate	F: ACCAGTGCCACGTCACCAATCTC
	synthatase	R: TGGTGGACCTGTTTGGCTTTATCG
budC	Acetoin reductase	F: TGTTTCTGACCGCGACCAGGTATT
		R: CCCGCCGTGACCCTCTTTCTTA
pdhC	Puruvate	F: TTCTCCTGTTGCTGGGACGAT
	dehydrogenase	R: GTGCTACTCCGCCTTGCTTTG

F: Forward; R: Reverse

5.2.5. Enzyme assay and toxicity test

GDH enzyme activity was determined at room temperature using microplate spectrophotometer (EPOCH, BioTek) by measuring the initial reduction rate of NAD at 340 nm as described in our earlier report [13]. Protein was quantified by the standard protocol of Bradford [17], using bovine serum albumin as a standard protein.

For toxicity test, the minimum inhibition concentration (MIC) of glycerol were performed in batch culture. A series of MS-2 culture medium tubes were prepared with different concentrations

of glycerol ranging from 0 to 350 g/L, inoculated with 50 µL of a 20 h culture, and incubated at 37^{o}C for 120h. Following incubation, growth was evaluated spectrophotometrically based on the absorbance at 600 nm, and the dry cell mass was measured by using a standard curve (a linear correlation between the dry cell mass and absorbance of cell suspension). All tests were conducted in at least triplicates.

5.2.6. Quantification of biomass, glycerol and metabolic products

Biomass or cell dry weight (g/L) was calculated from absorbance at 600 nm using the calibration curve. Optical density at 600 nm (OD_{600}) obtained from microplate spectrophotometer (EPOCH, BioTek) was converted to cell dry weight using a calibration equation (y = 1.9175x + 0.0183, where y = OD_{600}, x = biomass g/L). This calibration equation was obtained from the standard curve of a linear correlation between the dry cell mass and absorbance (OD_{600}) of cell suspension. Metabolic products were identified by GC-MS (Varian 1200 Quadrupole). The concentrations of major metabolic products 2,3-BD, 1,3-PDO, acetoin and acetate, as well as glycerol were quantified using a GC-FID (Shimatzu GC 14A) under the following conditions: sample volume 1 µl; column temperature range from 45°C (2 min) to 240°C at the rate of 10°C/min; the injector and detector temperature 250°C; carrier gas nitrogen; column DB-WAXetr. The injecting sample was purified by centrifugation (Fisher Scientific, Germany, accu Spin Micro 17,) and membrane filter (0.22 µm pores size) respectively.

5.3. Results

5.3.1. Strain development, phylogenetic relationship and toxicity

An **a**dapted mutant strain *K. variicola* SW3 with increased tolerance toward pure and crude glycerol developed from wild type strain *K. variicola* SRP3 able to grow at 20% (200 g/L) of crude glycerol or pure glycerol. The partial sequence of 16S rDNA of the strain SW3 inputted into

NCBI Basic Local Alignment Search Tool (BLAST) showed 99% sequence similarity with its wild type strain SRP3. After submitted the partial sequence (16S rDNA) of SW3 in GeneBank, the accession number is released as KY437692.

Nevertheless, the 16S rDNA sequence of SRP3 and SW3, and other *Klebsiella* strains retrieved from GeneBank are presented as a phylogenetic tree (Fig. 5.2) for their phylogenetic relationship analysis. The phylogenetic tree was constructed using the Neighbor-Joining method. The evolutionary history was inferred using the Neighbor-Joining method through NCBI website. The result of phylogenetic relationship confirmed mutagenesis through the distance between the *K. variicola* SRP3 and its adapted mutant *K. variicola* SW3.

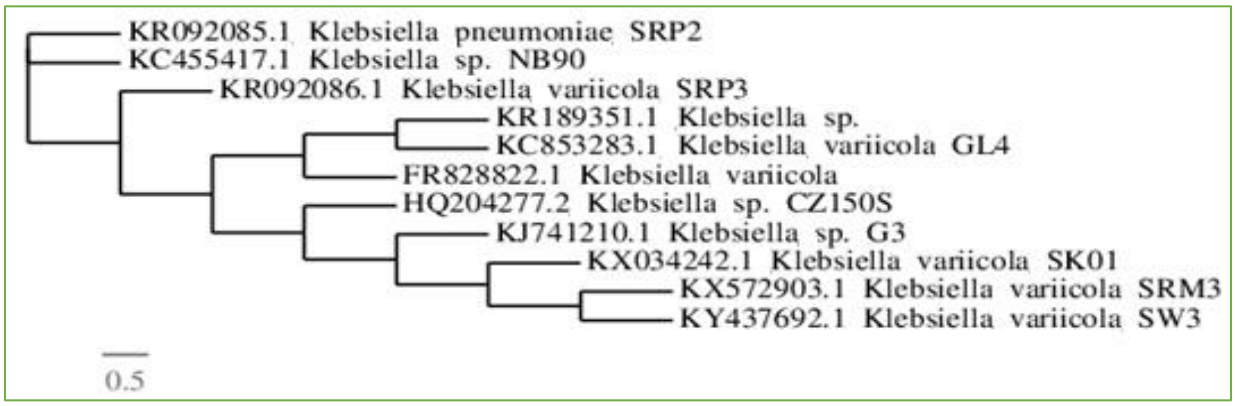

Fig 5.2. Evolutionary relationships of the strains: Phylogenetic tree drawn from sequence alignment program using Neighbor-Joining method with MEGA6, presented in TreeView. 16S rRNA gene sequences were retrieved by nucleotide BLAST searches in NCBI. The numbers that follow the names of the strains are accession numbers of published sequences. The scale bar represents 0.5 substitutions per nucleotide position.

To assess pure and crude glycerol utilization competence, toxicity test was performed. *K. variicola* SW3, an evolutionary adapted mutant was used for a toxicity test towards different concentrations of pure and crude glycerol (Table 5.2). Growth of SW3 was detectable after 120h incubation at the initial concentrations of 300 g/L glycerol. Based on this result, the strain SW3

appeared most tolerant to pure and crude glycerol. MIC of glycerol was determined as 325 g/L (Table 5.2).

Table 5.2. Minimum inhibitory concentration (MIC) of glycerol after 120 h incubation in MS-2 medium supplemented with glycerol.

Glycerol (g/L)	Biomass* (g/L)	Growth in broth medium	MIC (g/L)
0	0.485±0.021	+	
50	0.619±0.043	+	
100	0.513±0.032	+	
150	0.277±0.028	+	
200	0.129±0.017	+	325
250	0.044±0.006	+	
300	0.022±0.004	+	
325	0	-	
350	0	-	

*Dry weight of bacterial cell biomass

5.3.2. Quantitative real-time PCR analysis and enzyme activity

To demonstrate the expression of the genes responsible for 2,3-DB accumulation in SRP3 and SW3 strains, the experiment was carried out with qRT-PCR. With the aim of target, the genes related to 2,3-BD accumulation and learn about the transcriptional changes in response to different concentrations of glycerol in a batch culture, a preliminary effort has been made to search differentially expressed genes. Therefore, the expression levels of genes accountable for important enzymes in 2,3-BD, acetoin, 1,3PDO and acetate metabolic pathway using real-time qPCR were studied. For gene expression study, all the bacterial cells were cultured for a constant period to minimize its effect on the analysis of RT-PCR. The quantitative relationship among the expression levels of these genes with the strains SRP3 and SW3 at different concentrations of glycerol are presented in Figure 5.3. In this study, we presented a comparison of expression levels of a key

gene (dhaD) and its enzyme (GDH) activity between the two strains SRP3 and SW3 at different concentrations of glycerol (Fig. 5.4a). However, for the productions of 2,3-BD and biomass, the comparison between SRP3 and SW3 strains is shown in Figure 5.4b. As shown in figure 5.3, the results indicated that 50 g/L (5.0%) glycerol could stimulate relative expression levels of the genes dhaD, gldA, glpK, budB and budC in the 2,3-BD metabolic pathway, and the mutant strain SW3 displayed higher expression levels compared to that of its wild type strain SRP3.

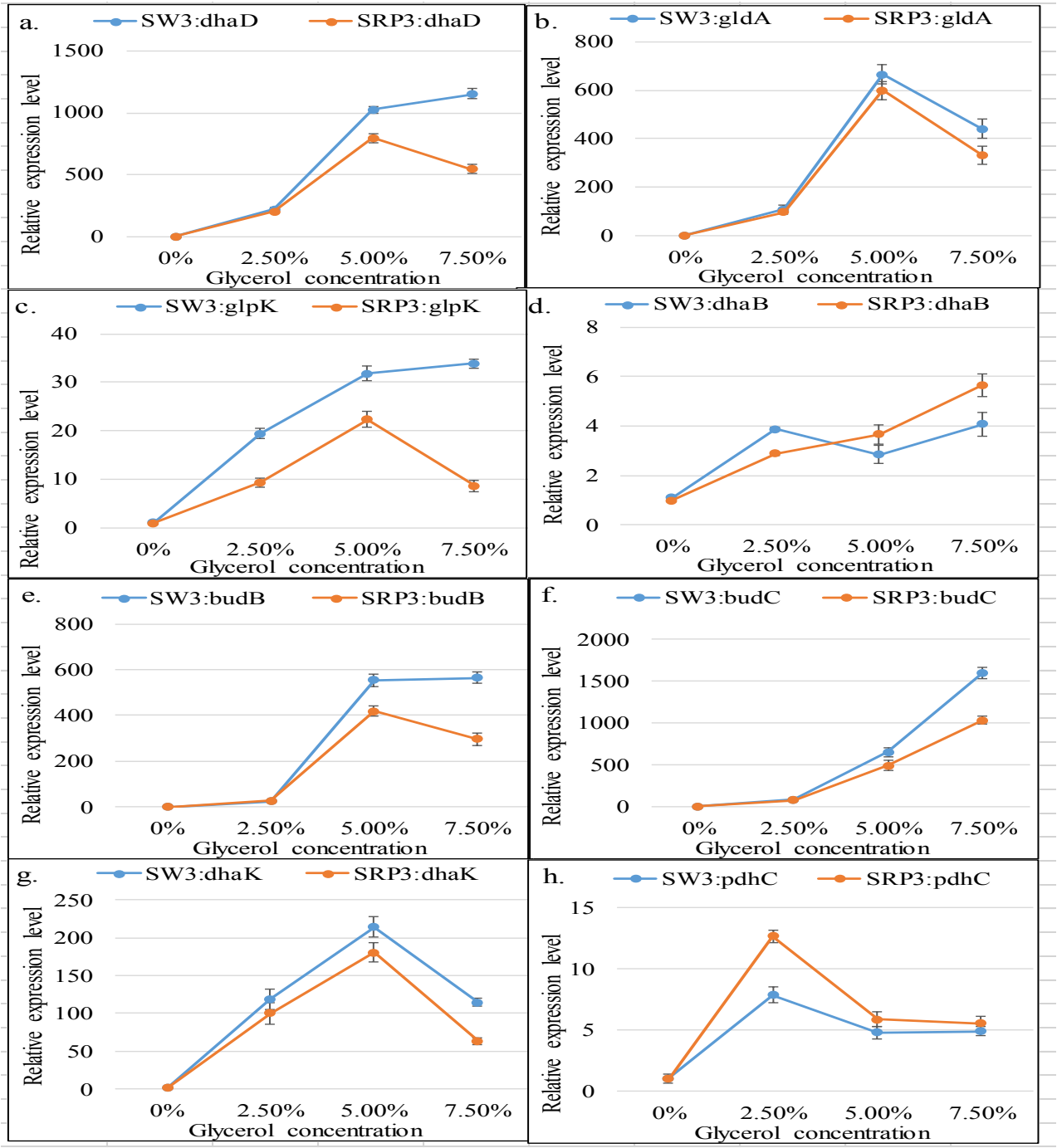

Fig 5.3. Expression level of glycerol dehydratase (dhaB), glycerol dehydrogenase (dhaD and gldA), dihydroxyacetone kinase (dhaK), glycerol kinase (glpK), acetolactate synthatase (budB), acetoin reductase (budC) and pyruvate dehydrogenase (pdhC) genes in glycerol metabolism.

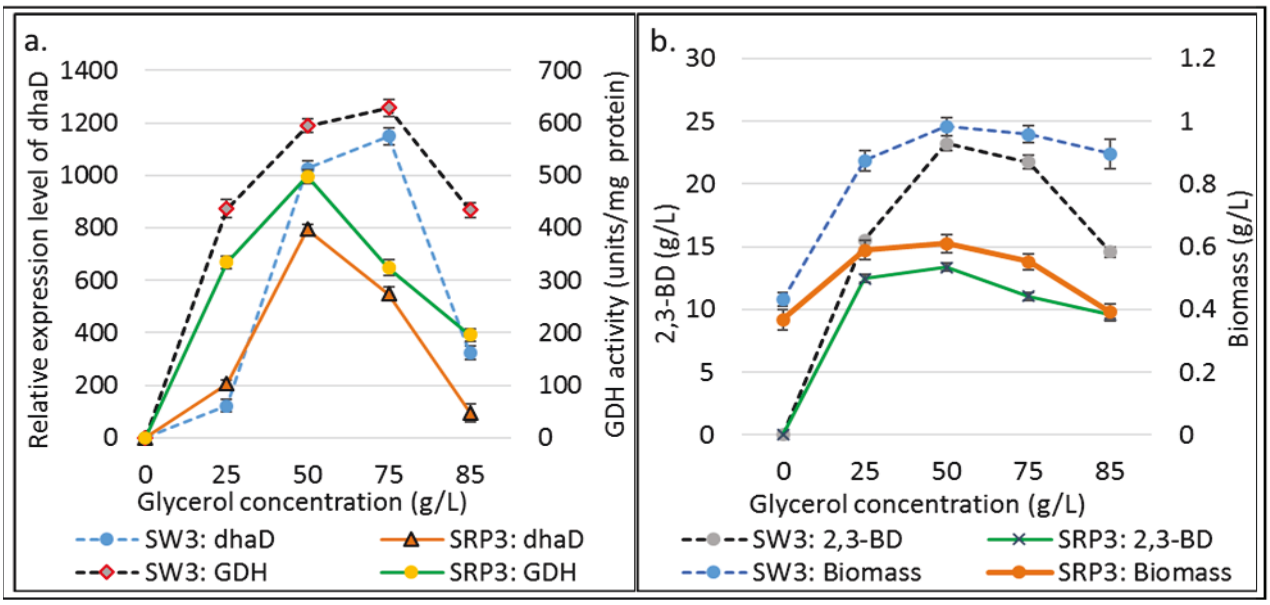

Fig 5.4. Comparison between SRP3 and SW3 strains: (a) GDH activity and relative expression level of dhaD; (b) 2,3-BD and biomass productions.

5.3.3. Biotransformation of glycerol in batch and fed-batch culture

To evaluate glycerol assimilation and 2,3-BD production using *K. variicola* SRP3 and *K. variicola* SW3, aerobic bioconversion was performed with pure and crude glycerol as the carbon sources. Biotransformation of glycerol to 2,3-BD and other metabolic products was carried out in shake flasks at initial pH 7.0. Batch and fed-batch process were carried out under optimized conditions for 2,3-BD production. Experiments were set at 37°C in a shaker incubator. MS-2 medium supplemented with specified concentrations of pure or crude glycerol were used for both batch and fed-batch cultures. This biotransformation process revealed two major metabolic products 2,3-BD and acetoin with other minor products viz., acetate and 1,3-PDO. The concentrations of glycerol (substrate) as well as end (metabolic) products as a function of incubation time in a batch culture process of pure glycerol biotransformation by the SRP3 and its adapted mutant SW3 are presented in the Figure 5.5a and 5.5b. The wild type *K. pneumoniae* SRP2 reported this article, used 100% of glycerol within 120 h, producing 22.72 g/L 2,3-BD, 2.11 g/L

1,3-PDO, 6.44 g/L acetoin and 0.82 g/L acetate in batch culture under aerobic condition. The maximum concentration and yield of 2,3-BD obtained after 120 h incubation were 22.72 g/L and 0.45 g/g respectively (Fig. 5.5a). However, a maximum 33.52 g/L of 2,3-BD was achieved from 50.0 g/L glycerol after 96 h incubation by SW3, and the product yield was 0.65 g/g (Fig. 5.5a). Conspicuously, 100% of glycerol consumed after 96 h incubation by the strain SW3, and the concentrations of foremost end products other than 2,3-BD obtained from our batch culture were acetoin, acetate and 1,3-PDO, with concentrations, 2.35 g/L, 0.72 g/L and 0.94 g/L respectively (Fig. 5.5b). Notably, as shown in Figure 5.5a, the adapted mutant strain *K. variicola* SW3 exhibited less concentrations of 1,3-PDO and acetate compared to that of *K. variicola* SRP3 in batch culture. The SW3 strain attained from SRP3 using adapted mutagenesis was capable to grow in a high concentration of pure glycerol or crude glycerol, up to 200 g/L. Consequently, this adapted strain *K. variicola* SW3 was further investigated as a possible effectual strain for high production of 2,3-BD using crude glycerol as a carbon source. Moreover, this SW3 strain was better adapted for utilizing crude glycerol, noticeably convert it to 2,3-BD compared to its wild type strain SRP3.

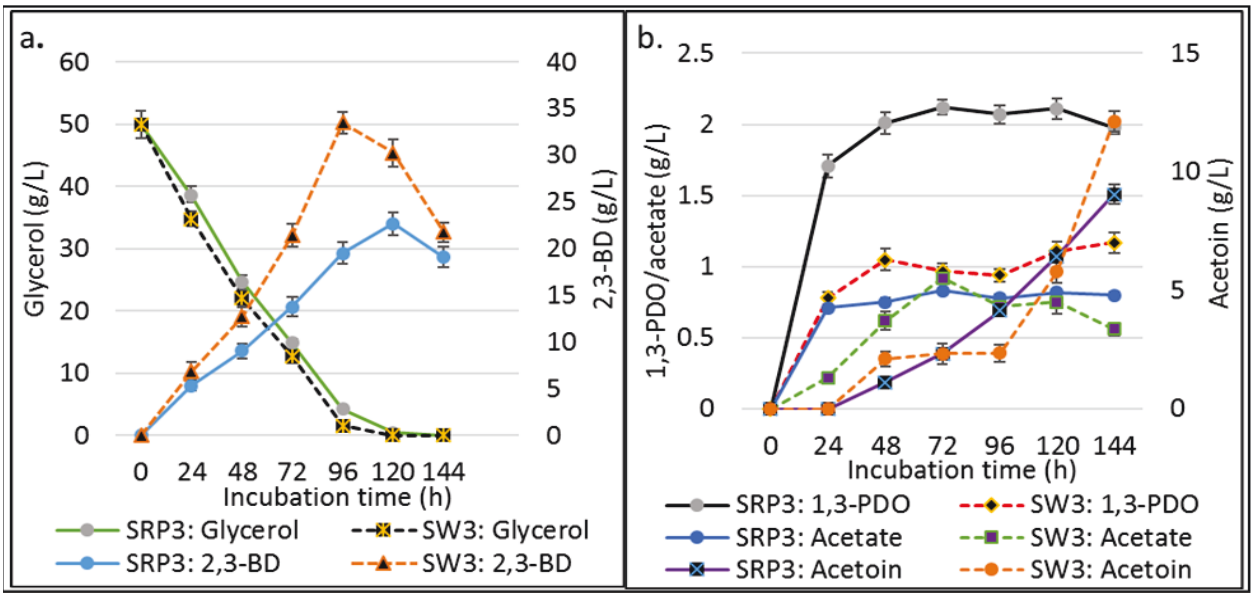

Fig 5.5. Comparison of time course data and metabolic products of *K. variicola* SRP3 and *K. variicola* SW3 in batch culture using pure glycerol: (a) glycerol, and 2,3-B concentrations; (b) concentrations of 1,3-PDO, acetate and acetoin. MS-2 medium with glycerol 50.0 g/L; incubation temperature 37^0C, and initial pH 7.0.

To explore the feasibility of crude glycerol utilization and a high concentration of 2,3-BD production in a batch culture by *K. variicola* SW3, the experiment was performed in shake flask (200 rpm) using MS-2 medium containing 100 g/L biodiesel-derived crude (raw) glycerol as a carbon source. To investigate whether the impurities present in biodiesel derived crude glycerol would inhibit 2,3-BD production as well as cell growth of *K. variicola* SW3, batch culture process using crude glycerol was performed, and the results are presented in Figure 5.6. When biotransformation was performed for 144h using crude glycerol, *K. variicola* SW3 effectively produced 2,3-BD at the concentration up to 29.25 g/L with the yield of 0.59 g/g (Fig. 5.6a). In case of *K. variicola* SRP3, the product concentration and product yield of 2,3-BD were obtained up to 26.51 g/L and 0.53 g/g respectively (Fig. 5.6a). Furthermore, the maximum biomass (cell dry weight) of *K. variicola* SW3 was also higher than that of *K. variicola* SRP3 (1.51 g L^{-1} vs. 1.13 g

L^{-1}). The foremost metabolic products other than 2,3-BD were acetoin and 1,3-PDO with little acetic acid (Fig. 5.6b).

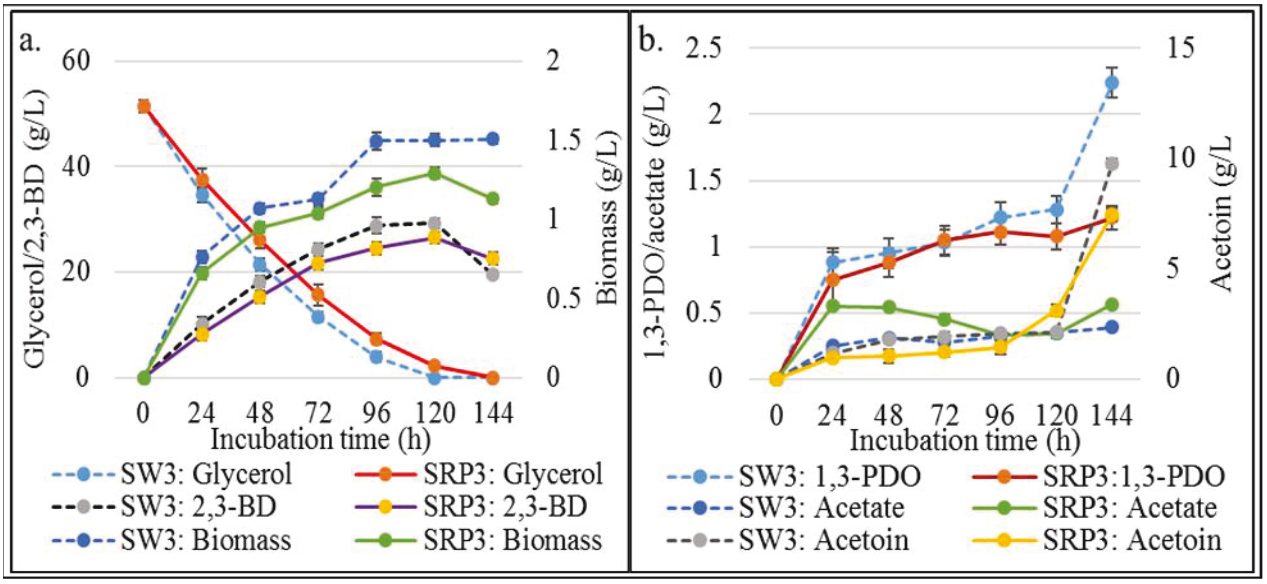

Fig 5.6. Comparison of time course data and metabolic products of *K. variicola* SRP3 and *K. variicola* SW3 in batch culture using crude glycerol: (a) glycerol, 2,3-B and biomass concentrations; (b) concentrations of 1,3-PDO, acetate and acetoin. MS-2 medium with raw (crude) glycerol 100.0 g/L; incubation temperature 37^0C, and initial pH 7.0.

In fed-batch process, the biotransformation kinetics displayed that SW3 produced high concentration of the principal product 2,3-BD in aerobic condition. Table 5.3 compares the production of principal product 2,3-BD as well as other co-products in fed-batch process by *K. variicola* SRP3 and *K. variicola* SW3 using pure and crude glycerol. As shown in Table 5.3, a high concentration (82.5 g/L) of 2,3-BD was attained from fed-batch culture using pure glycerol, utilized 134.0 g/L glycerol by the adapted mutant strain *K. variicola* SW3. However, for the crude glycerol used in fed-batch culture, the product concentration of 2,3-BD was achieved up to 64.93 g/L by the same strain SW3, consumed 102.33 g/L glycerol. The product yields of the principal metabolic product 2,3-BD were obtained up to 0.62 g/g and 0.63 g/g using pure and crude glycerol respectively (Table 5.3).

Table 5.3. Comparison of fed-batch bio-transformations by *K. variicola* SRP3 and *K. variicola* SW3 using pure glycerol and biodiesel derived raw (crude) glycerol.

Culture parameters/ products	*K. Variicola* SRP3		*K. Variicola* SW3	
	Pure glycerol	Crude glycerol	Pure glycerol	Crude glycerol
Final pH	5.25	6.48	5.42	6.23
Dry weight of cell (g/L)	2.08	1.87	2.53	2.37
Glycerol utilized (g/L)	101.2	76.65	134.0	102.33
2,3-BD (g/L)	59.3	43.79	82.5	64.93
2,3-BD yield (g/g)	0.59	0.57	0.62	0.63
Acetoin (g/L)	8.1	5.71	5.9	6.32
Acetoin yield (g/g)	0.08	0.07	0.07	0.06
1,3-PDO (g/L)	3.12	4.83	2.4	2.85
1,3-PDO yield (g/g)	0.03	0.06	0.03	0.03
Incubation time (h)	192	168	216	168

All experimental values presented are mean values from triplicate experiments

5.4. Discussion

With the flourishing of biodiesel manufacturing plants, global oversupply of biodiesel derived crude glycerol is increasing dramatically [1,3]. A large amount (10% v/v) of crude glycerol generated from biodiesel production process is the main economical as well as environmental concern for development of biodiesel industries [3]. Therefore, given the difficulties and economic cost of recovering glycerol biomass produced from biodiesel production process, an appreciated and alternative approach to utilizing the glycerol as a renewable resource would be the bioconversion process using microorganisms. Consequently, biotransformation of glycerol to renewable energy like 2,3-BD, an expensive product used as a liquid fuel or fuel additive would lead to both environmental and economic dividends of biodiesel plant. However, in the recent

years, bioconversion of glycerol to value-added metabolic products is an interesting topic for many researchers [18-21]. Unfortunately, until now, there is no available effective strain nominated for utilizing of glycerol to produce 2,3-BD successfully. In the last few years, numerous attempts have been made on bio-conversion of glycerol to biofuels and other various products including ethanol, 1,3-PDO, 1,2-PDO, and H_2 under microaerophilic or anaerobic culture process [22-23]. However, very little studies have been done on aerobic bioconversion of glycerol to biofuels and other value-added metabolic products until now [24-26].

Considering aerobic biotransformation of glycerol to value-added metabolic products, *K. variicola* SW3, an efficient adapted mutant strain developed from a newly isolated strain *K. variicola* SRP3 was used for high production of 2,3-BD using pure and crude glycerol. Only two species of *Klebsiella* (*K. pneumoniae* and *K oxytoca*) has been reported to utilize glycerol with producing 2,3-BD and 1,3-PDO in aerobic and anaerobic processes respectively [18, 27-28]. There is no report except our earlier report [13] on the bioconversion of glycerol to value-added product by *K. variicola*. In our study, however, we have confirmed that the adapted mutant strain *K. variicola* SW3 developed from our newly isolated strain *K. variicola* SW3 could aerobically convert glycerol to substantial amount of biotechnologically important product 2,3-BD along with other minor co-products through a GDH-dependent oxidative pathway. Recently Yang et al. [23] and Cho et al. [29] reported high concentrations of 2,3-BD 83.3g/L by *Bacillus amyloliquefaciens* B10-127 and 131.5 g/L by *K. oxytoca* M1 respectively using molasses or casamino acid as a co-substrate (carbon source) in addition to glycerol. Nevertheless, when glycerol was used as the sole carbon source, *B. amyloliquefaciens* B10-127 produced only 43.1 g/L of 2,3-BD. Lately, Petrov and Petrova [30] reported the highest concentration (70.0 g/L) of 2,3-BD with a yield of 0.39 g/g in a fed-batch culture using pure glycerol as a carbon source by *K.*

pneumoniae G31. Table 5.3 compares the product concentrations of 2,3-BDO reported earlier and this study in batch and fed-batch cultures using glycerol as the only carbon source.

Interestingly, our adapted mutant strain *K. variicola* SW3 able to grow on and tolerated a high glycerol concentration of pure or crude glycerol up until 200.0 g/L. Moreover, in the meantime, keeping the conditions defined in this study, concentration (82.5 g/L) and yield (0.62g/g) of 2,3-BD were higher than that of previously published reports by other researchers. Likewise, 64.93 g/L with a yield of 0.63 g/g, a high production of 2,3-BD was also a significant amount obtained using biodiesel derived raw glycerol as the sole carbon source in our fed-batch process. Furthermore, *K. variicola* SW3, an adapted strain able to tolerate a high concentration of the glycerol up to 20% (v/v). Overall, our results confirmed that a high yield (0.63 g/g) of 2,3-BD obtained from crude glycerol biotransformation could be the new prospect for economic feasibility of biodiesel industries. Further improvement on 2,3-BD production would make it more feasible to produce 2,3-BDO from biodiesel-derived crude glycerol for industrial use. Consequently, a general viewpoint on the fundamental mechanism of 2,3-BD biosynthesis response to glycerol as a feedstock, quite a few genes that could play an important role in 2,3-BD biosynthesis were designated as promising candidates for metabolic engineering (Fig. 5.3). It was therefore, undoubtedly observed that the relative expression level of key enzyme (GDH) gene dhaD responsible for conversion of glycerol in SW3 strain was higher compared to that of SRP3. The overexpression of GDH displayed a significant enhancement on budB and budC gene expression levels (Fig. 5.3). However, at a concentration of 50.0 g/L (5.0%) glycerol, both strains SW3 and SRP3 exhibited higher expressions of dhaD/gldA. Moreover, the wild type strain SRP3 displayed higher expression levels for the genes dhaB and pdhC than that of its adapted mutant SW3 at the

same concentration (50.0 g/L) of glycerol. Information obtained from our enzyme genes expression study would indicate further study on metabolic engineering.

Evolutionary adaptation is a method for increasing tolerance towards an inhibitor of a microbial strain. Consequently, this increased tolerance may lead down-regulation of gene(s) responsible for the predictable product [31]. Our attainment with increased tolerance towards glycerol and increased 2,3-BD production by adapted mutagenesis confirmed the competence of evolutionary adaptation for development of *K. variicola* SW2 mutant. As shown in Fig 5.6, the utilization capability of crude glycerol in batch process by *K. variicola* SW3 was almost similar as compared to that for pure glycerol. As comparison with the efficacy of SRP3 and SW3 strains, the bio-conversion rate of pure and crude glycerol to 2,3-BD with the strain SW3 was much higher than that of SRP3. However, a high product yield (0.59 g/g) of 2,3-BD obtained from Batch culture process was the highest amount using crude glycerol as a feed stock until now. We have proved, however, that an adapted mutant *K. variicola* SW3 could utilize glycerol to produce 2,3-BD through oxidative pathway in a GDH-dependent manner. Evidently, the GDH enzyme plays a vital role in oxidative pathway of glycerol metabolism and 2,3-BD formation.

Table 5.4. Comparison of 2,3-BDO production using crude and pure glycerol as the sole carbon source.

Name of bacterial strains	Culture	Carbon source	Product (g/L)	Yield (g/g)	References
Klebsiella pneumoniae G31	Fed-batch	Pure glycerol	70.00	0.39	30
Bacillus amyloliquefaciens	Fed-batch	Crude glycerol	43.1	0.45	24
K. variicola SRP3	Batch	Pure glycerol	29.9	0.60	13
K. oxytoca	Batch	Crude glycerol	4.8	0.14	18
Klebsiella sp. Ana-WS5	Fed-batch	Pure glycerol	30.1	-	32
K. variicola SW3	Fed-batch	Pure glycerol	82.5	0.62	This study
K. variicola SW3	Batch	Pure glycerol	33.5	0.67	This study
K. variicola SW3	Fed-batch	Crude glycerol	64.9	0.63	This study
K. variicola SW3	Batch	Crude glycerol	29.3	0.57	This study

5.5. Conclusion

Even though the bioconversion of biodiesel derived crude glycerol to 2,3-BD offers a noteworthy advantage in relation to the use of pure glycerol, most of the research works have been directed using pure glycerol. Therefore, in our present report, the metabolic product 2,3-BD achieved from biotransformation of crude glycerol compared to that of pure glycerol showing that this biodiesel by-product could be used with a great potential to produce 2,3-BD. Our result also showed that it is possible to discover novel microbial strains to produce 2,3-BSD using crude glycerol. Moreover, the gene expression study of glycerol metabolism was a primary step to investigate the culture parameters influencing gene regulation in the syntheses of metabolic products. It is demonstrated that the adapted mutant *K. variicola* SW3 able to tolerate a high

concentration of biodiesel derived raw (crude) glycerol, have a high glycerol utilization rate, and high product yield of 2,3-BD. Therefore, further studies with our newly developed adapted strain SW3 are granted to boost the utilization rate of glycerol, and the production of 2,3-BD. Until now, the high production and product yield (0.63 g/g) of 2,3-BD reported in this study using glycerol as the sole carbon source is the highest amount obtained from biotransformation process.

Acknowledgement

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Chapter 6: High Production of 2,3-butanediol by a Mutant Strain of the Newly Isolated *Klebsiella pneumoniae* SRP2 with Increased Tolerance Towards Glycerol

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Abstract

Biodiesel, a renewable fuel produced by transesterification of animal fats and vegetable oils, generates about 10% (v/v) of crude glycerol as a core by-product. The high volume of this non-biodegradable glycerol is becoming of a great environmental and economical concern due to its worldwide ever-growing surplus. Herein we report a high production of 2,3-butanediol (2,3-BD) from pure and biodiesel derived crude glycerol using a mutant *K. pneumoniae* SRM2 obtained from a newly isolated strain *Klebsiella pneumoniae* SRP2. The mutant strain SRM2 withstanding high glycerol concentration (220 g L^{-1} of medium) could rapidly convert glycerol aerobically to 2,3-BD, a versatile product extensively used in chemical, pharmaceutical and fuel industries Our study revealed that an increased GDH activity led to a substantially enhanced production of 2,3-BD. The mutant strain exhibited 1.3-fold higher activity of GDH than that of parent strain (500.08 vs. 638.6 μmol min^{-1} mg^{-1} protein), yielding of 32.3 g L^{-1} and 77.5 g L^{-1} 2,3-BD with glycerol in batch and fed-batch process respectively. However, in batch culture with crude glycerol, cell growth and glycerol consumption were expressively boosted, and 2,3-BD production was 27.7 g L^{-1} from 75.0 g/L crude glycerol. In this report, the optimal conditions for high production of 2,3-BD were defined in a completely aerobic process, and 0.59 g g^{-1} product yield of 2,3-BD was attained by the mutated strain *K. pneumoniae* SRM2, which is the highest amount obtained from

batch biotransformation process of glycerol metabolism till today. These results indicated that our newly developed mutant can tolerate high concentration of glycerol, have a high glycerol utilization rate, and high product yield of 2,3-BD. It is demonstrated that the mutant strain *K. pneumoniae* SRM2 has an ability to produce fewer co-products at trace concentrations at higher glycerol concentrations, and could be a potential candidate for 2,3-DB production in an industrial bioconversion process.

Keywords: Glycerol dehydrogenase, Crude glycerol, 2,3-Butanediol, Bioconversion, *Klebsiella pneumoniae*.

6.1. Introduction

Biodiesel has become one of the vibrant renewable fuels produced from animal fats and vegetable oils by reacting with a primary alcohol in the presence of an alkali or acid catalyst. During the last few years, biodiesel production has increased dramatically, and a significant amount (10% v/v) of crude glycerol is generating from a typical biodiesel production process. Crude glycerol produced from bio-refineries is a worldwide overflow problem due to a nonexistence of refining capacity. Thus, a high volume of crude glycerol is the key problem for growth of biodiesel industry. The economic feasibility of the biodiesel industry has been crucially affected due to a high volume (by worldwide surplus) of crude glycerol generated from biodiesel production process [1]. Therefore, with increasing biodiesel production plants, a large number of glycerol production plants will be shut down within few years due to price drop by oversupply of glycerol [2,3] that require new commercial uses. Glycerol, a core by-product of biodiesel production has become an inexpensive and easily obtainable product for which new applications have to be discovered [1]. Several species of Enterobacteriaceae are capable for converting glycerol to produce value-added bio-products [5-7]. Until now, there is no effectual microorganism

which can convert glycerol efficiently to produce industrially important high-priced products. Most of the studies have been carried out on bioconversion of glycerol using anaerobic fermentation process [1,7]. Our primary focus is to enhance the production of 2,3-BD from glycerol by effective bacterial strain under completely aerobic process. However, for glycerol metabolism, the metabolic pathways of *K. pneumoniae* has been explored recently [8]. In *K. pneumoniae*, the metabolic process of glycerol bioconversion is accomplished by a complex two-branch (oxidative and reductive) pathways (Fig. 6.1), which results in the synthesis of major products including dihydroxyacetone (DHA), 2,3-BD, ethanol, acetoin, acetate and 1,3-propanedion (1,3-PDO). In the oxidative branch, an important cell-bounded (intracellular) and NAD^+-dependent enzyme GDH converts glycerol to DHA, producing NADH as a reducing equivalent [4,8]. Subsequently, dihydroxyacetone phosphate (DHAP) is generated from DHA by ATP or phosphoenolpyruvate-depended DHA kinase, which is then further metabolized to various products through pyruvate [4,8]. Meanwhile, NADH (reducing equivalent) produced after receiving electrons from oxidation of substrate organic compounds could stimulates 1,3-PDO production [8]. Moreover, glycerol is firstly converted to 3-hydroxypropionaldehyde (3-HPA) by the coenzyme B_{12}-dependent glycerol dehydratase through the parallel reductive branch of glycerol metabolism, and it is then reduced to the major product 1,3-PDO by the NADH-linked 1,3-PDO dehydrogenase, thereby regenerating NAD^+ [4,8].

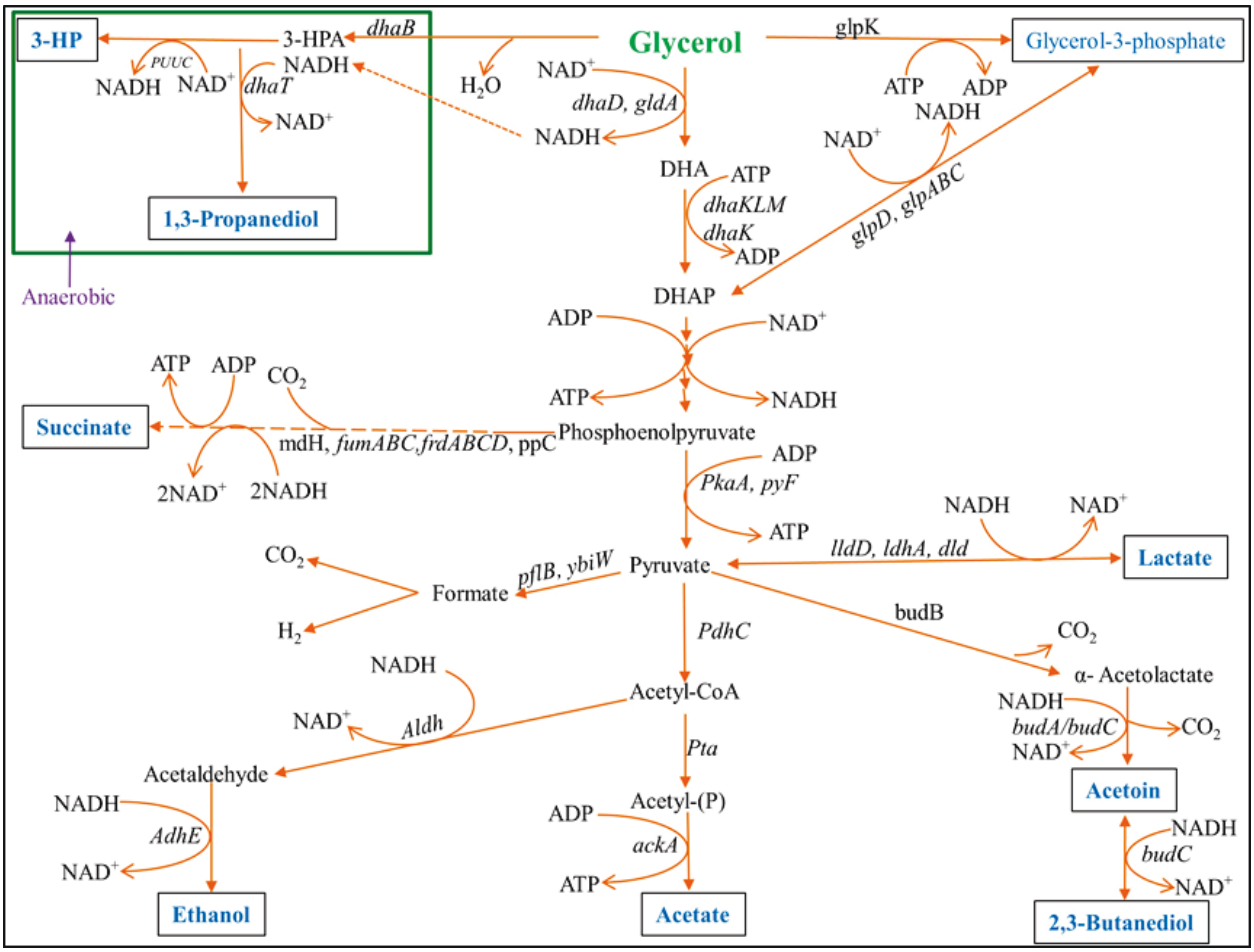

Figure 6.1. Initial steps of the glycerol dissimilation pathway together with 2,3-BD, 1,3-PDO, acetate and acetoin formation in *Klebsiella*. 3-HP: 3-Hydroxypropionic acid**.**

The two important platform chemicals 2,3-BD and acetoin can be obtained from oxidative pathways of glycerol metabolisms of many bacteria [6,9]. Acetoin, an important metabolic product of microorganisms is commonly used for the preparation of cosmetics, food, and many chemicals [10]. However, 2,3-BD is an expensive chemical which is used as a liquid fuel and fuel additive [11]. As an industrially important chemical, 2,3-BD is also extensively used for the preparation of pharmaceuticals and many synthetic materials [11,12].

Recently, many species (*Klebsiella planticola*, *K. oxytoca* and *K. pneumoniae*) of the genus *Klebsiella* have been reported to produce 1,3-PDO as a principal product through bioconversion

of glycerol, whereas 2,3-BD was the minor [9, 13-15]. In this study, metabolic products of batch and fed-batch fermentation of glycerol using a newly isolated strain *K. pneumoniae* SRP2 and its ethyl methane sulfonate (EMS) mutant was evaluated. Our goal was to optimize the culture conditions to gain expression of GDH as well as increase metabolic product yield of 2,3-BD to make a process relevant for industrial application. Here, we report a high product concentration of 2,3-BD from glycerol as a sole carbon source using our newly isolated strain *K. pneumoniae* SRP2 and its EMS mutant *K. pneumoniae* SRM2.

6.2. Materials and Methods

6.2.1. Isolation of glycerol degrading bacterial strain

K. pneumoniae SRP2 wild type and an EMS mutant SRM2 strains were used throughout this study. The strain *K. pneumoniae* SRP2 was isolated from paper mill waste of Resolute Forest Products, Canada. In order to isolate the glycerol degrading bacterial strain SRP2, the method described our earlier work [6] was followed. This isolated strain was further screened for their glycerol assimilation, GDH activity and 2,3-BD production. This strain was maintained at 4°C on Luria-Bertani (LB) agar slant and sub-cultured every two weeks.

6.2.2. Identification of the bacterial strain

The strain SRP2 was identified by 16S rDNA sequencing, confirmed by its phenotypic and biochemical characteristics. The gene encoding 16S rRNA was amplified from the genomic DNA of SRP2 strain by PCR (polymerase chain reaction) using 16S rDNA universal bacterial primer pairs, which were HDA-1 (5′-GACTCCTACGGGAGGCAGCAGT) and E1115R (5′-AGGGTTGCGCTCGTTGCGGG), and a 726 bp fragment was obtained. The product obtained after PCR was purified using PCR purification Kit (Geneaid, Canada) following the manufacturer's protocol. The purified PCR sample was sent to Euroffins Genomics (USA) for

sequencing. For identification of the strain SRP2, sequence analysis was performed using NCBI blast tool, compared with sequences of the Gene Bank database search (http://www.ncbi.nlm.nih.gov/genbank/). The phylogenetic relationship was analyzed using sequence alignment program ClustalX and TreeView software. The species identification was confirmed using phenotypic and biochemical features [16].

6.2.3. Optimization of fermentation parameters

Seed culture for batch and fed-batch processes was prepared from stock slant culture by inoculating into LB broth medium. The LB broth seed culture was incubated at 37°C and 180 rpm for 20 h. Following incubation, the seed culture was inoculated into appropriate medium for optimization of culture conditions such as incubation temperature and time, medium initial pH, glycerol concentration, and nitrogen sources. All the growth parameters were performed in triplicates. For GDH enzyme and 2,3-BD assay, batch fermentations were maintained in 250 ml ml Erlenmeyer flasks (100 ml medium and 100 µl of 20 h culture as the inoculum) at different initial pH (4, 5, 6, 7, 8 and 9), temperatures (25- 40°C), and at different concentrations of glycerol (25 - 65 g L^{-1}), yeast extract (1.0 - 5.0 g L^{-1}) and peptone (4.0 - 6.5 g L^{-1}). The minimal salt-1 (MS-1) medium (KCl, 0.1 g L^{-1}; K_2HPO_4, 0.1 g L^{-1}; $MgSO_4$.$7H_2O$, 0.05 g L^{-1} and $NaNO_3$, 0.1 g L^{-1}) supplemented with specified concentration of glycerol or crude glycerol and nitrogen sources was used throughout the experiments when indicated. Medium pH was adjusted using HCl(1 M)/NaOH (1 M). Biodiesel byproduct (crude glycerol) supplied by CARES (Centre for Agricultural Renewable Energy and sustainability) biodiesel plant (Guelph, Canada) was used as a feedstock (sole carbon source) for microbial growth throughout this study. This crude glycerol contains 50.0±4.6% glycerol.

6.2.4. Batch and fed-batch fermentation under optimized conditions

Batch and Fed-batch fermentations were carried out in 250 ml Erlenmeyer flasks containing 100 ml medium (initial pH 8.0) with 100 µl of 20h culture as the inoculum, incubated at 37°C under aerobic condition at 180-200 rpm. The medium MS-2 used for batch and fed-batch cultures contained KCl (0.1 g L^{-1}), K_2HPO_4 (0.1 g L^{-1}), $MgSO_4$.$7H_2O$ (0.05 g L^{-1}), $NaNO_3$ (0.1 g L^{-1}), glycerol (55.0 g L^{-1}) or crude glycerol (75.0 g L^{-1}), yeast extract (2.5 g L^{-1}) and peptone (4.5 g L^{-1}). In case of fed-batch culture, the glycerol concentrations were varied. In our fed-batch culture, an initial concentration of glycerol was 55 g L^{-1}, and then a concentrated solution containing 800 g L^{-1} of pure glycerol or crude glycerol was fed into the culture as required.

6.2.5. Enzyme Assay

The enzyme assay of membrane-bounded GDH was carried out by measuring the initial reduction rate of NAD (from NAD to NADH) at 340 nm absorbance according to the established method [17] with minor modifications [6]. The reaction mixture used for GDH assay contained ammonium sulfate (30 mM), glycerol (0.2 M), potassium phosphate buffer (50 mM) and NAD (1.2 mM), which was prepared just before the enzyme assay. A 96 well microplate (flat bottom), each well with 1 cm light path length containing 250 µl reaction mixture was used for GDH assay. The reaction was started by adding 50 µl cell free extract of bacterial cells. One unit of GDH activity is defined as the amount of enzyme required to oxidize 1 µmole of substrate per minute. However, the specific activity of GDH is stated as µmoles of substrate reduced/minute/mg of cell protein. Protein was quantified using standard protocol [18] with bovine serum albumin as a standard protein.

6.2.6. Strain development

An adapted strain was developed from the wild type strain SRP2 using the method described in our previous study [6]. This evolutionary developed (adapted) strain was able to grow at a concentration of 220 g L^{-1} glycerol. The evolutionary adapted strain was mutagenized with UV coupled with EMS [19]. To get UV mutant, the inoculated culture plate was exposed to UV light at 280 nm wavelength for 3 minutes and 40 cm away from the light source. For EMS mutagenesis, the UV treated culture was used, and a standard method described by Jensen et al [20] was followed. Briefly, 0.1ml of 24h LB broth culture was plated on MS-1 agar medium supplemented with 100 g L^{-1} glycerol. On the centre of the plate, a drop 6 µl EMS (99%) was placed, incubated at 37°C for 72h. Following incubation, a bacterial colony grown at the edge and middle of EMS inhibition zone was taken, streaked on MS-1 agar medium containing 100 g L^{-1} glycerol, and tested its 2,3-BD product concentration.

6.2.7. Toxicity test

For toxicity test, the minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC) of glycerol were performed in batch culture. A series of culture medium (MS-1 supplemented with yeast extract 2.5 g L^{-1}) tubes were prepared with different concentrations of glycerol ranging from 100 to 550 g L^{-1}, inoculated with 100 µL of a 20 h culture, and incubated at 37^0C for 120h. Following incubation, growth was evaluated spectrophotometrically based on the absorbance at 600 nm and the dry cell mass was measured by using a standard curve (a linear correlation between the dry cell mass and absorbance of cell suspension). For MBC, 100 µL culture broth from each tube was plated on LB agar medium, and incubated at 37^0C for 48h. After incubation, any growth was observed on the agar plate. All tests were conducted in at least triplicates.

6.2.8. Assay of biomass and metabolic products

Biomass was measured as absorbance at 600 nm using microplate spectrophotometer (EPOCH, BioTek) and converted to cell dry weight using a calibration equation (y = 2.0978x - 0.0346, where y = OD600, x = biomass g L^{-1}) obtained from standard curve of a linear correlation between the dry cell mass and absorbance of cell suspension. The identity of the end products was confirmed by GC-MS (Varian 1200 Quadrupole). Glycerol concentration (g L^{-1}) and major metabolic products including 2,3-BD, acetoin, 1,3-PDO and acetic acid were determined by GC-FID (Shimatzu GC 14A) under the following conditions: sample volume 1 µl; column temperature range from 45°C (2 min) to 240°C at the rate of 10°C/min; the injector and detector temperature 250°C; carrier gas nitrogen; column DB-WAXetr. The injecting sample was purified by centrifugation and membrane filter (0.22 µm pores size).

6.3. Results

6.3.1. Isolation and identification of bacterial strain

The isolation procedure was primarily based on tolerance and ability to utilize glycerol (100 g L^{-1}) as the sole carbon source to produce GDH enzyme under aerobic condition, and yielded a number of bacterial strains from soil and paper mill waste samples. Of these isolates, the strain SRP2 (*K. pneumoniae*) isolated from paper mill waste exhibited significant GDH activity. This highly active SRP2 strain was identified as genus *Klebsiella* (99% similarity) using 16S rRNA gene sequencing. By using phenotypic and biochemical characters shown in Table 6.1, the species *K. pneumoniae* (strain *K. pneumoniae* SRP2) was identified. On LB agar, the strain SRP2 was found to grow as large mucoid and slimy (old culture) colonies. The morphological features observed by light microscopy are: rod-shaped and encapsulated cells, 0.4 – 0.8 µm in diameter and 0.7-5.0 µm in length, arranged singly/ in pair/ in short chain.

The 16S rRNA sequences of the isolated strain SRP2 and its EMS mutant SRM2 have been submitted to the GenBank for accession numbers. The strains reported in this research article have been nominated as *Klebsiella pneumoniae* SRP2 (wild) and *K. pneumoniae* SRM2 (mutant), and their GenBank accession numbers are released as KR092086 and KX572902 respectively. However, for the phylogenetic relationship analysis, the 16S rRNA gene sequences of SRP2 and its mutant SRM2 were aligned using ClustalX UPGMA algorithm and uploaded into TreeView (Fig. 6.2). The phylogenetic tree was built by the Neighbor-Joining method using 16S rRNA gene sequencing. The result of phylogenetic relationship confirmed that the strains belong to the genus *Klebsiella* or species *K. pneumoniae*. In phylogenetic tree, the distance between the wild type (*K. pneumoniae* SRP2) and its mutant (*K. pneumoniae* SRM2) strains also confirmed mutagenesis.

6.3.2. Strain development

The adapted strain obtained from 220 g L^{-1} glycerol was used to develop EMS mutant. Following incubation, a zone of inhibition was developed on the plates around the drop of EMS (Fig. 6.3). All the colonies obtained from the edge and middle of the inhibition zone were streaked on MS-1 agar plates with 100 g L^{-1} glycerol, and tested for their 2,3-BD production. The EMS mutagenesis revealed a high yielding 2,3-BD mutant marked as strain No. SRM2.

6.3.3. Effect of growth factors on GDH activity and 2,3-BD production in batch culture

In the oxidative pathway, GDH is a key enzyme for converting glycerol to biofuels and value-added metabolic products including DHA, 2,3-BD, acetoin and organic acids. To optimize glycerol concentration, incubation temperature and time, initial pH and nitrogen sources for maximum enzyme activity, experiments were carried out in batch fermentation (Fig 6.4-6.7). In this aerobic batch fermentation, glycerol was only the carbon source for microbial growth to produce GDH and 2,3-BD. The results revealed optimum initial concentrations 45 g L^{-1} and 55 g L^{-1} of glycerol

for the strains SRP2 and SRM2 respectively, whereas the yeast extract concentration was 2.5 g L^{-1} at starting pH 7.0 and incubation temperature 37°C (Fig 6.4). To optimize the incubation temperature for maximum GDH activity and 2,3-BD production of the both strains SRP2 and SRM2, experiments were performed in batch culture at starting pH 7.0 under controlled temperatures ranging from 25 to 40°C. The maximum enzyme activities 344.5 and 467.7 unit/mg protein were achieved at 37°C after 48h incubation with the strains SRP2 and SRM2 respectively (Fig. 6.5). In case of 2,3-BD, the maximum concentrations 12.9 g L^{-1} and 15.0 g L^{-1} of 2,3-BD were attained by the strains SRP2 and SRM2 respectively at the same temperature under the same conditions (Fig. 6.5). The mutant strain SRM2 exhibited 1.35 and 1.16-fold higher production of GDH and 2,3-BD respectively compared to that of its wild type SRP2 strain under same incubation conditions.

The initial pH of the medium played an important role for the production of GDH and 2,3-BD by SRP2 and SRM2 strains. Experiments without pH control showed that the higher was the starting pH values, the greater was the GDH activity and 2,3-BD concentration (Fig. 6.6a). Before autoclaving, the medium pH values (4-8) were adjusted using HCl/NaOH. A time course of pH and cell biomass changes with starting pH 8.0 was performed over a period of 144 h (Fig. 6.6b). Fascinatingly, in the first 24 h, the pH of the medium speedily dropped and reached values from 8.0 to 4.6. This first 24 h incubation period was the exponential growth phase of the batch culture and the pH was dropped due to the production of acetate and/ other organic acids in the culture medium. After 24 h incubation, the pH values were slightly rose and dropped during incubation and reached values between 4.6 and 5.5 due to production of 2,3-BD. Figure 6.6a shows that the maximum GDH activity (522.5 unit/mg protein) and 2,3-BD (17.5 g L^{-1}) production were attained at initial pH 8.0 by SRM2.

In addition to yeast extract (2.5 g L^{-1}), experiments were carried out on the effect of other nitrogen sources viz., malt, peptone, NH_4NO_3, $(NH_4)_2SO_4$ and NH_4Cl for GDH and 2,3-BD production with the wild type strain SRP2 and its mutant SRM2.The peptone showed the highest GDH activity (616.7 unit/mg protein) and 2,3-BD product concentration (19.21 g L^{-1}) compared to that of other nitrogen sources with SRM2 strain (Fig 6.7a). The maximum GDH activity 638.6 unit/mg protein and 22.8 g L^{-1} were attained at a concentration of 4.5 g L^{-1} peptone after 48 h incubation by SRM2 (Fig. 6.7b).

6.3.4. Toxicity test

The mutated strain SRM2 was used for a toxicity test towards different concentrations of glycerol (Fig. 6.8). At an initial glycerol concentration of 300 g L^{-1}, growth of SRM2 was detectable after 120 h incubation. However, at an initial glycerol concentration of 450 g L^{-1}, viability of SRM2 strain was also detectable after cultured on LB agar. Based on these results strain SRM2 seemed most tolerant to glycerol. MIC and MBC of glycerol were determined as 350 g L^{-1} and 500 g L^{-1} respectively (Fig. 6.8).

6.3.5. Batch and fed-batch process under optimized conditions

Batch and feed-batch cultivation processes were carried out under optimized conditions for maximizing 2,3-BD production. To investigate glycerol utilization and 2,3-BD production by *K. pneumoniae* SRP2 and its EMS mutant *K. pneumoniae* SRM2, batch cultures were performed with pure glycerol or crude glycerol as the sole carbon source at the initial concentration of 55.0 g L^{-1} and 75.0 g L^{-1} respectively. For quantification of bio-products, experiments were set at initial pH 8.0 and temperature 37°C. The medium used for both batch and fed batch cultures was MS-2. The above culture conditions revealed two major products 2,3-BD and acetoin with other minor products including 1,3-PDO and acetate. In our batch process, a typical outline for aerobic

bioconversion of pure glycerol by SRP2 and its mutant SRM2 at different incubation time, and production of end products are summarized in the Fig. 6.9. The *K. pneumoniae* SRP2 in this paper, utilized 100% of glycerol after 144 h, generating 20.55±1.22 g L^{-1} 2,3-BD, 4.34±0.87 g L^{-1} 1,3-PDO and 8.52±0.76 g L^{-1} acetoin in batch process. The maximum concentration and yield of 2,3-BD attained after 120 h incubation were 28.53±1.34 g L^{-1} and 0.52 g/g glycerol respectively (Fig. 6.9a). Nevertheless, a maximum 32.3 g L^{-1} of 2,3-BD was obtained from 55 g L^{-1} glycerol after 120 h incubation by SRM2, and the product yield was 0.59 g g^{-1} glycerol. The strain SRM2 utilized 100% of glycerol after 120 h incubation and the concentrations of major bio-products other than 2,3-BD determined in this batch culture after 120 h incubation were acetoin and 1,3-PDO, with concentrations, 6.7±0.64 g L^{-1} and 3.22±0.25 g L^{-1} respectively (Fig. 6.9b). SRM2 obtained from SRP2 by EMS mutagenesis was capable of grow in a high concentration (220 g L^{-1}) of glycerol. Thus, the better adapted mutant strain SRM2 could efficiently convert glycerol to 2,3-BD and acetoin compared to that of its wild type strain SRP2. The maximum product concentrations and glycerol consumption were achieved from 96h to 120 h incubation. In our batch culture, the highest concentrations of 1,3-PDO and acetoin obtained after 144 h were 3.56± 0.73 g L^{-1} and 8.3±1.07 g L^{-1} respectively for SRM2, and 4.34± 0.82 g L^{-1} and 8.52±1.14 g L^{-1} respectively for SRP2 (Fig. 6.9a and 6.9b). The major metabolic products obtained from this batch process of SRP2 and SRM2 were 2,3-BD, acetoin and 1,3-PDO, with a minor product acetic acid.

To evaluate glycerol utilization and 2,3-BDO production from biodiesel derived crude glycerol (glycerol 50±4.6%, MONG 36.2±3.3, ash 4.2±0.3% and water 6.7±1.05%,) by strains SRP2 and SRM2, batch process was carried out at initial concentrations of 50.0 -100.0 g L^{-1} of crude glycerol. The optimal concentration of this crude glycerol was attained as 75.0 g L^{-1} for maximum product concentration of 2,3-BD. The effect of incubation time on SRP2 and SRM2 strains for

accumulation of major products in a batch culture process of crude glycerol are presented in the Fig. 6.10. As shown in Figure 6.10, the performance of the 2,3-BD production by SRM2 was higher than compared to that of wild type strain SRP2. Overall, the mutated strain SRM2 could produce 27.7±0.83 g L^{-1} 2,3-BDO as the principal product using 38.25 g glycerol after 120h incubation (Fig. 6.10a). In this batch culture process, the highest concentrations of acetoin, 1,3-PDO and acetate obtained after 144 h were 5.78±0.21, 2.79±0.2 and 0.402±0.019 g L^{-1} respectively with the strain SRM2 (Fig. 6.10b). However, the strain SRP2 exhibited the highest 2,3-BD production (25.73±1.04 g L^{-1}) after 120h incubation using 35.4 g glycerol (Fig. 6.10a).

In fed-batch process without pH control, the bioconversion kinetics showed that SRM2 produced high concentration of the principal product 2,3-BD in aerobic condition. Comparison between the final values of glycerol or biodiesel derived crude glycerol consumed, the biomass and major end products obtained by wild type strain SRP2 and EMS mutant SRM2 in fed-batch process are presented in Table 6.2. Table 6.2 shows that 51.1±1.7 g L^{-1}, a high production of 2,3-BD was obtained after 240 h incubation, consumed 77.4 g glycerol from 148.8 g L^{-1} biodiesel derived crude glycerol by SRM2. In case of pure glycerol, the highest concentration of 2,3-BD (77.51±2.03 g L^{-1}) obtained after 240 h incubation by consumed 132.0 g glycerol. However, the wild type strain SRP2 exhibited 54.3±1.1 g/L 2,3-BD by consumed 91.1 g glycerol after 192h incubation.

6.4. Discussion

With the thriving of biodiesel industry, global surplus of glycerol is increasing [1,21]. Due to oversupply of crude glycerol from biodiesel refinery the glycerol price would be significantly affected, and several glycerol production plants will be shutdown [22]. Therefore, bioconversion of glycerol to value-added bio-products would lead to both environmental and economic dividends

of biodiesel plant. Microbial conversion of glycerol to valuable chemicals is a subject of interest in the last few years [23,24]. Considering the glycerol bioconversion into biofuels and value-added bio-products, most of the recent studies focused on anaerobic fermentation process, and many microorganisms are able to utilize glycerol as a sole carbon source anaerobically [20,24]. Recently, several attempts have been made to produce value-added metabolic products including ethanol, 1,3-PDO and H_2 from glycerol under anaerobic fermentation process [23,25], but very limited studies have been done for aerobic processes. There is no any external electron acceptor in anaerobic fermentation, and the microbial growth is hampered. To overcome this problem in an anaerobic process, this study may be a viewpoint for high production of 2,3-BD as a major product of glycerol conversion in an aerobic process, and turning 1,3-PDO into a minor by-product. Considering economic utilize of glycerol, a goal was achieved to isolate an effectual bacterial strain, and develop the strain for high yield of 2,3-BD from crude and pure glycerol conversion.

K. pneumoniae, an enteric bacterium has long been considered to produce 1,3-PDO from glycerol as a feedstock in an anaerobic process [7,15]. Recently, a few works have been done on 2,3-BD production in an aerobic process [12,26]. We have demonstrated, however, that our newly isolated strain *K. pneumoniae* SRP2, and its mutants SRM2 could aerobically metabolize glycerol in a GDH-dependent oxidative pathway, and produce significant amount of industrially important product 2,3-BD together with other minor co-products. Furthermore, keeping the conditions defined in this study, the high production 32.3 gL^{-1} and 77.51 gL^{-1} of 2,3-BD was achieved from batch and fed-batch processes respectively by the mutant SRM2, as these amounts were the highest obtained from glycerol conversion reported. Therefore, in this study, the batch process with 55.0 g $L^{-1}$of initial glycerol was the optimal concentration for maximum GDH activity and 2,3-BD production, which is the highest glycerol concentration in batch culture (Fig. 6.5). Recently, Petrov

and Petrova [26] studied on glycerol bioconversion by *K. pneumoniae* in fed-batch process, and showed the highest concentration 70.0 gL^{-1} of 2,3-BD as well as 0.53 g/g product yield. Interestingly, the mutant strain grew on and tolerated a high glycerol concentration up until 220.0 gL^{-1}. Moreover, the glycerol, a feed stock for 2,3-BD production is less toxic to the mutant due to its high MIC and MBC values. This research confirmed that a high glycerol concentration, up until 55 g/L of feedstock concentration displayed maximum production yield of the metabolic product 2,3-BD, which could be the new prospect of glycerol bioconversion field.

Chemical mutagenesis is a well-known and extensively used method for increasing tolerance towards an inhibitor of a microbial strain. Therefore, this increased tolerance may lead down-regulation of gene(s) accountable for the expected product [27]. EMS, a widely used chemical mutagen induces base replacements or deletions of DNA in a bacterium [28,29] that may then gain an ability of higher productivity of a targeted product [23,31]. Our achievement with increased tolerance towards glycerol and increased 2,3-BD production by EMS mutagenesis confirmed the competence of EMS for development of *K. pneumoniae* SRM2 mutant.

In our batch process of *K. pneumoniae* SRM2, maintained at different initial pH levels (pH 4-9), discovered that slightly alkaline pH 8 was more favorable for the synthesis of 2,3-BD. This aerobic bioconversion of glycerol evidenced that the amount of the produced 2,3-BD from glycerol depends on the initial pH as well as other factors like temperature, initial concentration of glycerol and nitrogen sources.

We have demonstrated, however, that *K. pneumoniae* can utilize glycerol to produce 2,3-BD through oxidative pathway in a GDH-dependent manner. Clearly, the GDH enzyme plays a key role in oxidative pathway of glycerol metabolism and 2,3-BD formation. The establishment of optimal conditions including pH, incubation temperature and nitrogen sources for 2,3-BD

synthesis must comprise enhancement of GDH in oxidative pathway of the bioconversion process of glycerol.

6.5. Conclusion

Under aerobic conditions, 2,3-BD production from glycerol by *K. pneumoniae* mutant resulted in increased product yield and concentration in culture medium. The mutant strain *K. pneumoniae* SRM2 can tolerate high concentration of glycerol, have a high glycerol utilization rate, and high product yield of 2,3-BD. It is demonstrated that *K. pneumoniae* SRM2 has an ability to produce fewer co-products at trace concentrations at higher glycerol concentrations. Thus, the mutated strain SRM2 is a better organism for the aerobic conversion of glycerol to 2,3-BD. Therefore, we can conclude that it can metabolize high concentrations of glycerol and effectively convert the same to 2,3-BD and acetoin.

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Competing interests

The authors have declared that no competing interest exists.

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Table 6.1. Morphological and physiological characters of strain *K. pneumoniae* SRP2

Characters	**Results**
Gram staining (24h)	Gram -
Capsule	+
Indole production	-
H_2S production	-
Methyl red	+
Voges-Proskauer	+
Citrate	+
Urea hydrolysis	+
Gas/acid from Lactose (44^0C)	+/+
Gas/acid from glucose	+/+
Gelatin hydrolysis (22^0C)	-
KCN growth	+
Nitrate reduction	+
Lysine decarboxilate	+
Oxidase	-
Catalase	+
Acid from cellobiose, maltose, mannitol, arabinose, sucrose and sorbitol.	+

+, positive; -, negative

Table 6.2. Comparison the final values of major products obtained from metabolized of glycerol by *K. pneumoniae* SRP2 and SRM2 strains in feed-batch process at initial pH 8.

Strain	Duration (Hour)	Final pH	Biom. (g/L)	Glycerol utilized (g/L)	2,3-BD		Acetoin		1,3-PDO	
					(g/L)	Yield (g/g)	(g/L)	Yield (g/g)	(g/L)	Yield (g/g)
SRP2	192	5.46	1.49	91.1	54.3±1.1	0.60	6.1±0.6	0.07	2.22±0.06	0.02
SRM2	240	5.74	1.68	132.0	77.5±2.0	0.59	7.8±0.5	0.06	1.76±0.05	0.01
[C]SRP2	192	6.52	1.63	68.82	40.2±1.7	0.58	4.4±0.2	0.06	1.23±0.04	0.02
[C]SRM2	240	6.12	2.17	77. 4	51.1±1.7	0.66	5.5±0.4	0.07	1.85±0.12	0.02

All experimental values presented are mean values from triplicate experiments;
Biom.: Dry weight of biomass;
[C]Crude glycerol was used as carbon source for this strain

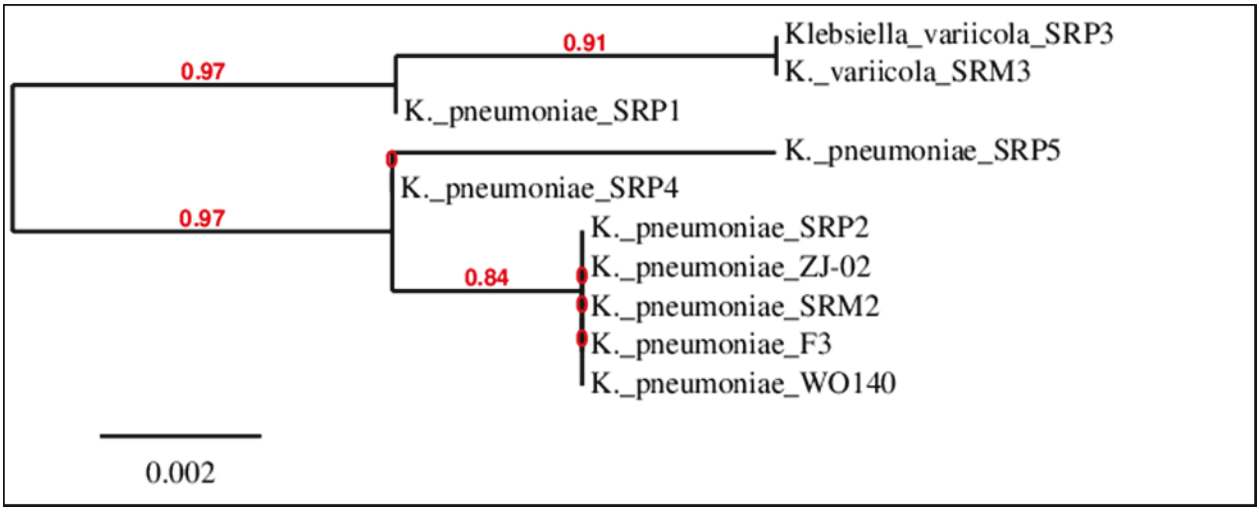

Figure 6.2. Phylogenetic tree drawn from ClustalX alignment, presented in TreeView.

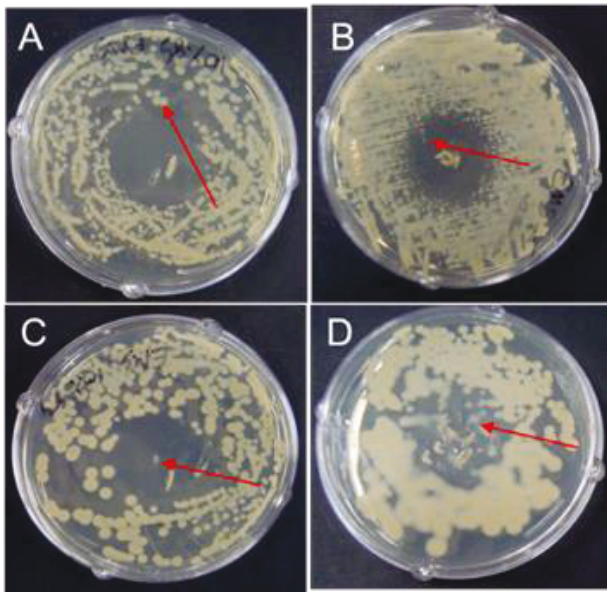

Figure 6.3. EMS treated culture plates A, B, C and D for possible mutant.

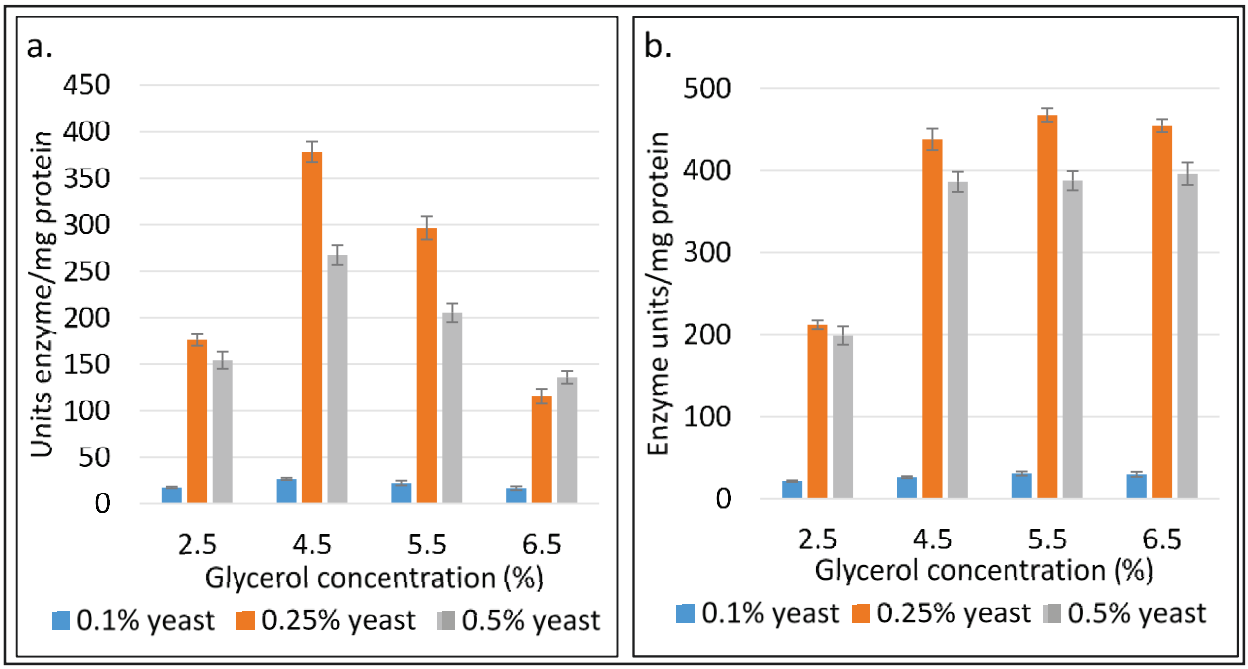

Figure 6.4. Influence of the concentrations of glycerol and yeast extract on GDH activity after 48h at 37^{0}C and initial pH 7.0. (a) Wild type *K. pneumoniae* SRP2 (b) Mutant SRM2.

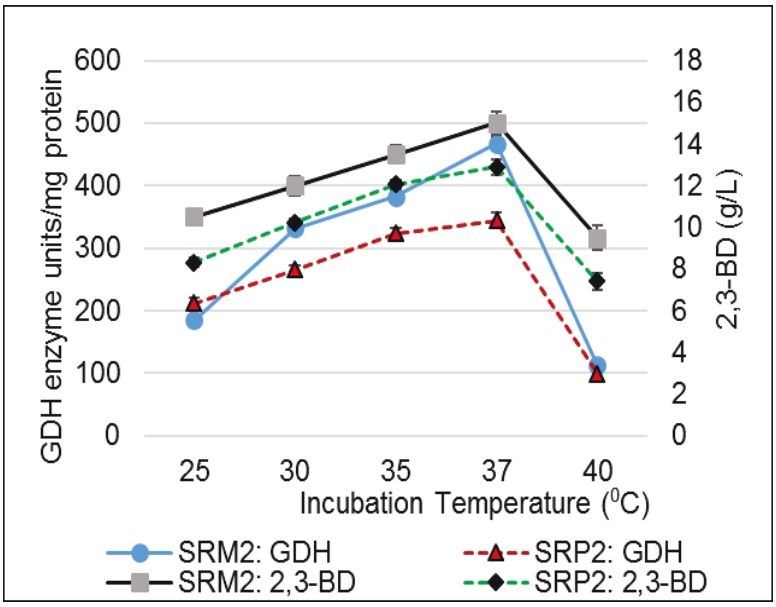

Figure 6.5. Influence of temperature on GDH activity and production of 2,3-BD of *K. pneumoniae* SRP2 and its mutant SRM2 after 48h at 37^{0}C. MS-1 medium with glycerol 55.0 g L^{-1} and yeast extract 2.5 g L^{-1} was used. Medium initial pH was 7.0.

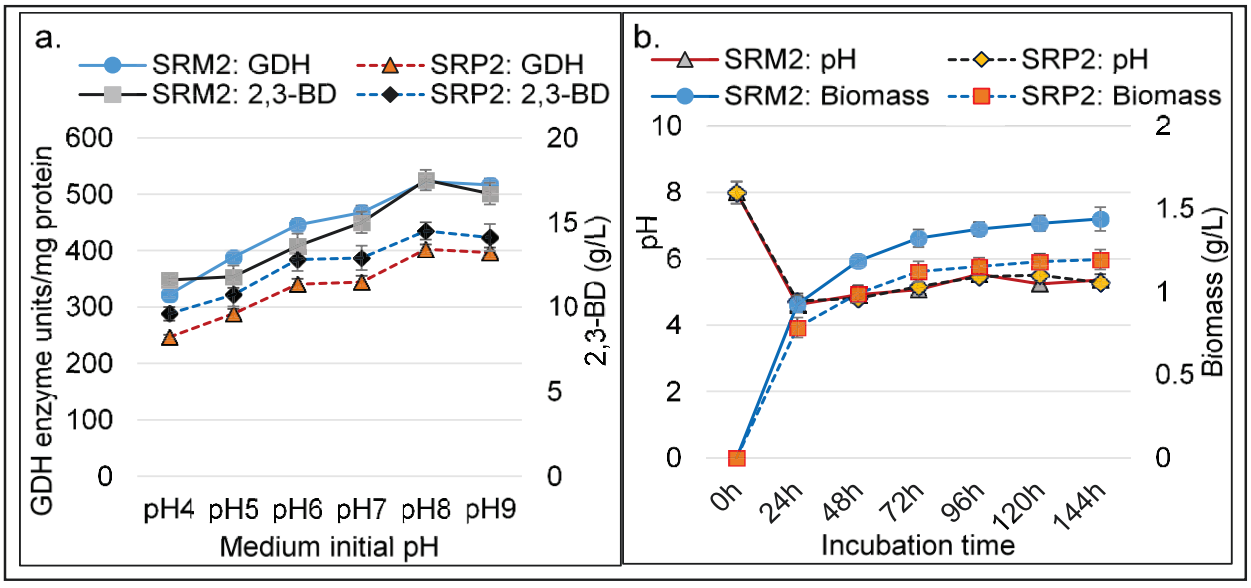

Figure 6.6. Effect of initial (starting) pH on *K. pneumoniae* SRP2 and its mutant SRM2 at 37^{0}C. (a) GDH and 2,3-BD production after 48h incubation. (b) Time profile of pH fluctuation and biomass production. MS-1 medium with glycerol 55.0 g L^{-1} and yeast extract 2.5 g L^{-1} was used.

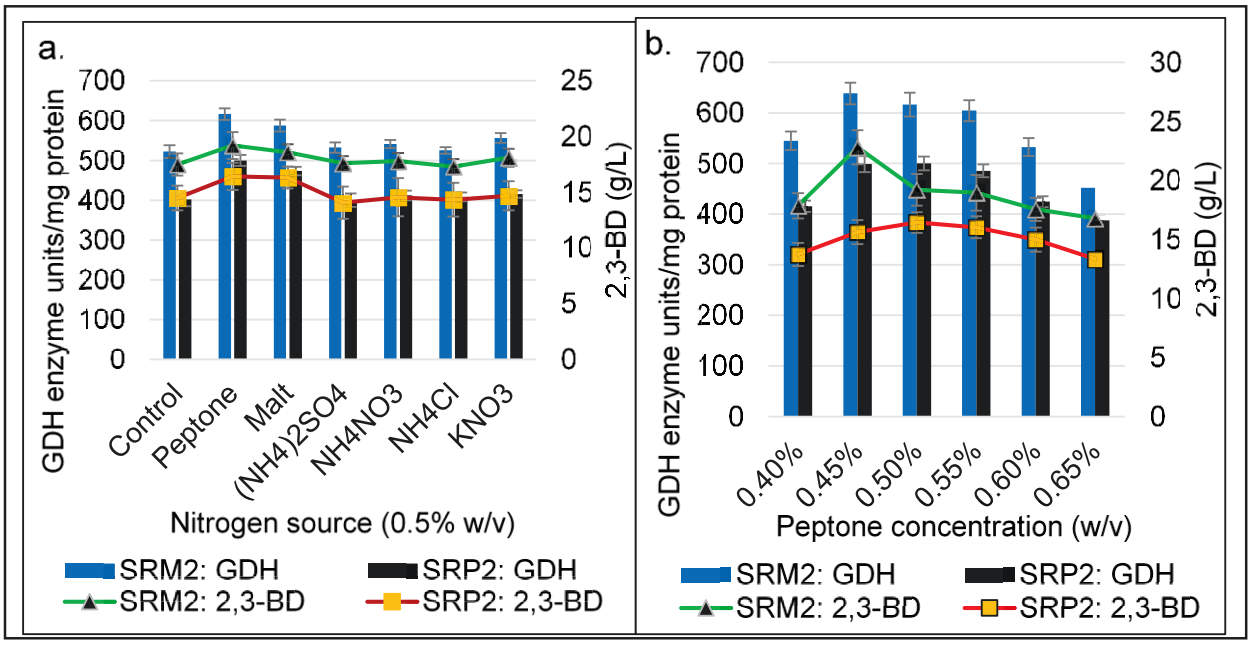

Figure 6.7. Influence of different nitrogen sources and peptone concentration on GDH and 2,3-BD production of *K. pneumoniae* SRP2 and its mutant SRM2 after 48h at 37^{0}C. (a) Nitrogen sources. (b) Concentrations of peptone. MS-1 medium with glycerol 55.0 g L^{-1} and yeast extract 2.5 g L^{-1} was used. Medium initial pH was 8.0.

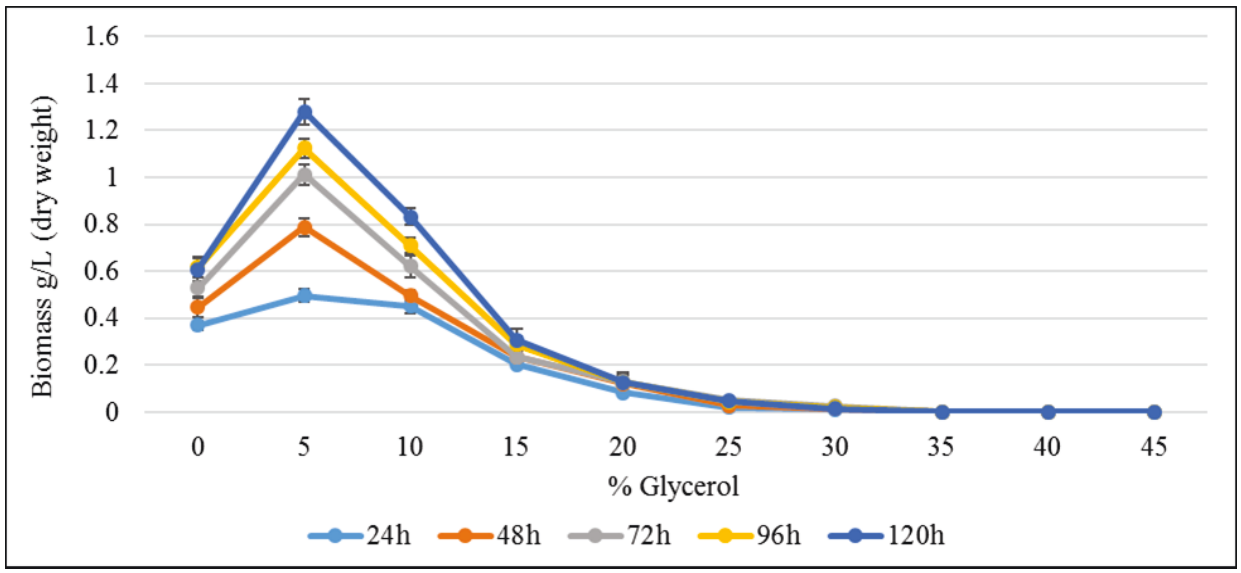

Figure 6.8. Toxicity [Minimum inhibitory concentration (MIC)] of glycerol against *K. pneumoniae* mutated strain SRM2 at initial pH 8.0. Incubation temperature was 37^{0}C. MS-1 medium with yeast extract 0.25% and peptone 0.45% was used.

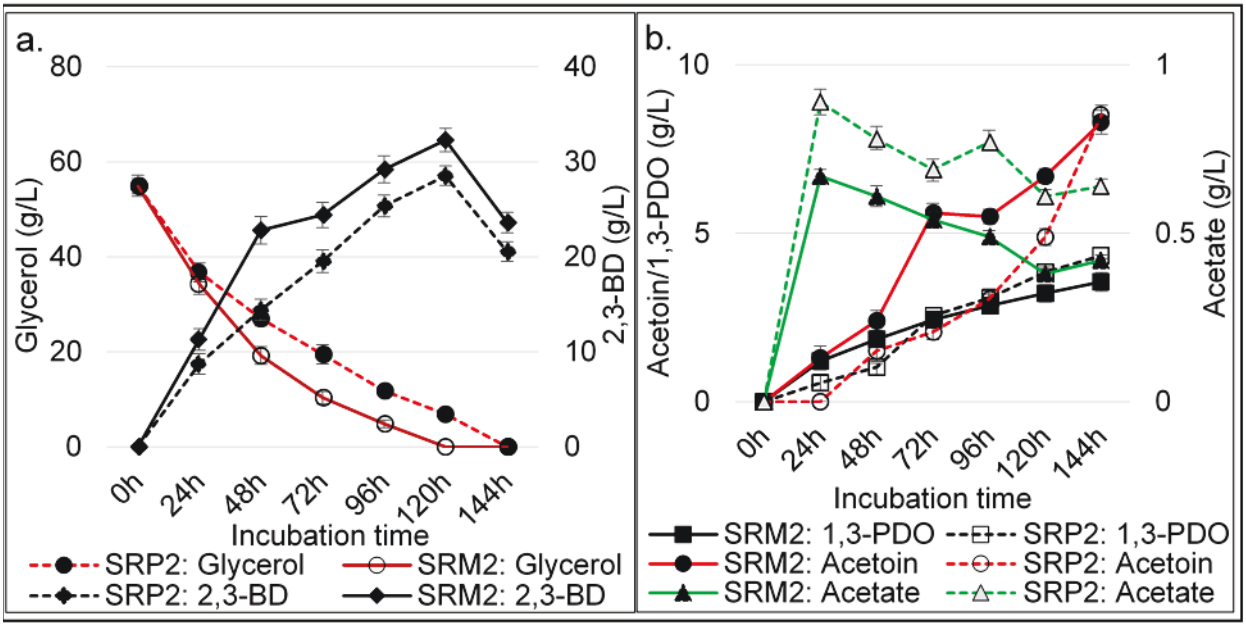

Figure 6.9. Time course data and metabolic products of batch cultivation at 37^{0}C by wild type strain SRP2 and its mutant SRM2 of *K. pneumoniae*. (a) Glycerol and 2,3-BD concentrations. (b) Acetoin, 1,3-PDO and acetate concentrations. MS-2 medium with pure glycerol 55.0 g L^{-1} was used. Medium initial pH was 8.0.

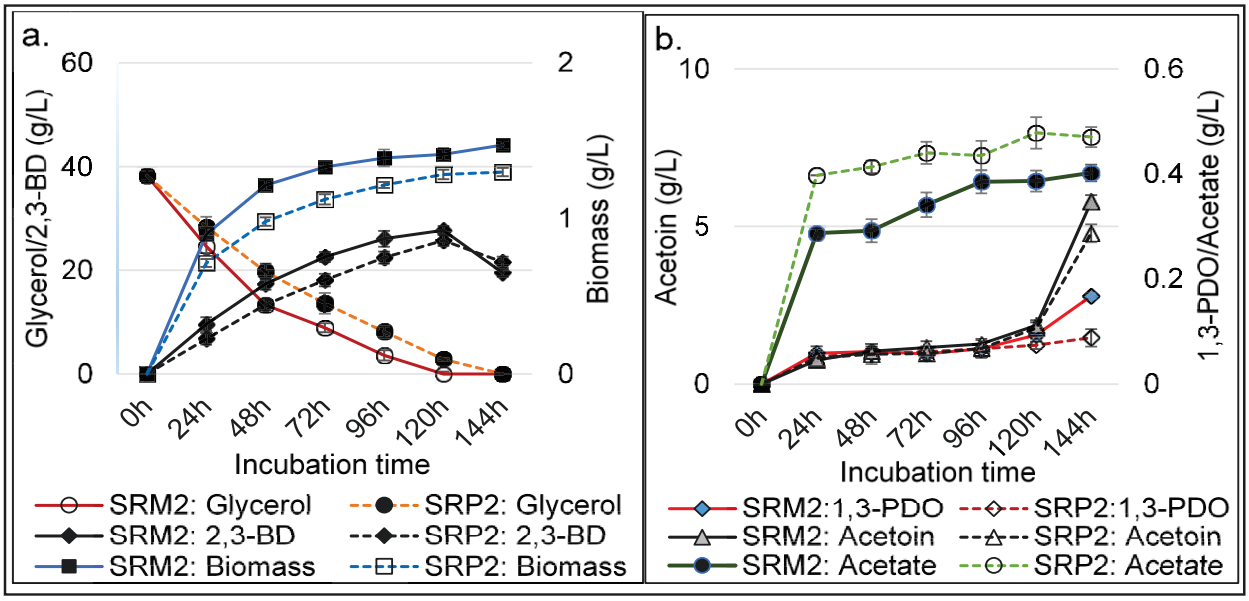

Figure 6.10. Time course data and metabolic products of batch cultivation at 37^{0}C by wild type strain SRP2 and its mutant SRM2 of *K. pneumoniae*. (a) Glycerol and 2,3-BD concentrations. (b) Acetoin, 1,3-PDO and acetate concentrations. MS-2 medium with biodiesel derived crude glycerol 75.0 g L^{-1} was used. Medium initial pH was 8.0.

Chapter 7: Engineering of *Escherichia coli* for construction of a novel expression system to produce 2,3-butanediol using gene from *Klebsiella pneumoniae* SRP3

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Abstract

Biofuels production is restricted by *Escherichia coli* due to its toxicity to bacterial cells. Therefore, a platform and fuel additive 2,3-Butanediol (2,3-BD) with low toxicity to microbes, could be a promising alternative for biofuel production by recombinant *E. coli*. A novel expression system of *E. coli* was developed to expressed dhaD gene encoding glycerol dehydrogenase (GDH) to produce value-added metabolic products by aerobic biotransformation of glycerol. The dhaD gene obtained from *Klebsiella pneumoniae* SRP3 was expressed in *E. coli* BL21(DE3)pLys using an *Escherichia coli-K. pneumoniae* shuttle vector pJET1.2/blunt consisting of chloramphenicol-resistance gene under the control of T7lac promotor. An Rt-PCR analysis and dhaD overexpression confirmed that 2,3-BD synthesis pathway gene was expressed on RNA and protein levels, and the recombinant *E. coli* exhibited a 38.9-fold higher enzyme activity (312.57 units/mg protein), yielding 8.97 g/L 2,3-BD is 2.4-fold increase of with respect to the non-recombinant strain. The metabolically engineered strain *E. coli* BL21(DE3)pLys/pJET1.2/blunt-dhaD (*E. coli* BL21(DE3)pLys/dhaD), carrying the 2,3-BD pathway gene dhaD from our newly isolated *Klebsiella pneumoniae* SRP3 strain, displayed the best ability to synthesize 2,3-BD. The value of expression of an important glycerol metabolism gene dhaD is the highest ever achieved with an engineered *E. coli* strain. In addition to the 2,3-BD production, the systematic approach could be used in the production of other important value-added products using glycerol as the substract

through recombinant *E. coli* strains. From these results, the first reported dhaD expression system has paved the way for improvement of 2,3-BD production and efficient for another heterologous gene expression in *E. coli*.

7.1. Introduction

With increasing fossil fuels price and environmental concern, alternative and renewable energy sources have become attractive. Biodiesel, a renewable and promising combustion fuel is synthesized from vegetable oils and animal fats. However, biodiesel synthesis process (transesterification) generates 10% crude glycerol as a core by-product, which is the cheapest feedstock or negative-value biomass to produce a high-value green product 2,3-BD [1]. 2,3-BD is an important platform chemical, and it is also known as an excellent building block in the synthesis of valuable chiral chemicals [2-4]. Optically pure 2,3-BD production through chemical biosynthesis processes is hard to control, complicated and expensive. Thus, the biotransformation process has considered as the preferred method for the production of optically pure 2,3-BD [2-4]. Several engineered strains of microorganisms including *Saccharomyces cerevisiae, Enterobacter cloacae, Bacillus licheniformis* and *E. coli* have been used for production of optically pure 2,3-BD [5-8].

In the past few years, several microorganisms including cyanobacteria, fungi and bacteria have been proved to produce biofuels and fuel additives using different biomass [9, 10]. *E. coli* is extensively used as a model organism for biofuel production using pentose and hexose sugars from lignocellulosic biomass [11]. Now, it has been proved that recombinant *E. coli* can produce numerous biofuels including ethanol, acetone, butanol, α-pinene, isoprenol, isobutanol and fatty alcohols through biosynthesis pathways [11-15]. Nevertheless, these biofuels are highly toxic to *E. coli,* and the isolation of new end-products which are less or non-toxic to microbial cells is

needed to attain high product yield [16] using low-cost or negative-cost biomass. Thus, less toxic metabolic products like, 2,3-BD, 1,3-PDO and acetoin can be produced through biotechnological routes [17]. Moreover, an industrially important platform chemical 2,3-BD could be produced through oxidative pathway of recombinant *E. coli* [18]. A high heating value (27,200 J/g) bulk chemical 2,3-BD could be used as liquid fuel or fuel additive [19], which has very low toxicity to bacterial cells [20]. Therefore, 2,3-BD could be a promising alternative for biofuel production through recombinant *E. coli* strains.

Several bacterial species including *Klebsiella pneumoniae*, *K. variicola, K. oxytoca*, *Serratia marcescens* and *Enterobacter cloacae* have been used to produce 2,3-BD with high yields through optimization of culture conditions or genetic engineering [21, 22], but these strains are pathogenic or opportunistic pathogenic and have been categorized under microorganisms-2 for unsuitable of industrial scale biotransformation [17, 22]. However, the strain *E. coli* BL21(DE3)pLys has been known to be a non-pathogenic that does not carry any virulence factor or pathogenic mechanisms causing infections [23]. Consequently, *E. coli* BL21(DE3) strain could be the best candidate for safe synthesis of bioproducts [14, 23]. Therefore, in this research work, a preliminary attempt has been made to establish a method for 2,3-BD production efficiently using *E. coli* BL21(DE3). Moreover, in oxidative pathway of glycerol metabolisms, there are three key enzymes viz., glycerol dehydrogenase (GDH), α-acetolactate synthase and acetoin reductase, which are involved in 2,3-BD biosynthesis [14, 21]. Consequently, GDH is the first enzyme in oxidative path way for converting glycerol to dihydroxyacetone(DHA), and then 2,3-BD is produced through pyruvate [1]. Several reports have been studied on the production of 2,3-BD by recombinant *E. coli* through metabolic engineering of the genes budB and budC responsible for α-acetolactate synthase and acetoin reductase enzymes respectively [24], but there is no report on

dhaD gene which is responsible for GDH enzyme production. Therefore, it is important to construct an efficient 2,3- BD biosynthesis pathway that includes the related gene cluster to improve the 2,3-BD production. In this backdrop, my aim was to construct a novel dhaD expression system in *E. coli* and its application for 2,3-BD production under completely aerobic condition. In this metabolic engineering work, a systematic approach has been taken to construct and optimize 2,3-BD production by an efficient engineered *E. coli* BL21(DE3)pLys. Also, a recombinant *E. coli* was constructed through expressing the GDH, and 2,3-BD was produced from glycerol. This research work is the 1st step for systematic metabolic engineering, which we successfully made a novel expression system of dhaD gene, and a high enzyme activity (GDH) was achieved through batch biotransformation process using glycerol as the sole carbon source.

7.2. Materials and methods

7.2.1. Enzymes and chemicals

2,3-BD (99.0%) was purchased from Sigma Aldrich (Canada). The restriction enzymes, FastPfu DNA polymerase and T4 DNA ligase were purchased from Thermo Fisher Scientific, Canada. Isopropyl-β-D-thiogalactoside (IPTG), ampicillin and chloramphenicol were obtained from BioShop, Canada. All other chemicals used in this research work were analytical-grade reagents and commercially available.

7.2.2. Bacterial strains and vector

Newly isolated *K. pneumoniae* SRP2 was used for amplification of dhaD gene. E. *coli* JM109 and *E. coli* BL21(DE3)pLys strains were used as the hosts for gene cloning and expression respectively. *E. coli* BL21 (DE3)pLys was used as a host strain for 2,3-BD production. Cloning

vector pJET1.2/blunt (Thermo Fisher Scientific, Canada) and *E. coli* BL21(DE3)pLys were contained ampicillin and chloramphenicol resistant genes respectively.

7.2.3. Media and growth conditions

K. pneumoniae SRP3 and *E. coli* JM109 were grown in the Luria-Bertani (LB) medium contained (g/L): peptone 10.0 g/L, yeast extract 5.0 g/L and sodium chloride 5.0 g/L. The LB broth medium was supplemented with 100 μg/ml ampicillin and 100 μg/ml ampicillin plus 34 μg/ml chloramphenicol for plasmid containing *E. coli* JM109 and *E. coli* BL21 (DE3) pLys/dhaD respectively when necessary to maintain the plasmids. For biotransformation or expression study of *E. coli* BL21 (DE3) pLys/dhaD, MS-2 medium (K_2HPO_4 0.1 g/L, $NaNO_3$ 0.1 g/L, $MgSO_4.7H_2O$ 0.05 g/L, KCl 0.1 g/L, yeast extract 2.5 g/L and peptone 5.0 g/L) supplemented with 25.0 g/L glycerol, 100 μg/ml ampicillin and 34 μg/ml chloramphenicol was used. The initial pH of the medium was adjusted to 7.0 by adding NaOH/HCl. The biotransformation was carried out in a 250-ml flask containing 50.0 ml medium with 5.0 ml seed culture using rotary shaker incubator at 200 rpm and 37°C under aerobic condition. All the seeds and culture medium of the *E. coli* BL21 (DE3) pLys/dhaD were supplemented with ampicillin (100 μg/ml) and chloramphenicol (34 μg/ml) to maintain the plasmid.

7.2.4. Construction of plasmid for dhaD gene

The dhaD gene encoding glycerol dehydrogenase was amplified through PCR using *K. pneumoniae* SRP2 genomic DNA as the template. The primers used in PCR were: Forward primer- GGATCCATGCGCACTTATTTGAGGGTGA (with BamH1 restriction site) and reverse primer - AAGCTTACGCGCCAGCCACTGGCCT (with HindIII restriction site). The PCR conditions were as follows: initial denaturation at 94ºC for 3 minutes; then 30 cycles of 30s at 94ºC, 30s at 58º C and 1 min at 72º C; and final extension at 72ºC 10 min. The amplified product was ligated

into cloning vector pJET1.2/blunt at PCR product site, then the ligation mixture was transformed into *E. coli* JM109 by the calcium chloride heat shock method [25], resulting in recombinant plasmid designated as pJET1.2/blunt-dhaD. The ampicillin resistant colonies were selected on the LB agar plate supplemented with 100 μg/ml ampicillin and purified. The plasmid containing dhaD gene was extracted from *E. coli* JM109, purified, and transferred to the competent cell *E. coli* BL21 (DE3) pLys. The recombinant *E. coli* BL21 (DE3) pLys/ pJET1.2/blunt-dhaD designated as *E. coli* BL21 (DE3) pLys/dhaD was obtained using heat shock transformation method [25].

7.2.5. Expression and SDS-PAGE analysis of dhaD

The transformed *E. coli* BL21 (DE3) pLys containing plasmid pET-28a-dhaD was grown at 37^{o}C in LB medium supplemented with 100 μg/ml ampicillin and 34 μg/ml chloramphenicol for 24h with shaking at 200 rpm. This overnight culture was inoculated into fresh LB medium containing antibiotics to an OD600 of 0.05–0.1(1:50 dilution of the overnight culture). One set of glycerol stock culture was stored at -80^{o}C until getting the clone that best expresses targeted protein. The cultures were grown until they reach mid-log phase (OD600 =0.4-0.5, 2 to 3 hours). Then, the cultures were induced by adding IPTG to a final concentration of 0.5 mM and cultured for an additional 2–4 hours, took time points to analyze for optimal expression of targeted protein. Clones were then used for enzymatic assay to determine which clone best expresses the protein of interest. Cells were harvested after 4-7h of incubation and 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), centrifuged (3-5 min at 15,000 X*g*). The supernatant was kept at low temperature (4°C) and used for protein, and GDH enzyme assays. Protein samples were separated by SDS-PAGE using 12% (w/v) SDS-polyacrylamide gel, and identified by staining with Coomassie Brilliant Blue R-250 (Merck). SDS-PAGE analysis was carried out using the Laemmli method

[26]. The operation was performed on the Mini-Protean Tetra System Electrophoresis (BioRad, Canada). The Color Burst Marker Proteins (Sigma) and the Broad Range Protein Molecular Markers (Fermentas) were used to estimate the molecular weight of proteins.

7.2.6. Expression: RT-PCR analysis and enzyme assay

To study the expression of the dhaD gene responsible for glycerol utilization in IPTG induced strain *E. coli* BL21 (DE3) pLys/dhaD, the experiment was carried out with qRT-PCR (Quantitative Real Time Polymerase Chain Reaction). Total RNA was extracted using the PureLink^{Tm} RNA extraction kit (Ambion, Thermo Fisher Scientific, USA), following the manufacturer's instructions. The expression of the dhaD gene was evaluated via RT-PCR. First strand cDNA was prepared by reverse transcription, using cDNA synthesis kit (Tetro cDNA synthesis Kit, Bioline, UK) and RNA as a template. Quantitative gene expression was carried out using SensiFAST^{TM} SYBR No-ROX Kit (Bioline, UK) on C1000^{TM} thermal cycler quantitative real-time PCR (qRT-PCR) detector system (BioRad, USA). The 16S rRNA, obtained based on the primers 5′-GCGGTTGTTACAGTCAGATG-3′ and 5′-GCCTCAGCGTCAGTATCG-3′ was used as an internal standard. The primer sequence of glycerol metabolism gene dhaD used in this study is upstream: AGCGGGCGAGTTTGAAGAGTATCTG and downstream: GCCAGCAGCGTATCATAGCACAGG. The $2^{-\Delta\Delta CT}$ method was used to analyze the fold change gene expression over control [27].

However, the intracellular GDH activity 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 described by Ahrens et at [28] with slightly modification [1]. Briefly, 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 µmoles of substrate/minute/mg of cell protein and represent averages for at least three cell preparations. The Bradford method was used for determination of protein concentration [29] and bovine serum albumin served as the standard protein.

7.2.7. Analytical methods

Biomass concentration was measured from absorbance at 600 nm. Optical density at 600 nm (OD_{600}) was obtained from microplate spectrophotometer (EPOCH, BioTek). Metabolic product was identified by GC-MS (Varian 1200 Quadrupole). The concentrations of major metabolic product 2,3-BD, as well as glycerol were quantified using a GC-FID (Shimatzu GC 14A) under the following conditions: sample volume 1 µl; column temperature range from 45°C (2 min) to 240°C at the rate of 10°C/min; the injector and detector temperature 250°C; carrier gas nitrogen; column DB-WAXetr. The injecting sample was purified by centrifugation (Fisher Scientific, Germany, accu Spin Micro 17,) and membrane filter (0.22 µm pores size) respectively.

7.3. Results and Discussion

7.3.1. Construction of plasmid for dhaD

The cloning vector pJET1.2/blunt was used for cloning the dhaD gene encoding glycerol dehydrogenase. After ligation the amplified product (dhaD) into cloning vector pJET1.2/blunt, the vector was transferred into *E. coli* JM109, resulting in recombinant *E. coli* JM109/pJET1.2/blunt-dhaD. The ampicillin resistant colonies were selected from LB agar plates supplemented with 100 µg/ml ampicillin, purified and the plasmid of transformed *E. coli* JM109/pJET1.2/blunt-dhaD

strain was extracted for confirmation of dhaD. Transformation of the plasmid containing dhaD gene into *E. coli* JM109 was confirmed by Agarose gel electrophoresis analysis (Fig. 7.1).

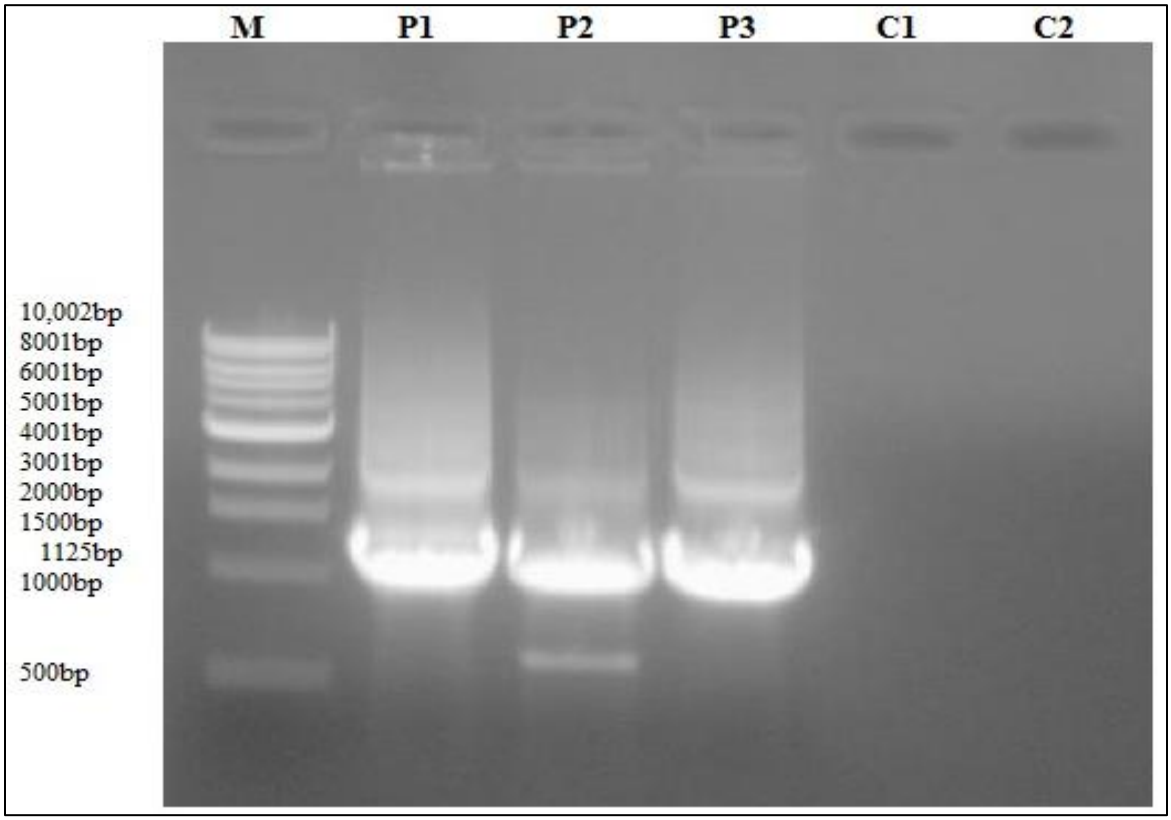

Fig 7.1. Agarose gel electrophoresis analysis of the whole cell lysate samples from the competent *E. coli* JM109 cell cultures containing plasmid dhaD. Lane P1, P2 and P2 cell lysates from the competent bacteria containing the dhaD gene construct; lane C1 and C2- negative control (no plasmid); lane M – molecular mass markers. Recombinant glycerol dehydrogenase (dhaD) from *K. pneumoniae* SRP2 is indicated by 1125bp.

7.3.2. Selection of clone of engineered E. coli BL21(DE3)pLysS/dhaD strain

However, for selection of the efficient clone, total six transformed *E. coli* BL21(DE3)pLysS/dhaD clones were tested for their GDH enzyme activity after 3h of IPTG induction. Among the six clones tested herein, the clone 3 exhibited highest activity of GDH which was 312.57 U/mg protein (Table 7.1). Thus, the clone 3 was finally selected for expression study,

and the clone nominated as *E. coli* BL21(DE3)pLysS/dhaD strain. The clone 3 also exhibited the highest biomass production. However, for confirmation of dhaD gene inserted into *E. coli* BL21(DE3)pLysS agarose gel electrophoresis analysis as well as gene sequencing were performed. The open reading frame (ORF) of dhaD gene was 1125 bp (Fig. 7.2). The dhaD gene was blasted in NCBI and the highest homologous gene was dhaD from *K. pneumoniae* TUAC01 (Fig.7.3)

Table 7.1. Glycerol dehydrogenase (GDH) activities of the clones constructed with dhaD containing plasmid

E. coli BL21 (DE3) pLys/dhaD	GDH activity (U/mg protein)	Biomass (OD_{600})
Clone 1	253.56±12.85	0.97±0.03
Clone 2	245.44±12.33	0.87±0.02
Clone 3	312.87±14.81	1.27±0.06
Clone 4	298.97±8.94	1.08±0.03
Clone 5	233.33±11.47	0.98±0.05
Clone 6	277.54±13.33	0.88±0.02
E. coli BL21 (DE3) pLys (Control)	11.54±0.97	0.55±0.03

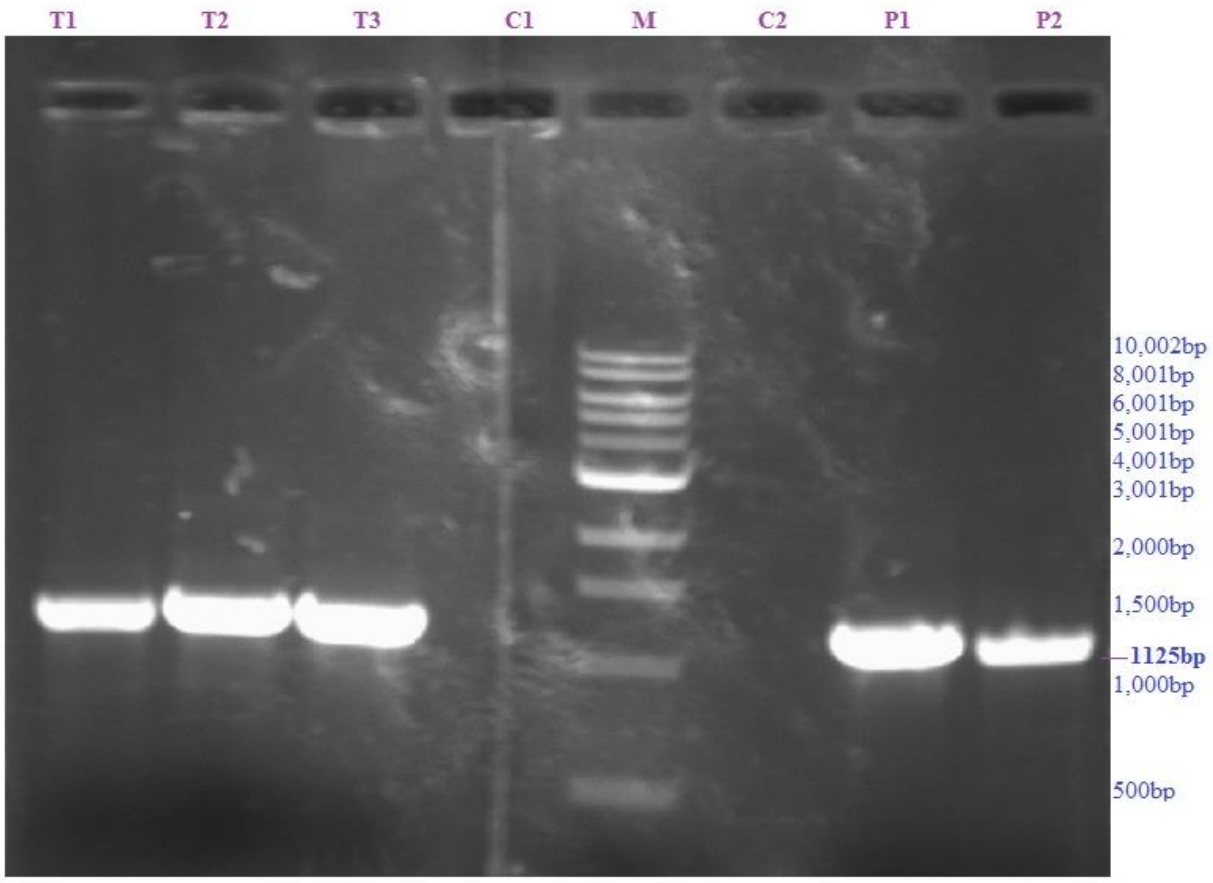

Fig 7.2. Agarose gel electrophoresis analysis of the whole cell lysate samples from the expression *E. coli* BL21(DE3)pLys cell cultures containing plasmid dhaD. Lane T1, T2, T3, P1 and P2 cell lysates from the competent bacteria containing the dhaD gene construct; lane C1 and C2- negative control (no plasmid); lane M – molecular mass markers. Recombinant glycerol dehydrogenase (dhaD) (1125 bp) (lane P2) from *K. pneumoniae* SRP2 is indicated by a purple line.

>SR12_dhaD Sample_Name=SR12_dhaD Chromat_id=5971413 Read_id=5914674 Version=1 Length=254

CGGCGCGCACGCTTTCCGGGATCACCGCAAACGGCATATTGTGGATGGTCTCCCCTTCCGCGCAGGTGGCTTTTGC
CACCGCGGTGATTTTCGCGTCGATCCCCTCTTTGACGCCCATCTGCGCGAGGGTCACCGGCAGGCCGACGCGCTG
GCAGAAGCCCAGCACGGTTTCAATCTCGTCCATCGGGCTGTTCTGCAGCACCAGCTGCGCCAGGGTGCCAAAGGC
CACT

Klebsiella pneumoniae strain TUAC01 glycerol dehydrogenase (dhaD) gene, complete cds
Sequence ID: EF424559.1Length: 1098Number of Matches: 1
Related Information
Range 1: 822 to 1051GenBankGraphicsNext MatchPrevious Match

Score	Expect	Identities	Gaps	Strand
387 bits(209)	8e-104	223/230(97%)	0/230(0%)	Plus/Minus

```
Query  1     CGGCGCGCACGCTTTCCGGGATCACCGCAAACGGCATATTGTGGATGGTCTCCCCTTCCG  60
             ||||| ||||||| |||||| ||||||||||||||||||||||||||| ||||||||||
Sbjct  1051  CGGCGTGCACGCTCTCCGGGGTCACCGCAAACGGCATATTGTGGATGGTTTCCCCTTCCG  992

Query  61    CGCAGGTGGCTTTTGCCACCGCGGTGATTTTCGCGTCGATCCCCTCTTTGACGCCCATCT  120
             |||||||||||| |||||||||| ||||||||||||||||||||||||||||||||||||
Sbjct  991   CGCAGGTGGCTTTCGCCACCGCGGCGATTTTCGCGTCGATCCCCTCTTTGACGCCCATCT  932

Query  121   GCGCGAGGGTCACCGGCAGGCCGACGCGCTGGCAGAAGCCCAGCACGGTTTCAATCTCGT  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  931   GCGCGAGGGTCACCGGCAGGCCGACGCGCTGGCAGAAGCCCAGCACGGTTTCAATCTCGT  872

Query  181   CCATCGGGCTGTTCTGCAGCACCAGCTGCGCCAGGGTGCCAAAGGCCACT  230
             |||||||||||||||||||||||||||||||||||| |||||||||
Sbjct  871   CCATCGGGCTGTTCTGCAGCACCAGCTGCGCCAGGGTGCCGAAGGCCACT  822
```

Fig 7.3. NCBI BLAST result: Comparison of sequences between the cloned dhaD gene amplified from *E. coli* BL21(DE3)pLysS/dhaD and dhaD gene from *K. pneumoniae* TUAC01.

7.3.3. Enzyme activity

To compare the expression levels of *E. coli* BL21(DE3)pLysS and *E. coli* BL21(DE3)pLysS/dhaD strains, GDH enzyme activities were determined *in vitro*. As shown in Figure 7.4, when the GDH-expressing plasmid pJET1.2/blunt-dhaD was introduced into *E. coli* BL21(DE3)pLysS, however, the resultant strain *E. coli* BL21(DE3)pLysS/dhaD displayed a

significant increase in GDH activity which was 312.57 U/mg protein, 38.9 times more than those of *E. coli* BL21(DE3)pLysS (Fig 7.4). Similarly, the overexpression of GDH gene in the *E. coli* BL21(DE3)pLysS did not lead to a significantly enhanced GDH activity after induced by IPTG (Fig. 7.4). Similar effects were observed previously in *Gluconobacter oxydans* MF1 when the gene coding for glucose dehydrogenase or gluconate-5-dehydrogenase was overexpressed [31].

Moreover, the GDH enzyme activity, was evaluated for *E. coli* BL21(DE3)pLysS and its plasmid containing strain *E. coli* BL21(DE3)pLysS/dhaD, and was found to be an average 38.9.00-fold increased in *E. coli* BL21(DE3)pLysS/dhaD which was directly correlated with an increase in expression of GDH from plasmid pJET1.2/blunt. As shown in Figure 7.4, the GDH activity increased during late log and stationary phases of growth for *E. coli* BL21(DE3)pLysS/dhaD, and were found to be much more greater than *E. coli* BL21(DE3)pLysS.

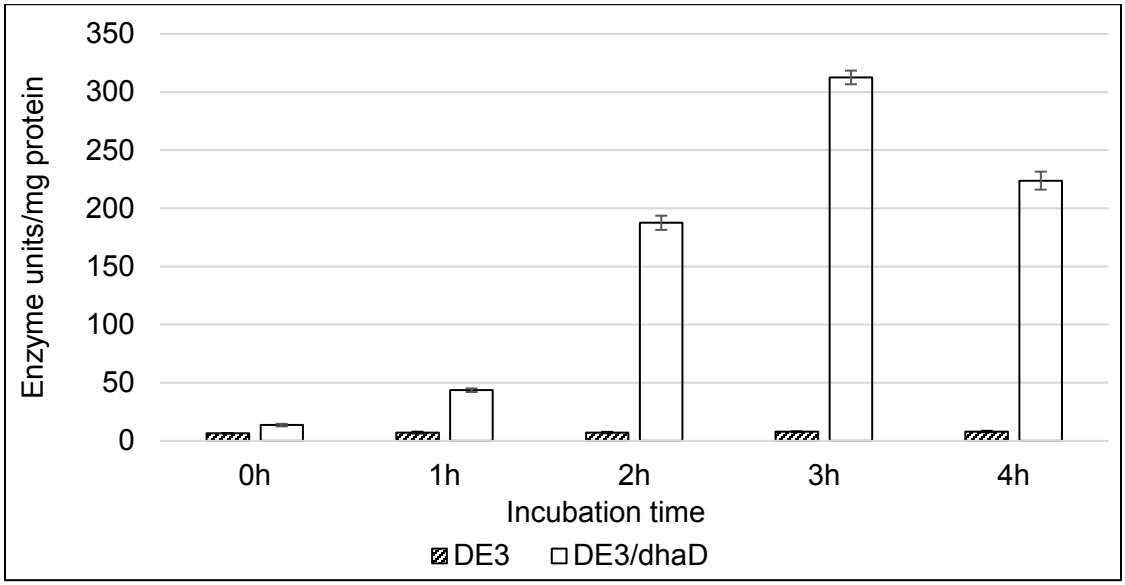

Fig 7.4. Comparison of GDH enzyme activities between the strains *E. coli* BL21(DE3)pLysS (DE3) and *E. coli* BL21(DE3)pLysS/dhaD (DE3/dhaD)

7.3.4. RT-PCR analysis

However, for demonstrating the expression of GDH gene in *E. coli* BL21 (DE3) pLys and *E. coli* BL21 (DE3) pLys /dhaD strains, detection of the mRNA was carried out with real-time RT-PCR. The expressions of gene dhaD in *E. coli* BL21 (DE3) pLys/dhaD and *E. coli* BL21 (DE3)pLys were determined using qRT-PCR. RT-PCR analysis was performed on bacteria having undergone glycerol transformation by dhaD gene expression construct which was obtained from *K. pneumoniae* SRP2. The use of two antibiotics (ampicillin, chloramphenicol) allowed for stable maintenance of plasmid in the recombinant bacterium. The qRT-PCR analysis exhibited the expression of dhaD gene from *K. pneumoniae* after added IPTG as an inducer (Fig. 7.5). As gene expression often varied significantly in different growth phases [30], all bacterial cells used for RNA isolation were cultured for constant period to minimize its effect on RT-PCR analysis. Taking 16S rRNA as the internal standard, it was clearly observed that the transcription level of GDH gene in *E. coli* BL21 (DE3) pLys/dhaD was much higher than that of the control strain *E. coli* BL21 (DE3) pLys (Fig. 7.5). In *E. coli* BL21 (DE3) pLys/dhaD, the expression level of GDH gene was about 84 times more abundant than that in *E. coli* BL21(DE3)pLys after 3h of IPTG induction, which was due to GDH overexpression. Therefore, the results confirmed expression of dhaD in *E. coli* BL21(DE3)pLys/dhaD expression system.

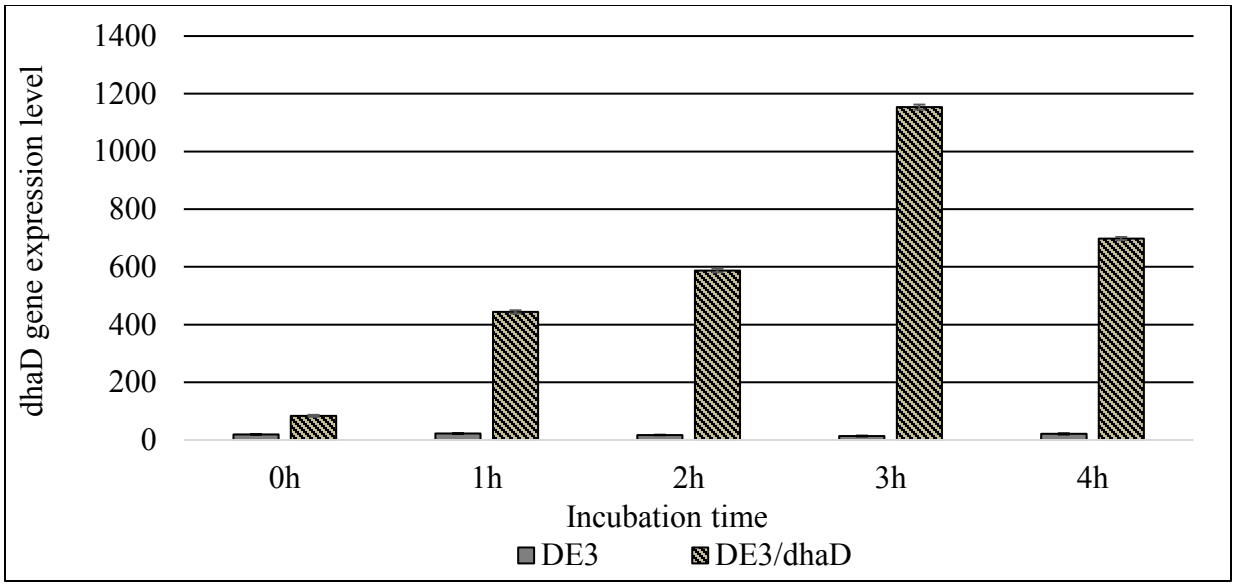

Fig 7.5. Comparison of expression levels of dhaD between the strains *E. coli* BL21(DE3)pLysS (DE3) and *E. coli* BL21(DE3)pLysS/dhaD (DE3/dhaD)

7.3.5. Overexpression of the pJET1.2/blunt-dhaD construct

The *E. coli* BL21(DE3)pLysS strain contains a fragment of the DE3 phage genome in the genome system, containing a T7 RNA polymerase gene under the control of the lacUV5 promoter. Logically, the bacterium contains lacI gene in chromosome and expression plasmid, which is encoding a repressor that binds to the T7lac promoter on the expression plasmid and the lacUV5 operator–promoter, thereby blocking the expression of the gene encoding RNA polymerase. This repression is induced by IPTG, which allowing induced transcription from the T7lac promoter. The gene dhaD transcribed in the similar way and controlled by the T7lac promoter. The results indicate efficient overexpression of the dhaD gene from *K. pneumoniae* SRP2 in *E. coli* BL21 (DE3) pLys. SDS-PAGE analysis of the expression products from genes inserted with the pJET1.2/blunt-dhaD construct indicates a higher level of the expressed protein in *E. coli* BL21(DE3)pLysS strain.

7.3.5. Batch fermentation by the recombinant E. coli BL21(DE3)pLysS/dhaD strain

After transformed *E. coli* BL21(DE3)pLysS strain by pJET1.2/blunt construct, the presence of the inserted plasmid was confirmed via the PCR reaction with primer specific for the sequence of dhaD encoding enzyme. The purpose of the culture was both to assess the production of 2,3-BD and to determine concentrations of other glycerol catabolism products, the efficiency and selectivity of the process, and glycerol consumption. The concentration of the desired metabolite (2,3-BD), as well as of unused glycerol in the culture medium was analyzed using GC-FID. IPTG was added in the culture medium (MS-2 medium supplemented with 25.0 g/L glycerol) after 6 h. As the GDH activity was increased when the GDH gene was overexpressed, a detailed study of the time course of glycerol oxidation by *E. coli* BL21 (DE3) pLys/pET-28a-dhaD as well as its corresponding control strains was carried out in shake flasks. The results of the culture are shown in Fig. 7.6. The recombinant bacterial strain containing the pET-28a-dhaD gene construct was shown to have produced 8.97 g/L of 2,3-BD by consumed 24.67 g/L of glycerol after 48h incubation. This product yield of 2,3-BD is not high as the strain *K. pneumoniae* SRP2. The strain *E. coli* BL21 (DE3) pLys/dhaD might be produced metabolic products other than 2,3-BD. Therefore, the other two important genes budB and budC responsible for 2,3-BD production should be inserted in to glycerol metabolism pathway of *E. coli* BL21 (DE3) pLys/dhaD stain to get high yield of 2,3-BD.

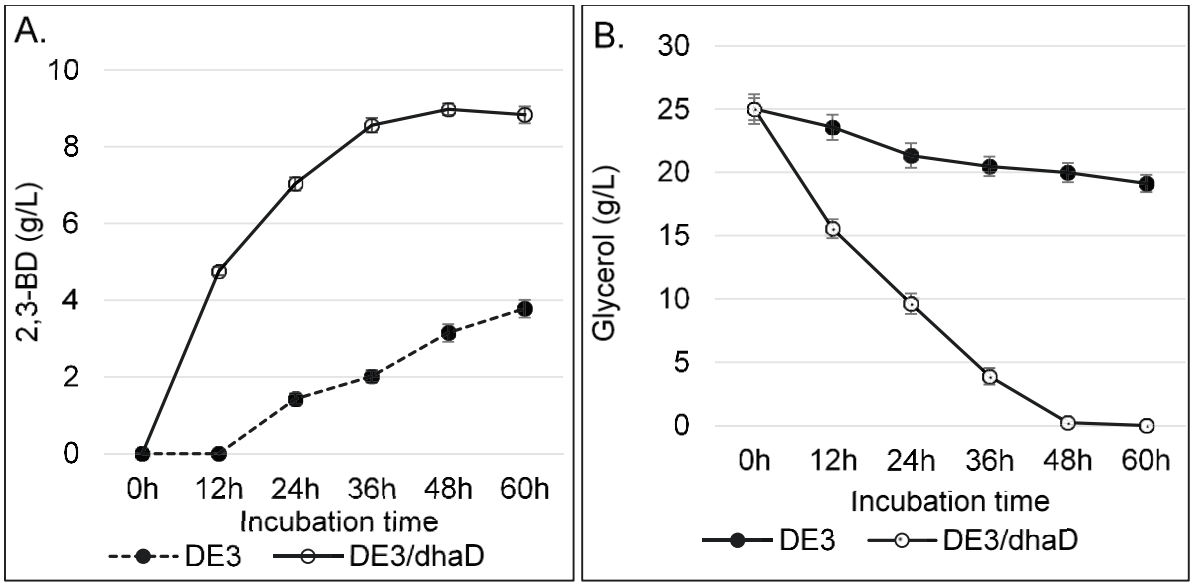

Fig 7.6. Comparison of 2,3-BD product yield and glycerol consumed between the strains *E. coli* BL21(DE3)pLysS (DE3) and *E. coli* BL21(DE3)pLysS/dhaD (DE3/dhaD)

We have demonstrated that overexpression of GDH in a *E. coli* strain led to a significant improvement of GDH activity and 2,3-BD production. The increased GDH activity led to a 2.4-fold increase in 2,3-BD product yield compared with *E. coli* BL21 (DE3) pLys in a batch biotransformation when 25.0 g/L glycerol was supplied and shortened the reaction time (Fig. 7.5 and 7.6). It is also possible that higher levels of GDH could be increased the tolerance of the strain against product(s) inhibition, as suggested by Gatgens et al. [32]. Since in utilized a sufficient amount of glycerol, a relatively low yield of 2,3-BD was attained (Fig. 7.6A and 7.6B), possibly produced bioproducts other than 2,3-BD due to lack of sufficient activities of other genes in the metabolic pathway of 2,3-BD, further improvements in yield will have to construct recombinant strain by introducing budB and budC genes from highly efficient strain and involve changes in fermentation procedures such as those described by Hekmat et al. [33] who designed a reactor system consisting of a shaking tank and a permeable column harboring immobilized cells.

The current experiments were carried out with pure glycerol. In a commercial setting, most likely biodiesel-derived glycerol would have to be used. This type of glycerol typically contains various impurities that could cause severe inhibition at high concentrations, or even are toxic to the cells [34]. It remains to be determined if our recombinant strain will be able to provide high yields under those conditions.

7.4. Conclusion

A promising strain for industrial production of 2,3-BD was developed by overexpressing the GDH gene in the *E. coli* BL21 (DE3) pLys strain. Taking advantage of the elevated activity of GDH, the recombinant *E. coli* BL21 (DE3)pLys/dhaD strain can produced 2,3-BD at an acceptable concentration and displayed a substantially increased GDH activity when IGTG inducer was used. The recombinant strain thus has potential for industrial production of 2,3-BD.

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Chapter 8: Discussion and Future Directions

Global climate change, uncertain sources of petroleum and rising cost of fossil fuels have sparked a worldwide search for "greener" energy replacement. Biodiesel is the most abounded renewable biofuel which has been rapidly gaining attention as a greener energy. However, the rapid growth of biodiesel industries is generating a large amount of biodiesel-derived crude glycerol which is now considered as a waste instead of product. Therefore, the biodiesel industries are currently facing several challenges for its economical development. For one, the purification of this large amount of crude glycerol to pure glycerol is also a costly process. Furthermore, the impurities present in biodiesel-derived crude glycerol negatively influence the bioconversion to value-added bioproducts by microorganisms. Consequently, there are no single microorganisms which can convert this biodiesel waste crude glycerol to efficiently produce biofuel and value-added product. Thus, high-value application of this crude glycerol is the urge of situation to support the sustainability of the development of biodiesel industry all over the world. In this situation, biotransformation of glycerol as well as biodiesel-waste crude glycerol is a promising way in which this low-value can be converted to high-value bioproducts. Fuel and chemical industries are among the fields where a large amount of glycerol could be utilized as its derivatives. All of the work presented here in this thesis, approaches these challenges in the current production of 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 strains. There were several reasons for this choice, namely bacteria can be easily cultured, can be found in habiting unlimited environments and can survive in extreme environmental stresses. These attributes lend to the potential exploitation of hardier bacterial strains for the industrial biotransformation of a large volume of glycerol or crude glycerol to biofuels and other high-value

products. Also, these attributes could allow for development of a conventional way of bioconversion processes which can work efficiently to use industrial level.

This PhD research work led to discovery of new glycerol degrading bacteria and development of novel strains with potential in future studies for advancement in bioconversion processes. Moreover, this work lends knowledge to the future study and use of our novel bacterial strains in the simultaneous biotransformation of glycerol or biodiesel-derived crude glycerol to biofuels and bioproducts.

In this research work, pure and crude glycerol were used as the sole carbon source in batch and fed-batch flask cultures to produce 2,3-BD efficiently in different bioconversion processes using newly isolated or developed bacterial strains. Likewise, the effect of process parameters was studied, and the biotransformation conditions were optimized. The efficiency of the processes also assessed using pure and crude glycerol as feedstocks.

First of all, the research work presented in this thesis was focused on isolation and characterization of efficient glycerol degrading novel bacteria. Regarding this purpose, I could develop an efficient and economical method for screening a large number of bacteria from different environmental and commercial (paper mill waste) sources to find the most efficient and potentially unique glycerol dehydrogenase (a key enzyme of glycerol bioconversion process) producing bacterial strains, have potential for downstream application in the industrial production of 2,3-BD in completely aerobic process. In this research work, four isolates P1, P2, P4 and P5, both different strains of *Klebsiella pneumoniae*, one isolate P3 (*K. variicola*) were found to have the greatest glycerol dehydrogenase (GDH) activity. Moreover, another two isolates S2 and S3, both *Serratia* species also exhibited moderate to high GDH activity towards pure glycerol, but they did not grow more than 30^{o}C. However, until now, in biotechnological applications like production of high-

value products from glycerol looking for efficient GDH enzymes producing bacteria, traditional microbiological isolation techniques are still important. Several studies have been done on GDH activity bacterial strains [1-3]. Therefore, in this study, we have demonstrated a total 13 bacterial strains isolated from soil and paper mill waste which represent a foundation for the exploitation of GDH producing microorganisms which might be more efficient in the industrial environment. Consequently, the future work is required to characterize as well as development those isolates exhibiting greatest GDH activity. Considering for doing such work, we need a smaller sample size, and only two bacterial strains *K. pneumoniae* SPR2 (P2) and *K. variicola* SRP3 (P3) have been selected which show the greatest GDH activity.

However, in my 2nd study, an environmental consortium obtained from paper mill waste was investigated, and a stable co-culture of *pneumoniae* SPR2 and *K. variicola* SRP3 was developed for the 1st time which could improve united biotransformation in the production of 2,3-BD and acetoin. These two strains reported in this co-culture study working synergistically have the potential to complete responsibilities such as in this case, the biotransformation of both pure and crude glycerol, ultimately increase 2,3-BD yields. Thus, the co-culture of *K. pneumoniae* SRP2 and *K. variicola* SRP3 was capable of converting crude glycerol competently to produce a high yield 0.73 g/g (27.87 g/L) of 2,3-BD using 37.0 g/L glycerol under aerobic conditions in batch culture, showing great potential for biotransformation bioprocess. This co-culture also exhibited resistance towards toxic impurities present in crude glycerol. The results obtained from the co-culture constructed for converting biodiesel-derived crude glycerol to 2,3-BD in similar way to results stated in other researchers where the culture was environmental consortium for the production of 1,3-propanediol [4,5] Furthermore, the co-culture system developed here also advances knowledge for future improvements, and it is also a new finding on crude glycerol

bioconversion to 2,3-BD production. Therefore, it is exceedingly valuable to the aerobic biotransformation of biodiesel waste crude glycerol studies presented here if through mutation and metabolic engineered strains with increased resilience to bacterial inhibitory agent present in crude glycerol.

In my 3rd study, I used a newly isolated novel and highly active bacterial strain *K. variicola* SRP3 for the aerobic bioconversion of pure glycerol to value-added bioproducts. That is the first-time report to obtain a high production of 2,3-BD and acetoin with the bacterium *K. variicola* which have a great potential on the overall biodegradation of glycerol having activities towards high concentration of glycerol. Moreover, an adapted mutant strain *K. variicola* SRM3 obtained from this wild type stain SRP3 capable of growing at a high concentration (200 g/L) of glycerol which is the new report of glycerol bioconversion research study. The mutated strain SRM3 displayed 1.3-fold increased GDH activity, yielding bioproduct as 0.79 g/g glycerol (79.0% glycerol converted to bioproducts). However, in our report, the optimal conditions for maximal GDH enzyme activity and 2,3-BD product yield were defined, and a high yield of products was achieved by the muted strain *K. variicola* SRM3, which is the highest amount obtained from batch culture using glycerol as a sole carbon source until now.

Several works have been done on bio-conversion of pure glycerol to produce 1,3-PDO, 1,2-PDO, and H_2 under anaerobic or microaerophilic fermentation process [6,7], but very few work have been done on aerobic process for 2,3-BD production. Recently, it was reported that *Klebsiella pneumoniae and K. oxytoca* could produce 1,3-PDO anaerobically [8] and 2,3-BD aerobically [9]. Still now there is no any report for the bioconversion of glycerol by *K. variicola*. Lately, Petrov and Petrova [9] claimed the highest product yield 48.47% of 2,3-BD in feed batch fermentation. However, our study exhibited the product yield 59.8% of 2,3-BD attained by an adapted mutated

strain *K. variicola* SRM3 was the highest amount and reported till now. However, impurities like methanol, salts, soaps, non-glycerol organic matter and catalysts of raw (crude) glycerol, and by-products like acetate, lactate and succinate produced during glycerol biotransformation process can negatively influence of 2,3-BD production [10] [31]. Thus, future work will be focused on the investigation of expression of genes responsible for glycerol metabolisms to carryout metabolic engineering work for increasing product yield of 2,3-BD. Also, the further work is required to attain the highly efficient strain by mutagenesis for the utilization of crude glycerol and increase product yield of 2,3-BD. Bioreactor and optimized process parameters could be used to scale up product yield of 2,3-BD.

A new EMS mutant *K. pneumoniae* SRM2 has been developed for the 1st time for efficiently converting pure and crude glycerol to 2,3-BD and acetoin. EMS mutagenesis is widely used for increasing tolerance towards the inhibitors of a microbial strain, lead down-regulation of gene(s) responsible for the expected product [11-13], that may then gain an ability of higher productivity of a targeted product [14,15]. Our attainment with increased tolerance towards glycerol and increased 2,3-BD production by EMS mutagenesis confirmed the development of *K. pneumoniae* SRM2 mutant.

The mutant SRM2 obtained from newly isolated strain *K. pneumoniae* SRP2 withstanding high glycerol concentration (220 g L^{-1} of medium) could rapidly convert glycerol aerobically to 2,3-BD. There are very few studies that have reported crude glycerol utilizing bacteria and thus they have been readily considered a co-substrate (2nd carbon source) in addition to crude glycerol to produce 2,3-BD. [16,17]. However, the results presented in this thesis was obtained using pure or crude glycerol as the only substrate or sole carbon source for high production of 2,3-BD. Moreover, my study revealed that the mutant strain can tolerate high concentration of glycerol,

have a high glycerol utilization rate, and high product yield (77.5 g/L and 75.0 g/L using pure and crude glycerol respectively) of 2,3-BD in a fed-batch culture. Recently, Petrov and Petrova [9] reported a high production (70.0 gL^{-1} and 0.53 g/g) of 2,3-BD by *K. pneumoniae* using pure glycerol under control of fed-batch culture's pH. Additionally, our results on both pure and crude glycerol biotransformation are much higher than that of this recent report. This study also confirmed that a high glycerol concentration (55.0 g/L) of displayed maximum production yield of 2,3-BD, which could be the new prospect of glycerol bioconversion field. Consequently, keeping the conditions defined in this study the mutant strain *K. pneumoniae* SRM2 exhibited fewer co-products at trace concentrations at higher glycerol concentrations, and could be a potential candidate for 2,3-DB production in an industrial bioconversion process. Thus, future work will be focused on the investigation of genes expression in the metabolic pathway of glycerol metabolisms for metabolic engineering work to increase product yield of 2,3-BD.

Furthermore, another innovative adapted mutant strain *K. variicola* SW3 has been developed for the 1st time for efficiently converting biodiesel-derived crude glycerol to produce 2,3-BD. This mutant strain is resistant to all inhibitory impurities present in biodiesel-derived raw glycerol, and capable of converting raw glycerol at a concentration as high as 200.0 g/L. A product yield 0.63 g/g (64.9 g/L) of 2,3-BD obtained using crude glycerol as a sole carbon source or only the substrate was the highest amount reported until today. However, until now, there are only two reports on biotransformation of crude glycerol to produce significant amount of 2,3-BD using co-substrate in addition to crude glycerol [18,19]. Thus, my report on bioconversion of biodiesel-derived raw glycerol to produce high concentration of 2,3-BD led a more cost-effective process where low-value or negative-value raw glycerol was only the substrate. Furthermore, our report has for the first time proved that this *K. variicola* species can efficiently convert biodiesel derived crude

glycerol, and also 1st time report on biosynthesis gene expression study of glycerol metabolisms for metabolic engineering. The gene expression study revealed that the overexpression of GDH gene dhaD resulted in an increased GDH enzyme activity, led to a markedly boosted 2,3-butanediol (2,3-BD) production. Moreover, our report has for the first time proved that three important key genes dhaD, budB and budC have the great role on 2,3-BD product yield through oxidative pathway of glycerol metabolisms, and this finding could be a future direction of metabolic engineering for improving product yield of 2,3-BD. Thus, it is also our great achievement for glycerol bioconversion research work. Therefore, if future work can combine more resistant and efficient strains, produced via dhaD, budB and budC or mutagenesis, with increased copy numbers of glycerol dehydrogenase, acetolactate synthatase, and acetoin reductase (2,3-butanediol dehydrogenase) a more industrially viable system could be applied for 2,3-BD production, and could be incorporate into co-cultures such as that developed in my 2nd study.

However, an attempt was made to construct metabolically engineered strain suitable for industrial biotransformation of glycerol, and a novel expression system was developed in order to improve 2,3-BD production using a non-pathogenic *Escherichia coli* strain. The dhaD gene encoding glycerol dehydrogenase (GDH) obtained from *Klebsiella pneumoniae* SRP2 was successfully transferred to *E. coli* BL21(DE3)pLys, and expressed under the control of T7lac promoter. Thus, GDH was over-expressed (68.9-fold), and the engineered *E. coli* displayed 84.0-fold increased expression of dhaD gene, increased 2.4-fold in yield of 2,3-BD. It is the 1st time report on construction of high expression system of dhaD gene has paved the way for improvement of 2,3-BD production and will be used for other bacterial strain. Although, the construct utilized a sufficient amount of glycerol, a relatively low yield of 2,3-BD was obtained.

Thus, further improvements in yield will have to construct engineered strain by over expressing budB and budC genes, and involve changes in fermentation procedures [20].

Last of all, the key economic barrier to scaling-up and commercialization of microbial production of 2,3-BD may not be the fermentation, but somewhat the separation and purification of the end-product from the fermented broth media, although high concentration of end-product 2,3-BD in fermentation broth is not a problem for the fermentation because 2,3-BD is less toxic to microorganisms compared with other alcoholic products. Recovery and purification of 2,3-BD from fermented broth may represent one of the major technological challenges and an economical obstacle for efficient microbial production of 2,3-BD in large scale. The principal methods for recovery of this product include steam stripping, distillation, reverse osmosis, pervaporation, and solvent extraction [21-24]. There is no single method proved to be simple and efficient enough. Firstly, the recovery process of 2,3-BD from fermented broth is difficult due to its high boiling point, great affinity for water, and the presence of solid and dissolved constituents of the fermented broth. It was found out that the separation of 2,3-BD from fermented broth could make more than 50% of the total costs in the production, and it was estimated that the costs for the recovery of 2,3-BD from a model medium on a production scale of 500 tons per year by using single distillation, reverse osmosis followed by distillation and combination of distillation and extraction were to be 0.73, 0.69 and 0.67 USD/kg 2,3-BD respectively [25]. Therefore, large-scale microbial production of 2,3-BD is challenging in terms of energy consumption for separation and purification of the end-product from the fermented broth media. Innovation in separation technology is needed to address this challenge, and improvements are especially needed with regard to yield, purity, and energy consumption. For further development, classic separation techniques need to be improved or combined with other new technologies [26]. For instance, evaporation may be improved by

adopting multi-stage evaporation instead of single-stage evaporation. Moreover, the novel aqueous two-phase extraction method with short chain alcohols or hydrophilic organic solvents deserves attention in the future.

Details of the new and mutated strains could be retrieved from the website: https://blast.ncbi.nlm.nih.gov/Blast.cgi **of NCBI GeneBank by inputting following Accession numbers in Nucleotide Blast:**

Strain No.	Strain Name	GeneBank Accession No.	Type of Strain
SRP1	*Klebsiella pneumoniae* SRP1	KU550763	Wild
SRP2	*Klebsiella pneumoniae* SRP2	KR092085	Wild
SRM2	*Klebsiella pneumoniae* SRM2	KX572902	EMS mutant
SRP3	*Klebsiella variicola* SRP3	KR092086	Wild
SRM3	*Klebsiella variicola* SRM3	KX572903	Mutant
SW3	*Klebsiella variicola* SW3	KY437692	Mutant
SRP4	*K. pneumoniae* SRP4	KU550764	Wild
SRP5	*K. pneumoniae* SRP5	KU550765	Wild
S2	*Serratia liquefaciens* SRWQ2	KX602659	Wild
S3	*S. proteamaculans* SRWQ1	KX602658	Wild

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List of publications from my PhD works:

1. Md. Shafiqur Rahman, Chunbao (Charles) Xu, Kesen Ma, Malaya Nanda, Wensheng Qin. High Production of 2,3-butanediol by a Mutant Strain of the Newly Isolated *Klebsiella pneumoniae* SRP2 with Increased Tolerance Towards Glycerol. International Journal of Biological Sciences. (2017) 13:308-318.

2. Md. Shafiqur Rahman, Zhongshun Yuan, Kesen Ma, Chunbao (Charles) Xu, Wensheng Qin. Aerobic conversion of glycerol to 2,3-butanediol by a novel *Klebsiella variicola* SRP3 strain. Journal of Microbiology and Biochemical Technology. (2015). 7: 298-304.

3. Md. Shafiqur Rahman, Chunbao (Charles) Xu, Kesen Ma, Haipeng Guo, Wensheng Qin. Utilization of by-product glycerol from bio-diesel plants as feedstock for 2,3-butanediol accumulation and biosynthesis genes response of *Klebsiella variicola* SW3. Renewable Energy, (2017), 114: 1272-1280.

4. Md. Shafiqur Rahman, Chunbao (Charles) Xu, Wensheng Qin. Biotransformation of biodiesel-derived crude glycerol using newly isolated bacteria from environmental consortia. Process Biochemistry, (2017, Article in press).

5. Md. Shafiqur Rahman, Chunbao (Charles) Xu, Wensheng Qin. Engineering of *Escherichia coli* for construction of a novel expression system to produce 2,3-butanediol using gene from *Klebsiella pneumoniae.* Manuscript under preparation, (2017).