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Media optimization, scale-up and repeated fed-batch operations for the production of 1,3-propanediol from glycerol by halanaerobium hydrogeniformans

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Media Optimization, Scale-up and Repeated Fed-Batch Operations for the Production of 1,3-Propanediol From Glycerol by *Halanaerobium hydrogeniformans*

by

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This thesis is prepared in the style of three journals. The first paper has been submitted to *Life- an open access journal* and published. The second paper has been prepared for submission to the *Journal of Biotechnology*. The third paper has also been prepared for the submission to the *Journal of Biotechnology*. The Introduction in Section 1 and the Conclusion have been added for the purposes normal to thesis writing.

ABSTRACT

The development of renewable energy sources, such as biofuels, is of great interest in today's world due to depletion of fossil fuels and concerns about climate change. The production of the main by-product, crude glycerol, in biodiesel production presents an opportunity for the production of industrially relevant commodities. Crude glycerol in biodiesel waste is highly impure and possesses high salt concentrations and alkalinity, making it infeasible for direct industrial utilization. Moreover, current glycerol purification methods are highly cost-intensive, necessitating a search for cost-effective approaches that are feasible to employ. Genome data analysis of *Halanaerobium hydrogeniformans*, revealed the metabolic capability of this bacterium to convert glycerol into 1,3-propanediol (PDO), a relevant industrial commodity. To develop an optimum growth medium for this glycerol conversion, assays with different combinations of twenty-four media components were performed. Data analysis using HPLC demonstrated enhanced production of 1,3-propanediol with specific additions of glycerol, pH, vitamin B12, ammonium-ions, sodium sulfide, cysteine, iron and cobalt. Reactor studies were also performed to monitor the production of PDO, as a function of scale up in reactor volume. In addition, the impact of different glycerol concentrations and retained fraction volumes on PDO productivity was determined through repeated fed-batch reactions. Fed-batch operation provided a 39.2 percent increase in PDO concentration and a 61.7 percent increase in the productivity in comparison to the batch operation. Ongoing enzyme kinetics studies are being performed in order to better understand the PDO production pathway. This information will be helpful in modelling the dynamics of culture to maximize the yield and productivity of PDO production.

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SECTION

1. INTRODUCTION

1.1. BIOENERGY

The limited supply of non-renewable energy resources, such as coal, oil and natural gas, have brought interest to the utilization of renewable energy resources such as solar, biomass, wind and water [1]. Over the last decades,' great attention has been focused on the development of technologies to increase the efficiency of renewable energy sources [2]. Common types of biofuels thus far developed are ethanol and biodiesel. According to the US EPA, biodiesel and renewable energy market produced 2.8 billion gallons in the United States in 2016 [4]. However, biodiesel production generates high amounts of glycerol (almost 10% (w/w)) as its waste product [5]. Although pure glycerol has various industrial purposes, glycerol obtained from biodiesel is in crude form and possesses high pH (10), salt concentrations (3%) and impurities such as methanol, minerals, NaOH, and esters [6,7]. Therefore, prior to its utilization, purification and neutralization of crude glycerol are essential, leading to the increased cost of biodiesel production. To reduce such costs, it is crucial to develop effective ways for crude glycerol to be used. Various studies have been performed on the conversion of glycerol to 1,3-propanediol (PDO) by using bacteria such as; *Clostridium butyricum*, *Citrobacter freundii* and *Klebsiella pneumoniae* [8,9,10]. 1,3-propanediol (PDO) is used to make the polymer, polytrimethylene terephthalate, used in the carpet and leather industries [11]. Moreover, it is a chemical intermediate for the synthesis of lubricants, cosmetics, perfumes, and laminates [12]. Nevertheless, neutralization of crude glycerol is essential prior to its utilization by these non-extremophilic microorganisms in PDO production.

1.2. HALOALKALIPHILE

This project entails working with a haloalkaliphilic bacterium, *Halanaerobium hydrogeniformans*, isolated from Soap Lake in Washington state [13]. The genome analysis of *H. hydrogeniformans* indicated that this bacterium possesses genes actively involved in the conversion of glycerol to 1,3-propanediol. Optimum growth conditions

for this bacterium are 30˚C, pH 11 and 7.5% salt concentration, with the ability to grow at broad pH (7.5-12) and salt (2.5-15%) ranges [14]. This ability of the bacterium makes it ideal for the conversion of crude glycerol into PDO, as it removes the need for neutralization and can possibly make 1,3-propanediol production a cost-effective approach.

Glycerol is used as a carbon and energy source for the growth of microorganisms. Under anaerobic conditions, glycerol assimilation in the cell occurs via two pathways, oxidative and reductive, as shown in Figure 1.1 [15]. Through the oxidative pathway, glycerol is dehydrogenated by the enzyme glycerol dehydrogenase to dihydroxyacetone, which is further converted to pyruvate and further into various end-products such as acetate, ethanol, and lactic acid. In the reductive pathway, the conversion of glycerol to PDO occurs in two steps. In the first step, a water molecule is removed from glycerol and 3- hydroxy-propionaldehyde (3-HPA) is formed. This reaction is catalyzed by a vitamin B_{12} dependent enzyme, glycerol dehydratase. In the consecutive step, 3-HPA is reduced to PDO by the enzyme 1,3-propanediol dehydrogenase that oxidizes $NADH_2$ and regenerates NAD^+ needed by cell. In the cell, PDO is produced for the regeneration of NAD^+.

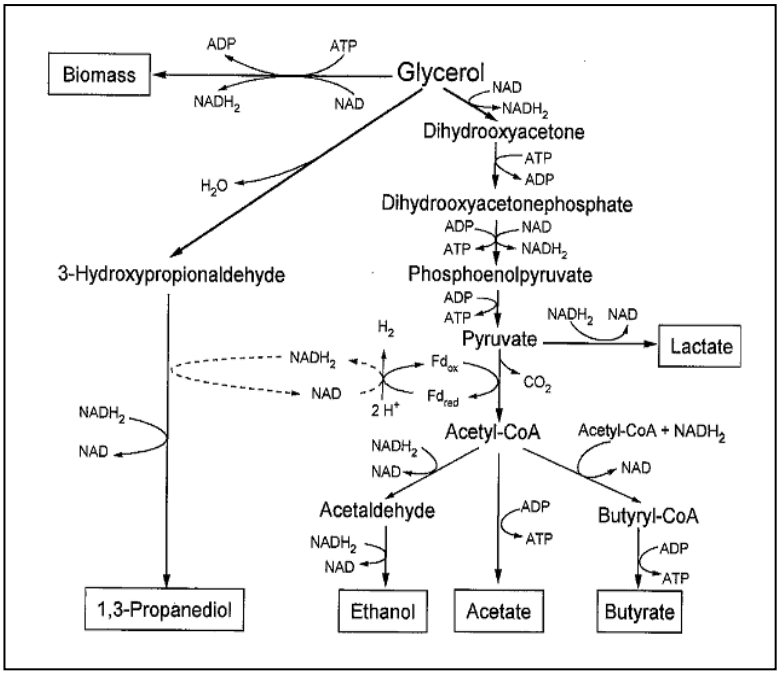

Figure1.1. Glycerol fermentation pathway modified from Zeng et al. [15]

In the initial studies on PDO production, media screening tests were designed and performed to identify the media components that have significant impact on PDO production. These tests showed that glycerol, ammonium ion, sodium sulfide, cysteine, iron, and cobalt had significant impact on the productivity [16]. Under these optimized media conditions, reactor studies were performed with this bacterium to determine the PDO yield. Reactors were run under batch and fed-batch conditions. HPLC analysis demonstrated the formation of PDO along with high amount of acetate, another fermentation byproduct. In order to increase the amount of PDO but minimize the concentration of by-products, it is essential to understand the kinetics of enzymes involved in PDO production.

1.3. GOALS AND OBJECTIVES

1.3.1. Media Optimization for 1,3-propanediol Production. The primary objective was to determine the factors that could have significant impact on the PDO production from glycerol by *H. hydrogeniformans*. A fractional factorial design approach was used to determine the effect of 24 factors, evaluated on the basis of standard media composition. This approach revealed the enhanced production of PDO with the addition of eight factors: glycerol, pH, ammonium ions, vitamin B_{12}, sodium sulfide, cysteine, iron and cobalt.

1.3.2. Scale up Reactor Studies. The objective was to measure the yield and productivity of PDO as a function of scale up in the reactor volume from 2 liters to 20 liters under batch conditions. In both of the reactor runs, three products were routinely found through HPLC analysis, PDO, acetate, and formate. As the reactor volume increased from 2 L to 20 L, both PDO concentration and productivity decreased by 10%.

1.3.3. Fed-Batch Reactor Studies to Maximize Productivity. This study was performed to identify the effect of high glycerol concentration as well as the impact of retained volume fraction on the PDO production. PDO production and productivity at five different retained volume fractions were measured. With increase in retained volume fraction, increase in the PDO concentration and productivity was observed. Moreover, the effect of five different glycerol feed concentrations on the PDO production was also

measured. Here too, increase in the glycerol concentration had resulted in the enhancement of PDO concentration and productivity.

1.3.4. Ongoing Enzyme Kinetics Study. This study is aimed at the activity analysis of the enzymes involved in PDO production by *H. hydrogeniformans*. Genes coding for each of the enzyme will be extracted from the bacterium. The genome data suggested that there is a total of six genes involved in the PDO production. The amplification of each gene will be done by using specifically designed primers. The amplified genes will be ligated into vectors and cloned into competent *E. coli* cells. Resulting recombinant proteins or enzymes will be purified from the transformed competent *E. coli* cells for activity analysis. Optimum conditions for the enzymes will also be determined. This information will be helpful in modelling the dynamics of the culture to maximize the yield and productivity of PDO production and for genetic modification of industrially relevant microorganisms.

PAPER

I. THE USE OF A FRACTIONAL FACTORIAL DESIGN TO DETERMINE THE FACTORS THAT IMPACT 1,3-PROPANEDIOL PRODUCTION FROM GLYCEROL BY *HALANAEROBIUM HYDROGENIFORMANS*

Published in Life- an open access journal.

ABSTRACT

In recent years, biodiesel, a substitute for fossil fuels, has led to the excessive production of crude glycerol. The resulting crude glycerol can possess a high concentration of salts and an alkaline pH. Moreover, current crude glycerol purification methods are expensive, rendering this former commodity a waste product. However, *Halanaerobium hydrogeniformans*, a haloalkaliphilic bacterium, possesses the metabolic capability to convert glycerol into 1,3-propanediol, a valuable commodity compound, without the need for salt dilution or adjusting pH. Experiments were performed with different combinations of twenty-four medium components to determine their impact on the production of 1,3-propanediol by using a fractional factorial design. Tested medium components were selected based on data from the organism's genome. Analysis of HPLC data revealed enhanced production of 1,3-propanediol with additional glycerol, pH, vitamin B_{12}, ammonium ions, sodium sulfide, cysteine, iron, and cobalt. However, other selected components nitrate ions, phosphate ions, sulfate ions, sodium:potassium ratio, chloride, calcium, magnesium, silicon, manganese, zinc, borate, nickel, molybdenum, tungstate, copper and aluminum did not enhance 1,3-propanediol production. The use of a fractional factorial design enabled the quick and efficient assessment of the impact of twenty-four different medium components on 1,3-propanediol production from glycerol from a haloalkaliphilic bacterium.

Keywords: glycerol, 1,3-propanediol, haloalkaliphilic bacteria, fractional factorial design

1. INTRODUCTION

Humanity's increasing consumption of energy requires the expenditure of resources. However, primary resources i.e. fossil fuels are finite and limited. This problem has drawn interest from the scientific community to develop effective methods for generating renewable sources of energy. Biodiesel is one of these emerging alternatives. However, a fundamental problem with biodiesel is that it leads to large amount of waste products as almost 10% (w/w) glycerol is produced as the main byproduct [1]. Pure glycerol has numerous applications in food, pharmaceutical, and cosmetic industries. Moreover, it can be converted to various chemical intermediates such as 1,3-propanediol (PDO), poly-hydroxyalkanoates, epichlorohydrin, acrylic acid, polyhydroxybutyrate etc. [2]. On the other hand, glycerol obtained from biodiesel is in a crude form and has many impurities. Hansen et al. [3] performed a study to determine the composition of crude glycerol samples obtained from the different biodiesel plants. They demonstrated that glycerol content in the samples ranged from 38% to 96%, with some samples comprising up to 14% methanol and 29% ash. Besides, crude glycerol has high pH (10) and salt concentration (3%). Therefore, purification and neutralization of crude glycerol, prior to its industrial utilization, is essential and leads to increased costs of biodiesel production [4].

Subsequently, effective ways for the utilization of crude glycerol need to be developed to reduce the cost of biodiesel production, thus, enhance the growth of biodiesel industries. One of such process is the conversion of crude glycerol to a chemical commodity, 1,3-propanediol. 1,3-propanediol has various industry applications, as it is used as a chemical intermediate for the synthesis of cosmetics, lubricants, adhesives, perfumes and laminates [5]. It is also used to make polymer polytrimethylene terephthalate, is used in carpet and textile industries [6]. Previous studies have revealed the role of microorganisms such as *Klebsiella pneumoniae* [7] and *Clostridium butyricum* [8] can have in the production of PDO. However, neutralization of the crude glycerol has to be done before it can be metabolized by these organisms.

This study was performed with a haloalkaliphilc bacterium, *Halanaerobium hydrogeniformans*, isolated from Soap Lake in Washington State [9]. *H. hydrogeniformans* has the ability to convert crude glycerol into PDO. It should be noted that optimum growth conditions for this bacterium are pH of 11, salt concentration of 7.5%, and a temperature of 30˚C [10]. Moreover, this bacterium is capable of growing at a broader pH range (7.5-12) and salt range (2.5-15%) in comparison to other PDO-producing bacterial species. This property of *H. hydrogeniformans* makes it ideal for the conversion of crude glycerol into PDO. The use of this organism removes the need for the neutralization of crude glycerol and salt dilution, possibly making PDO production a cost-effective approach.

Genome data analysis of *H. hydrogeniformans* revealed that it possesses the genes for PDO production. In general, glycerol to PDO production pathway entails two steps. In the first step, a water molecule is removed from glycerol, forming 3-hydroxypropionaldehyde, catalyzed by the enzyme glycerol dehydratase. In the following step, enzyme 1,3-propanediol dehydrogenase, regenerates NAD^+ needed by cell through the oxidation of $NADH_2$ and produces PDO. Glycerol dehydratase is a vitamin B_{12} dependent enzyme. Moreover, *H. hydrogeniformans* possesses the vitamin B_{12} synthesis pathway. However, earlier experiments performed with *H. hydrogeniformans*, on glycerol fermentation, suggest that the production of PDO almost doubles when its growth medium is supplemented with vitamin B_{12}. Previous work indicated that *H. hydrogeniformans* had a 60.3% conversion of glycerol in our standard growth medium [11].

Therefore, primary objective of this study was to determine the components that could have significant impact on the production of PDO from glycerol by *H. hydrogeniformans*. Furthermore, potential sources of nitrogen and sulfur in the medium were evaluated. On the basis of standard medium composition, a total 24 factors were evaluated for their effect on PDO production. The experimental design for these factors was generated by using a statistical approach, fractional factorial design. This study

suggests that eight factors: glycerol, vitamin B_{12}, pH, ammonium ion, sodium sulfide, cysteine, iron, and cobalt, have significant impact on the production of PDO.

2. MATERIALS AND METHODS

2.1. Growth medium preparation

Cultures of *Halanaerobium hydrogeniformans* were maintained in the laboratory by using a standard medium composition i.e., 70 g sodium chloride (NaCl), 40 g sodium carbonate (Na_2CO_3), 6.3 g potassium phosphate dibasic (K_2HPO_4), 1 g yeast extract, glycerol 5 w/v% std (543 mM), 10 ml basal medium stock solution and 10 ml trace mineral solution in a 1 liter solution. The composition of 1 liter trace mineral solution was: 30 mg magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$), 23.6 mg disodium ethylene diaminetetraacetatedihydrate (EDTA), 10 mg sodium chloride (NaCl), 6.4 mg manganous chloride ($MnCl_2 \cdot 4H_2O$), 1.3 mg zinc chloride ($ZnCl_2$), 1mg ferrous sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$), 1mg calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$), 1mg cobalt chloride hexahydrate ($CoCl_2 \cdot 6H_2O$), 0.3 mg nickel sulfate hexahydrate ($NiSO_4 \cdot 6H_2O$), 0.25 mg sodium molybdate dihydrate ($Na_2MoO_4 \cdot 2H_2O$), 0.25 mg sodium tungstate dihydrate ($Na_2WO_4 \cdot 2H_2O$), 0.1 mg aluminium potassium sulfate hydrate ($AlK(SO_4)_2 \cdot 12H_2O$), 0.1 mg boric acid (H_3BO_3) and 0.07 mg cupric chloride dihydrate ($CuCl_2 \cdot 2H_2O$). One liter of basal medium stock solution contained 50 mg ammonium nitrate (NH_4NO_3), 8.5 mg magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$), 7.5 mg silicon oxide (SiO_2), 4.5 mg manganous sulfate monohydrate ($MnSO_4 \cdot H_2O$), 4.2 mg calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$), 4 mg methylene blue (as an oxygen indicator) and 1.8 mg ferrous sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$). The resulting medium was made anaerobic by boiling and degassing under a stream of N_2. As the temperature of the medium dropped, 0-100 µg vitamin B_{12}, 0.025 g Na_2S (as a reductant), 0.025 g cysteine (as a reductant) per liter were added. The flask containing cooled medium was transferred into an anaerobic chamber under a forming gas (N_2 (95%):H_2 (5%)) atmosphere. In the anaerobic chamber, 50 ml amounts of the medium were dispensed into 160 ml serum bottles, sealed with butyl rubber stoppers held in place with aluminum crimp seals. The medium was sterilized by autoclaving at 121˚C/15 PSI for 20 min. The headspace gas in the serum bottles was

exchanged aseptically with 100% nitrogen. The medium was inoculated with 10% inoculum from previous stock cultures. Cultures were incubated at 30˚C in a shaking incubator.

2.2. Experimental design

On the basis of standard medium composition, a total of 24 (23 + Na:K) factors were considered to evaluate their impact on PDO production. Table 1 lists the experimental variables or factors, each identified by using a capital letter. For screening purposes, two levels of each factor, either high or low, were used to evaluate its significance in PDO production, as shown in the Table 1.

Table 1: Factors for screening experiments.

Factor	**Name**	**Variable**	**Std Media**	**Low Value (-1)**	**High Value (+1)**
A	GLYC	Glycerol	542,947 µM	500,000 µM	2,000,000 µM
B	B12	Vitamin B_{12}	100 µg/L	0 µM, 0.0M	500 µg/L, 0.3689µM
C	pH	pH	10.85	9.0	10.85
D	NH_4	Ammonium ion	624 µM	0 µM	3,000 µM
E	NO_3	Nitrate ion	624 µM	0 µM	3,000 µM
F	SO_4	Sulfate ion	160 µM	160 µM	800 µM
G	PO_4	Phosphate ion	36,169 µM	40,000 µM	200,000 µM
H	NaK	Sodium:potassium ratio	27	12.5	50.0
J	NaS	Sodium sulfide	104 µM	50 µM	200 µM
K	Cys	Cysteine	142 µM	75 µM	300 µM
L	Cl	Chloride	1,198,371 µM	600,000 µM	2,400,000 µM

M	Ca	Calcium	35 µM	0 µM	500 µM
N	Mg	Magnesium	164 µM	0 µM	1000 µM
O	Si	Silicon	125 µM	0 µM	500 µM
P	Mn	Manganese	59 µM	0 µM	500 µM
Q	Zn	Zinc	9.5 µM	0 µM	100 µM
R	Fe	Iron	10.0 µM	0 µM	100 µM
S	Co	Cobalt	4.2 µM	0 µM	100 µM
T	BO_3	Borate	1.6 µM	0 µM	10 µM
U	Ni	Nickel	1.1 µM	0 µM	10 µM
V	MoO_4	Molybdenum	1.0 µM	0 µM	10 µM
W	WO_4	Tungstate	0.8 µM	0 µM	10 µM
X	Cu	Copper	0.4 µM	0 µM	5 µM
Y	Al	Aluminum	0.2 µM	0 µM	2 µM

2.3. Statistical design of experiments

A screening experiment was designed to determine the effect of each factor and all interactions among them by using a fractional factorial design approach. In this design, there are 2^{k-p} experiments where p defines the $1/2^p$ fraction of the total number of experiments. Therefore, for this study, a set of 32 experiments was designed to determine the effect of each factor. This design does not consider the effect of interfactor interactions on the PDO production. For *H. hydrogeniformans,* a significant interaction among the factor is not expected, as the bacteria either requires a factor or not. Therefore, a classical resolution III design, also labeled as 2_{III}^{24-19} design, was used. The interaction among factors was considered negligible.

These 32 experiments were performed in triplicates and were divided into four blocks. Therefore, each block included a total of 24 tests. Each of the 24 factors were taken in two concentrations either high or low level. In the Table 2, the pattern value of - represents the low level of a factor and + represents the high level of a factor for each of the 24 factors.

Table 2. Serum bottle and experimental block set-up with the average amount and standard deviation for three replicates for each of the tests run.

Test	Pattern	Block	Average PDO Yield	Std. Dev.
1,37,71	−−++−−++−−++−−++−−+++−−+	1,5,9	15.36	±0.62
2,40,68	−−−++−++−+−−+−++−+−−−++−	1,5,9	17.64	±0.78
3,34,66	+++−+−−−−−−−+++++++−−−−	1,5,9	19.73	±1.27
4,36,72	+++−−++++++++−−−−−−−−−−	1,5,9	19.90	±1.27
5,33,67	++−−−−−−−+++++++−−−+++++	1,5,9	18.87	±0.65
6,39,65	++−−+++++−−−−−−−−+++++++	1,5,9	20.10	±0.23
7,38,69	−−−+−+−−+−++−+−−+−++−++−	1,5,9	17.80	±0.67
8,35,70	−−++++−−++−−++−−++−−+−−+	1,5,9	20.34	±0.93
9,46,76	+−+−+++−−++−−−−++−−+++−−	2,6,10	17.72	±0.83
10,43,79	−+−+++−+−−+−++−+−−+−+−+−	2,6,10	16.06	±0.97
11,42,75	+−−−+−−++++−−++−−−−+−−++	2,6,10	16.82	±0.17
12,41,80	−+−+−−+−++−+−−+−++−++−+−	2,6,10	22.42	±0.89
13,47,78	+−+−−−−++−−++++−−++−++−−	2,6,10	18.12	±0.65
14,45,73	−++++−+−+−+−+−+−+−+−−+−+	2,6,10	19.64	±0.51
15,44,77	+−−−−++−−−−++−−++++−−−++	2,6,10	17.19	±1.01
16,48,74	−+++−+−+−+−+−+−+−+−+−+−+	2,6,10	20.45	±0.90
17,53,84	+−−+−−−++++−−−−++++−++−−	3,7,11	22.23	±1.37
18,50,85	−+−−−+−+−−+−+−+−++−+−+−+	3,7,11	17.60	±1.23
19,49,82	+−+++−−++−−++−−++−−+−−++	3,7,11	19.30	±0.64
20,56,87	−++−++−+−+−+−−+−+−+−+−+−	3,7,11	18.20	±1.08
21,55,88	+−++−++−−++−−++−−++−−−++	3,7,11	19.75	±0.96
22,52,83	+−−++++−−−−++++−−−−+++−−	3,7,11	15.61	±0.45
23,54,86	−+−−+−+−++−+−+−+−−+−−+−+	3,7,11	17.73	±1.15
24,51,81	−++−−−+−+−+−++−+−+−++−+−	3,7,11	18.16	±1.15
25,62,90	++−++−−−−++++−−−−+++−−−−	4,8,12	21.27	±0.82
26,58,93	−−+−−+−−++−−+−++−−++−++−	4,8,12	15.68	±0.49

27,59,91	++++++++++++++++++++++++	4,8,12	25.24	±1.56
28,60,94	++−+−++++−−−−+++++−−−−−−	4,8,12	20.77	±1.14
29,64,95	−−−−−−++−+−−++−−+−+++−−+	4,8,12	15.84	±0.62
30,57,92	−−−−++−−+−++−−++−+−−+−−+	4,8,12	16.74	±0.45
31,61,96	++++−−−−−−−−−−−−−−−−++++	4,8,12	17.86	±1.03
32,63,89	−−+−+−++−−++−+−−++−−−++−	4,8,12	16.52	±0.38

2.4. Optimization tests procedure

Test solutions were prepared to achieve the concentration of each tested component as prescribed in Table 1. The pH of these solutions was adjusted by using two different base stock solutions. One stock solution had a Na:K ratio of 12.5 and the other had a Na:K ratio of 50 to match the Na:K ratios to be tested. Test medium was made anaerobic by boiling and sparging with argon gas. Anaerobic medium was placed into the anaerobic chamber and 50 ml aliquots of medium were transferred to 160 ml serum bottle. In each serum bottle, cysteine and sodium sulfide solutions were added. Serum bottles containing test medium were then sealed with butyl rubber stoppers held in place with aluminum crimp seals and autoclaved. After cooling, the serum bottles were inoculated with 1 ml of inoculum and placed onto a shaking incubator at 30˚C for three days. After three days, serum bottles were removed and samples were analyzed for PDO production by using HPLC.

For HPLC analysis, samples were filter sterilized using 0.45µM PTFE filters. Filtered samples were injected onto an Aminex HPX-87H, 300x 7.8 mm column (BioRad, Hercules, CA). The column's temperature was maintained at 50˚C. Here, 2.5 mM H_2SO_4 was used as mobile phase and its flow rate was 0.6 ml/min at 2.2 MPa pressure. For the sample analysis, two types of detectors; a UV 231 (at 210 nm) and refractive index monitor were used.

3. RESULTS

The statistical experimental design resulted into 32 different screening tests, run in blocks (Table 2). Each block contained 8 different test conditions. Experiments were run in triplicates. For each screening test, the media were inoculated with *H. hydrogeniformans* and incubated for 3 days. The production of PDO in each culture was determined by HPLC analysis.

3.1. Screening tests and HPLC

The amount of PDO produced during each test is reported in Table 2. Yield was calculated as the moles of PDO produced per the moles of the initial glycerol concentration. Maximum PDO yield was observed when all the factors were added in their high concentration. The lowest yield was observed when low levels of glycerol, vitamin B_{12}, nitrate, sulfate, sodium sulfide, cysteine, magnesium, silicon, iron, cobalt, tungstate, and copper were provided. This indicates that these components are essential during the production of PDO by *H. hydrogeniformans*.

3.2. Effect tests

Based on genome data analysis, it was predicted that ammonium ion, nitrate ion, sulfate ion, Na:K ratio, zinc, iron, nickel, and tungstate might positively impact PDO production. To confirm these predictions, an experimental design was generated. This experimental design was created by using fractional factorial design approach, in which the individual effect of each variable or factor was determined. HPLC data for the PDO yield from the screening tests was fed into JMP Pro 12.1 software for multiple linear regression analysis. This model generates data for the effect test, on the basis of PDO yield obtained. This analysis works on the null hypothesis (P-value>0.05), that each factor has no significance and the alternate hypothesis (P-value<0.05), where each factor is significant. Here, results from the effect tests are presented in the Table 3 that lists all the 24 factors and their respective P-value. The P-value for glycerol, vitamin B_{12}, pH, ammonium ion, sodium sulfide, cysteine, iron, and cobalt was less than 0.05 for each. Therefore, the null hypothesis is rejected for each and the alternate hypothesis is

accepted, indicating that these factors have significant impact on the production of PDO at the 95 % confidence level. In contrast, P-value for nitrate ion, sulfate ion, phosphate ion, Na:K ratio, chloride, calcium, magnesium, silicon, manganese, zinc, borate, nickel, molybdenum, tungstate, copper, and aluminum was more than 0.05. Therefore, the null hypothesis is accepted and these factors did not have a significant impact on the production of PDO at the 95 % confidence level.

Table 3. Multiple linear regression analysis of PDO yield by using JMP Pro 12.1 software for each assay run

Factor	**Number of parameters**	**Degrees of Freedom**	**Sum of Squares**	**F Ratio**	**Prob > F**
GLYC	1	1	55.161176	67.9746	<.0001
B12	1	1	92.022084	113.3979	<.0001
pH	1	1	4.964051	6.1172	0.0164
NH4	1	1	67.318251	82.9556	<.0001
NO3	1	1	0.040426	0.0498	0.8242
SO4	1	1	0.259376	0.3196	0.5741
PO4	1	1	0.587501	0.724	0.3985
NAK	1	1	1.226276	1.5111	0.2241
NAS	1	1	60.055884	74.0063	<.0001
CYS	1	1	51.993984	64.0717	<.0001
CL	1	1	0.681751	0.8401	0.3633
CA	1	1	2.145026	2.6433	0.1096
MG	1	1	1.318359	1.6246	0.2077
SI	1	1	0.100751	0.1242	0.7259
MN	1	1	0.008626	0.0106	0.9183
ZN	1	1	0.106001	0.1306	0.7191
FE	1	1	46.078959	56.7826	<.0001
CO	1	1	86.165651	106.1811	<.0001

BO3	1	1	0.958001	1.1805	0.2819
NI	1	1	0.221376	0.2728	0.6035
MOO4	1	1	0.111384	0.1373	0.7124
WO4	1	1	0.090651	0.1117	0.7395
CU	1	1	0.197109	0.2429	0.624
AL	1	1	0.094376	0.1163	0.7344
Block	11	11	7.465253	0.8363	0.6052
GLYC*pH	1	1	0.469001	0.5779	0.4503
B12*pH	1	1	0.198926	0.2451	0.6225
pH*MOO4	1	1	0.098176	0.121	0.7293
NH4*MOO4	1	1	1.547876	1.9074	0.1727

There is another factor provided in Table 3, the Block factor. As previously mentioned that screening tests were divided into 4 blocks and each block included 8 experiments. These experiments were performed separately. The block factor is used to determine if any difference in results was due to performing these screening test on different days. P-value for block factor is 0.6052 i.e., more than 0.05. Thus, the block factor had no significant impact, indicating consistency in test measurements.

This experimental design was also able to identify the two factor interactions for some of the main factors. This interaction test whether high or low value of a factor has any impact on the other factor. This design works on the assumption that all the two factor interactions are not important, which may or may not be true. Previous studies have shown the significance of factors such as glycerol, vitamin B_{12} and pH on the production of PDO form glycerol by *H. hydrogeniformans*. The interaction between glycerol*pH, vitamin B_{12}*pH, pH*MoO_4 and NH_4*MoO_4 were identified. P-value for the each of these interactions was more than 0.05, indicating that these interactions are not significant.

4. DISCUSSION

Microbial mediated conversion of crude glycerol into PDO is an effective way to utilize waste glycerol. Previous experiments performed in our lab have showed significant production of PDO from glycerol by *H. hydrogeniformans* [11]. The primary objective of this study was to determine the impact of 24 different factors on the production of PDO. These factors were selected on the basis of standard medium components for the growth of *H. hydrogeniformans*. From the genome information of this bacterium, factors such as ammonium ion, nitrate ion, sulfate ion, Na:K ratio, zinc, iron, nickel and tungstate were anticipated to be important for the PDO production. It was anticipated that ammonium ion and nitrate ion are important sources of nitrogen, sulfate ion is the source of sulfur, and Na:K ratio is essential to maintain the ability for this organism to salting-in potassium and maintain its osmotic balance. The trace elements zinc, iron, nickel, and tungstate were thought to be crucial for the enzymatic activity.

Through statistical analysis, 8 of 24 factors were found to significantly enhance PDO production. These factors were glycerol, vitamin B_{12}, pH, ammonium ion, sodium sulfide, cysteine, iron, and cobalt. Glycerol is the initial substrate in this pathway, thereby, higher concentration of glycerol would lead to higher production of end-product PDO. In this pathway, glycerol dehydratase converts glycerol into an unstable intermediate 3-hydroxypropionaldehyde. This intermediate is the precursor for PDO and the reaction is catalyzed by PDO dehydrogenase enzyme. Glycerol dehydratase is a vitamin B_{12} dependent enzyme, indicating that vitamin B_{12} a significant factor here. Previous experiments performed with vitamin B_{12} have shown that *H. hydrogeniformans* can produce PDO even in the absence of B_{12}. It appears that this bacterium possesses vitamin B_{12} synthesis pathway as evidenced in its genome. However, the supplementation of vitamin B_{12} doubles the concentration of PDO produced [11].

In the standard growth medium, ammonium ion and nitrate ion are supplemented as potential nitrogen sources. In order to identify the nitrogen source utilized by bacteria,

ammonium ion and nitrate ion were kept as separate factors. Generally, microorganisms prefer ammonium over nitrate as the nitrogen source [12]. The exact mechanism involved in the effect of ammonium ion on the production of PDO is still unknown. However, the evidence for the impact of ammonia limited media on the glycerol dissimilation to produce PDO has also been provided by Zheng *et al*. [13]. Furthermore, *H. hydrogeniformans* is an anaerobic bacterium and requires a reducing environment. In the standard growth medium of this bacterium, sodium sulfide and cysteine were added as the reducing agents that reduce the oxidation-reduction potential of the media. This makes these components essential for the growth of the bacterium and thereby, the production of PDO. Additionally, *H. hydrogeniformans* grows preferentially at a pH of 11 and produces more PDO under highly alkaline conditions.

Microbial glycerol metabolism under anaerobic conditions occurs mainly via enzyme-mediated oxidative-reductive pathways [14]. In general, the activity of enzymes is attributed to the availability of cofactors or metal ions. Our results revealed the significance of two cofactors, iron and cobalt, in the production of PDO. As mentioned before, glycerol dehydratase is a vitamin B_{12} dependent enzyme and typically cobalt is an integral part of vitamin B_{12}'s structure. Therefore, concentration of cobalt in the medium affects the structure and synthesis of vitamin B_{12}, and subsequently, the production of PDO. Moreover, iron is an essential cofactor for the activity of many alcohol dehydrogenases. One such iron dependent alcohol dehydrogenase has been observed in the *C. acetobutylicum* [15]. In conclusion, selected medium components in higher concentrations significantly enhance the production of PDO by *H. hydrogeniformans*.

5. CONCLUSIONS

By using a fractional factorial design, this study effectively generated information for the medium optimization of PDO production. Such experimental designs can be crucial for the identification of significant factors among a large number of factors. Moreover, this statistical design reduced the number of tests from a large number to a

feasible set of experiments. Furthermore, results obtained from this medium optimization study can be utilized to produce PDO on a bioreactor scale.

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Author Contributions: O. Sitton and M. Mormile conceived and designed the experiments; J. Trager, S. Kalia, and O. Sitton performed the experiments; all authors analyzed the data; O. Sitton and M. Mormile contributed reagents/materials/analysis tools; all authors contributed to the writing of the paper.

Conflicts of Interest: The authors declare no conflict of interest.

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II. 1,3-PROPANEDIOL PRODUCTION FROM GLYCEROL BY *HALANAEROBIUM HYDROGENIFORMANS* IN SCALE UP REACTOR AND REPEATED FED BATCH OPERATION

To be submitted to Journal of Biotechnology.

ABSTRACT

The development of renewable energy sources, such as biofuels, is a crucial issue in today's world. For instance, the production and disposal of crude glycerol, biodiesel by-product, has become economically infeasible. Moreover, the high pH and salt concentration of crude glycerol makes it essential to purify and neutralize before utilization. However, the use of a haloalkaliphile microorganism could eliminate the need for biodiesel waste neutralization and make glycerol product use cost-effective. A haloalkaliphilic bacterium, *Halanaerobium hydrogeniformans*, capable of growing at a broad pH range (7.5-12) and salt concentration (2.5-15%) can possibly serve this purpose. *H. hydrogeniformans* can convert glycerol into 1,3-propanediol (PDO), a known chemical commodity. The impact of scale up in the reactor volume on the PDO production was investigated. An increase in reactor volume by 10X decreased the PDO productivity by 10% from 1.67 to 1.5 mmol/L-hr and PDO concentration by 10 percent from 327.8 to 300.75 mM. In order to enhance the PDO production and reactor productivity, repeated fed batch (RFB) operations were performed with different retained volume fraction and glycerol concentrations. In the RFB reactor, the optimal productivity of 2.7 mmol/L-hr and maximum PDO concentration of 456 mM was observed. These results were obtained at a retained volume fraction of 0.8 and glycerol feed concentration of 2000 mM. Overall, in comparison to the batch fermentation, RFB operation resulted in a 61.7 percent increase in productivity and a 39.2 increase in PDO concentration.

1. INTRODUCTION

A limited supply of primary energy resources has resulted in the development of renewable energy sources such as biofuels. Biodiesel is the most common biofuel and is made from animal fats or vegetable oils. In United States, biodiesel production increased

from 343 million of gallons in 2010 to 1359 millions of gallons in 2013 [1]. However, this increase in biodiesel production has led to the generation of large amount of crude glycerol as a waste product, almost 10% (w/w) [2]. Glycerol in its pure form is used in the food, pharmaceutical, and cosmetics industries. Glycerol can also be chemically or microbiologically converted to metabolites such as poly-hydroxyalkanoates, acrylic acid, polyhydroxybutyrate, 1,3-propanediol, fumaric acid, arabitol and dihydroxyacetone [3, 4, 5, 6]. Crude glycerol obtained from biodiesel plants has various impurities. A study performed on the composition of waste from biodiesel plants showed that glycerol concentration can range from 38% to 96%, methanol 14% and ash 29% [7]. Presence of methanol and ash in crude glycerol makes it unsuitable for utilization in chemical and pharmaceutical industries. Along with these impurities, biodiesel waste has high pH and salt concentration, up to 10 and 3%. Therefore, pre-treatment of waste glycerol is required prior to its utilization, resulting in an increase of the cost of biodiesel production and its use economically unfeasible [8]. Thus, crude glycerol is considered as a waste stream that is to be disposed [9].

Conversion of crude glycerol to a higher value chemical commodity can be an ecological and economical benefit to the biodiesel industry. One such product is 1,3-propanediol (PDO). 1.3-propanediol is a key monomer for the manufacture of polytrimethylene terephthalate (PTT), a polymer used in the textiles and carpet industry [10]. 1,3-propanediol is also used in the synthesis of cosmetics, perfumes, antifreezes, UV-cured coatings, and laminates [11]. Previous studies have assessed PDO production by bacteria such as *Clostridium butyricum*, *Citrobacter freundii* and *Klebsiella pneumoniae* [11, 12, 13]. However, these studies were performed with pure glycerol at neutral pH values. Haloalkaliphilic microorganisms, due to their ability to grow at high salt and pH level, could remove the need for glycerol pretreatment steps. Moreover, hypersaline medium limits the growth of most organisms, therefore, sterilization cost of biotechnological processes can be reduced [14].

An anaerobic haloalkaliphilic bacterium, *Halanaerobium hydrogeniformans*, isolated from Soap Lake in Washington State, was assessed for its ability to metabolize

glycerol to PDO [15]. Optimum growth conditions for *H. hydrogeniformans* are 30˚C, pH of 11 and a NaCl concentration of 7.5% [16]. The genome data analysis of this bacterium revealed that it has the ability to convert glycerol into PDO [15]. Another interesting feature is that this bacterium can grow at broad salt (2.5-15%) and pH (7.5-12) ranges [16]. 1,3-propanediol production from crude glycerol using this bacterium could remove the need for neutralization steps and salt dilution.

Under anaerobic conditions, glycerol assimilation occurs via two metabolic pathways, reductive and oxidative [10]. Through the oxidative pathway, glycerol is dehydrogenated by glycerol dehydrogenase to dihydroxyacetone and further converted to various end-products such as acetate, ethanol, and lactic acid. Through the reductive pathway, glycerol is converted into PDO in two steps. Initially, glycerol is dehydrated to 3-hydroxypropionaldehyde (3-HPA) by glycerol dehydratase. In the consecutive step, 3-HPA is reduced to PDO by 1,3-propanediol dehydrogenase, oxidizing $NADH_2$ and regenerating NAD^+. Previous work with *H. hydrogeniformans* had shown that it can convert 60.3% of glycerol in our standard growth medium [17]. However, a medium composition was also designed that contains the components that have significant impact on the PDO production [18]. The objectives of the current study is to measure the yield, titer, and production of PDO as a function of scale up in the reactor performance and to determine the optimal fed-batch operating parameters in order to maximize the yield, titer, and productivity of the PDO production.

2. MATERIALS AND METHODS

2.1. Microorganism used for study

A haloalkaliphilic bacterium strain, *Halanaerobium hydrogeniformans* was used to convert crude glycerol into 1,3-propanediol. The bacterium was isolated from Soap Lake, Washington State [17]. The complete genome of the bacterium has been deposited in NCBI with accession number NC_014654 [19].

2.2. Growth Medium

H. hydrogeniformans was grown under anaerobic conditions by using a medium composed of 70 g NaCl, 40 g Na_2CO_3, 6.3 g K_2HPO_4, 1 g yeast extract, glycerol 5 w/v% std (543 mM), 10 ml basal medium stock solution and 10 ml trace mineral solution in a 1 liter solution. The composition of the trace mineral solution per 1 liter water was: 30 mg $MgSO_4 \cdot 7H_2O$, 23.6 mg EDTA, 10 mg NaCl, 6.4 mg $MnCl_2 \cdot 4H_2O$, 1.3 mg $ZnCl_2$, 1 mg $FeSO_4 \cdot 7H_2O$, 1 mg $CaCl_2 \cdot 2H_2O$, 1 mg $CoCl_2 \cdot 6H_2O$, 0.3 mg $NiSO_4 \cdot 6H_2O$, 0.25 mg $Na_2MoO_4 \cdot 2H_2O$, 0.25 mg $Na_2WO_4 \cdot 2H_2O$, 0.1 mg $AlK(SO_4)_2 \cdot 12H_2O$, 0.1 mg H_3BO_3 and 0.07 mg $CuCl_2 \cdot 2H_2O$. One liter of basal medium stock solution contained 50 mg NH_4NO_3, 8.5 mg $MgCl_2 \cdot 6H_2O$, 7.5 mg SiO_2, 4.5 mg $MnSO_4 \cdot H_2O$, 4.2 mg $CaCl_2 \cdot 2H_2O$, 4 mg methylene blue (as an oxygen indicator) and 1.8 mg $FeSO_4 \cdot 7H_2O$.

The medium was made anaerobic by boiling and degassing with nitrogen gas. Once the medium cooled, 100 µg Vitamin B_{12} and reducing agents 0.025 g sodium sulfide and 0.025 g cysteine per liter were added. The resulting anaerobic medium was transferred to an anaerobic chamber under a forming gas (90% N_2 : 10% H_2) atmosphere. Aliquots of medium, 50 ml, was transferred into 160 ml serum bottles. These bottles were sealed with butyl rubber stoppers crimped into place with aluminum crimp seals. The medium was autoclaved at 121ºC and 15 PSI for 20 min. The medium was inoculated after exchanging headspace gas with 100% nitrogen. Cultures were incubated in shaking incubator at 30ºC.

2.3. Fermentation Medium

The fermentation medium composition was a modification of the medium above based upon the media screening tests for PDO production [18]. Media screening tests showed that out of all the standard medium components; glycerol, vitamin B_{12}, pH, ammonium ion, sodium sulfide, cysteine, iron and cobalt have significant impact on the PDO production. The resulting medium consisted of 1000 µM glycerol, 3000 µM ammonium ion, 200 µM sodium sulfide, 300 µM cysteine hydrochloride, 500 µg/L

vitamin B_{12}, 50 μM iron and 100 μM cobalt. The remaining components consisted of 160 μM sulfate, 40,000 μM phosphate and 600,000 μM chloride ion resulting in a Na:K ratio of 12.5. All other salts were kept at zero concentration.

2.4. Fermentation Experiments

Two bioreactors manufactured by Sartorius Stedim were used, two-liter (Biostat BPlus) and a twenty-liter working volume (Biostat C). Variables such as temperature, agitator speed, optical density, pH, liquid flow, liquid level, and gas level were controlled by a digital control unit. Before each run, the bioreactor was autoclaved at 121ºC for 45 minutes and sparged with argon gas for 30 minutes to maintain anaerobic conditions. All liquids entered the reactor through a sterilizing filter (0.2 μm) attached to the liquid inlet port and all gases passed through a sterilizing filter attached to the gas inlet port.

Fermentation medium was introduced into a feed vessel through a peristalic pump attached to one of the inlet port. Oxygen was removed from the medium by sparging with argon gas. The reducing agents, sodium sulfide and cysteine, were added to the feed vessel via injection to maintain a reducing environment.

For batch cycle runs, anaerobic and filter-sterilized medium from the feed vessel was fed into the reactor. The operating conditions were maintained at 30ºC, pH 11 and an agitator speed of 250 RPM. The flow of the argon for the head space gas exchange was set at 30 ml/min. As conditions stabilized, fresh inoculum was anaerobically added through an injection port in the reactor head. Liquid culture samples were collected every two hours using a ceramic filter (Flownamics, Madison, WI).

For fed-batch cycle runs, fresh medium was fed when liquid volume changed from maximum reactor volume, V_{max} (2 liters) to a specified initial volume (V_0). This reduction in liquid volume was measured with a low-level electrode and achieved by pumping liquid from the liquid sampling port. As liquid volume reached V_0, the peristalic pump on the feed line transferred fresh, sterile medium of a specified glycerol

concentration into the reactor. Medium addition was stopped as soon as the liquid volume reached apparent V_{max}. Cell concentration in the reactor was determined by monitoring the optical density of the culture fluid by using a Fundulus II (Sartorius Stedim) probe.

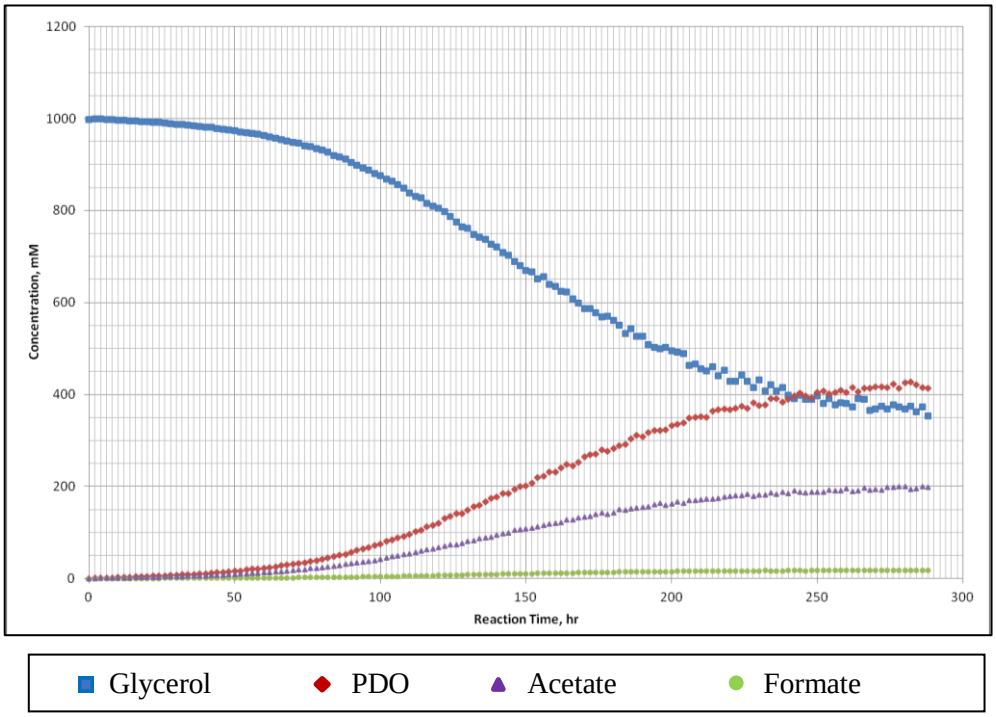
2.5. Component Analysis

Glycerol, PDO, and organic acids were analyzed by using HPLC. The analytical column was a Rezex-RHM ion exchange column (Phenomenex, Torrance, CA) placed on a HP1090 system running Chemstation A.03 software (Agilent, Santa Clara, Ca). Milli-Q water containing 0.005 N sulfuric acid was used as the mobile phase at a flow rate of 0.6 ml/min. The column temperature was controlled at 85ºC. UV-VIS diode array detector and a HP 1037 refractive index (RI) detector (Agilent, Santa Clara, Ca) were used. The UV-VIS detector detected organic acids by quantitating the UV cut-off absorbance at 210 nm. The RI detector was used to measure glycerol and PDO concentration. For all samples, the injection size was 10 µl. Samples were first filtered by using a membrane filter and then analyzed on the HPLC system.

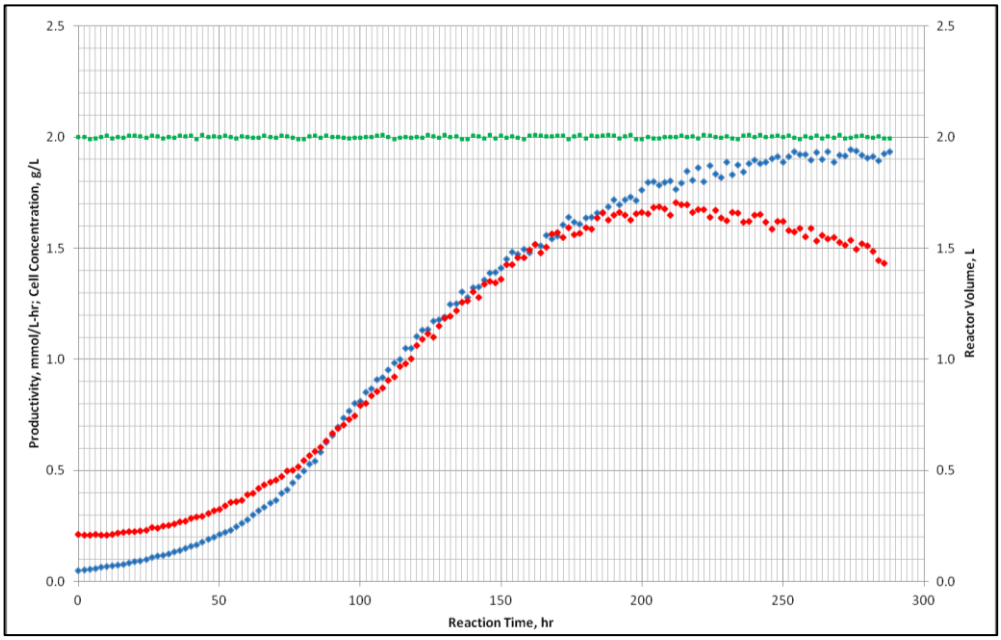
3. RESULTS

3.1. Batch fermentation

The impact of bioreactor scale-up on the production of PDO from glycerol by *H. hydrogeniformans* was investigated. The process was analyzed in two reactor volumes, 2 liter and 20 liter. In order to establish reproducibility, the reactor tests were performed in two replicates. Moreover, the 2-liter reactor was run for an extended time in order to find the optimal reaction times. Data obtained from each run were used to calculate the kinetic parameter, as shown in Table 1.



(a)

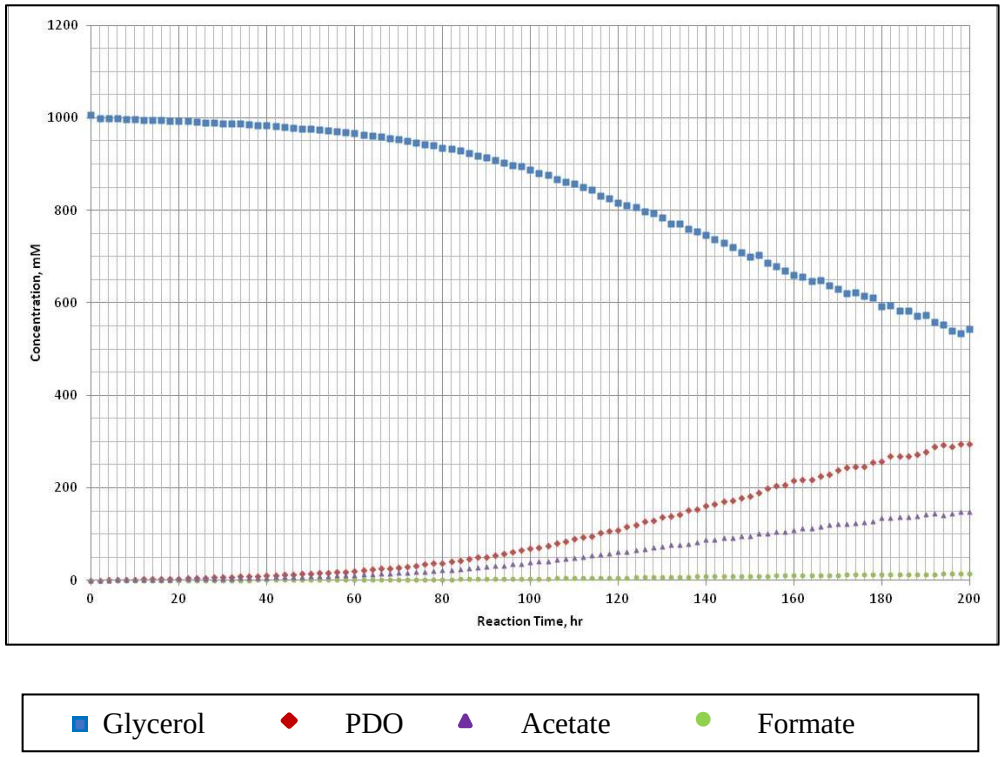


(b)

Figure 1. 2-liter batch reactor over extended time; (a) concentration profiles of metabolite (PDO, acetate and formate) production with glycerol as a carbon source; (b) reactor performance.

Figure 1a shows the concentration profiles generated in 2-liter batch reactor. The initial glycerol concentration in each batch reactor run was 1000 mM. After 200 hours, 323.1 mM of PDO was measured and its yield was 0.65 mol/mol. The productivity of PDO after 200 hours was 1.65 mmol PDO/L-hr. However, it was found to decrease with further reaction time. The concentration of acetate was 200 mM, approximately one-half the concentration of PDO. A small amount of formate was also observed.

These results were consistent with a second 2-liter reaction test performed under identical conditions. Results of this replicate are provided in Figure 1s in the supplementary material. Reaction conditions were run in the 20 L bioreactor as in the two-liter reactor test. Figure 2 shows the concentration profile and the performance of one 20 L batch reactor run. The concentration of PDO at 200 hours was 303 mM and its yield was 0.64 mol/mol. Though the PDO yield was similar under both reactor volumes, the productivity was 1.51 mmol/L-hr, which is 10 percent lower than 2-liter run. The concentration of acetate was observed 150 mM approx. one-half the concentration of PDO and 20 mM of formate was formed.



(a)

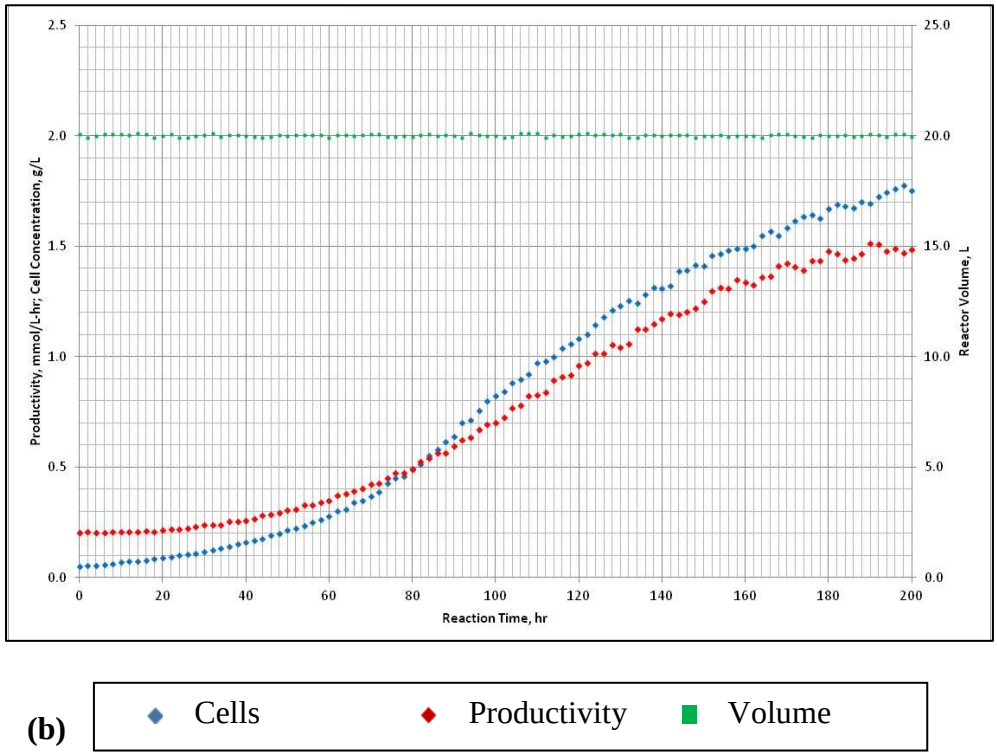


Figure 2. 20-liter batch reactor; (a) concentration profiles of metabolite (PDO, acetate and formate) production with glycerol as a carbon source; (b) reactor performance.

A second test for 20-liter reactor was performed under identical conditions. Figure 2s, supplementary material, provides the concentration profile and performance of the 20-liter replicate run. The results were consistent with the previous 20-liter run. The PDO productivity was 1.49 mmol/L-hr, approximately 10 percent lower from 2-liter reactor run.

Table 1 summarizes the results from the 2-liter and 20-liter reactor tests along with their replicates under batch conditions. At 200 hours, the PDO concentration was higher in the 2 L reactor runs than the 20 L reactor runs. A similar trend was seen with PDO productivity. Günzel et al. [20] performed the first scale-up experiments on PDO synthesis by *C. butyricum* DSM 5431. The fermentation process was assessed in reactor volumes ranging from 1.4 L to 2000 L. In air-lift bioreactors, the highest PDO concentration was observed in 30 L and 1200 L bioreactors. However, in stirred-tank bioreactors, the highest PDO concentration of 56 g/L was seen in the 30 L reactor.

Another study performed on scale up of PDO production by *Clostridium butyricum* DSP1 from crude glycerol showed almost similar concentration of PDO in 6.6 L, 42 L and 150 L, averaged 37 g/L [21].

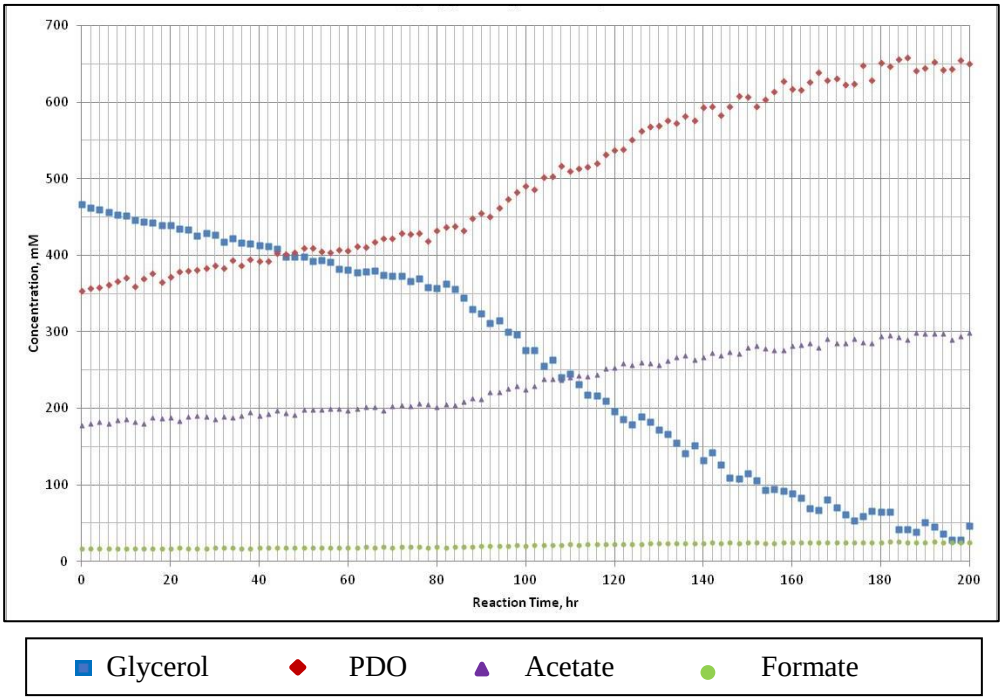
Table 1. Kinetic parameter values for PDO production from glycerol at ~200 hr in 2 liter and 20 liter bioreactors.

Bioreactor Volume	**2 L**		**20 L**	
Run #	**1**	**2**	**1**	**2**
PDO Concentration (mM)	323.1	332.5	303	298.5
Yield (mol PDO/mol Glycerol)	0.65	0.64	0.64	0.63
Productivity (mmol PDO/L-hr)	1.65	1.69	1.51	1.49

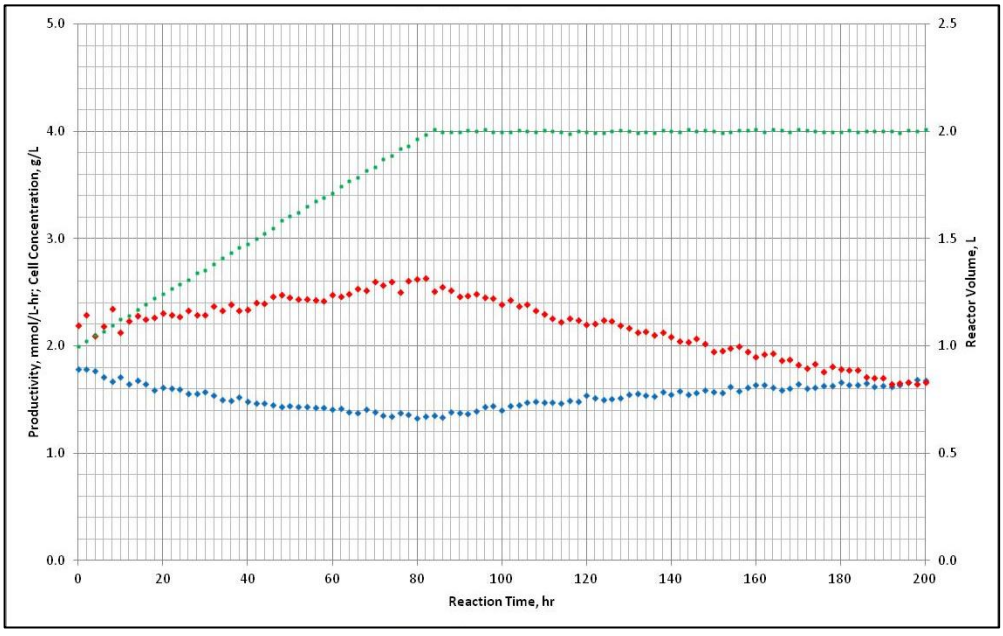
3.2. Fed-batch fermentation

Repeated fed-batch fermentation operations were run to identify the effect of high glycerol concentration as well as a range of retained volume fraction on the production of PDO. A 2-liter reactor volume was used as the maximum velocity (V_{max}). At the end of 2-liter repeated batch run, the reactor volume was drained to 1 liter, resulting in a 0.5 retained volume fraction (V_0/V_{max}). Fresh, sterile and anaerobic medium containing a glycerol concentration of 1000 mM was used as a feed. The feed flow was maintained at 0.2 ml/min until the reactor volume reached 2 liters. The feed flow was stopped for the rest of the test. The concentration profile and performance of one cycle of fed batch reaction at a retained volume fraction of 0.5 and 1000 mM glycerol feed concentration is shown in Figure 3. The reaction ran for 200 hours. However, the productivity was maximum around 75 hours near the time when the reactor volume was maximum. After 200 hours, the PDO concentration was 650 mM, acetate concentration was 300 mM and 25 mM of formate were formed. The reaction continued in batch mode and the productivity decreased gradually. For the remaining tests, the cycle time was set as the

reactor volume reaches apparent V_{max} and then the reactor contents were drained to V_0. Once this point was reached, feed flow started again to begin the next cycle.



(a) Glycerol PDO Acetate Formate

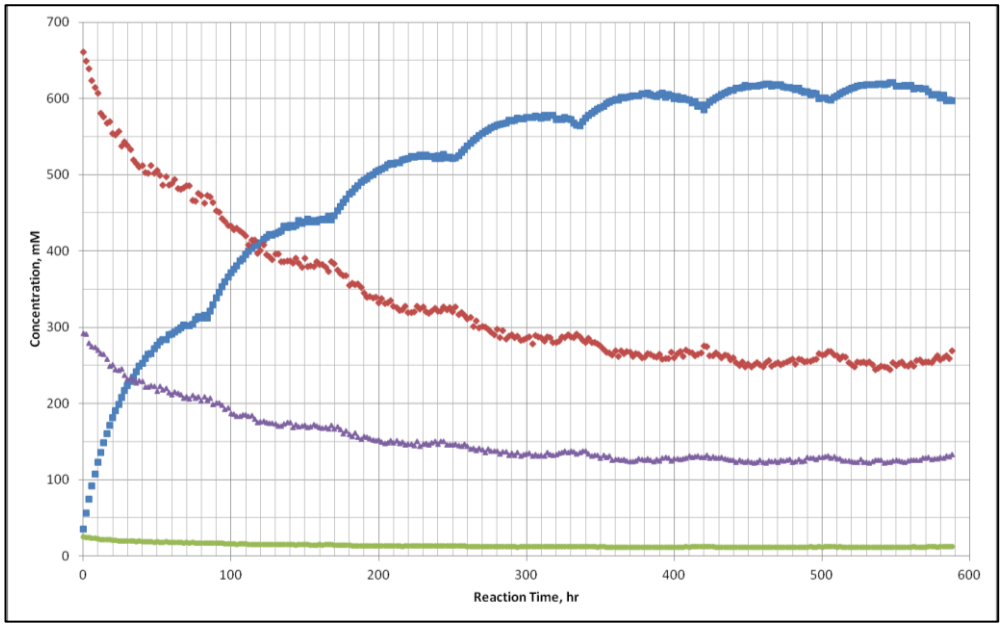


(b) Cells Productivity Reactor volume

Figure 3. 2-liter fed-batch reaction at 0.5 retained volume fraction (V_0/V_{max}) and 1000 mM Glycerol (S_{in}) in feed; (a) concentration profiles of metabolite (PDO,

acetate and formate) production with glycerol as a carbon source; (b) reactor performance.

Further tests were performed under repeated fed batch (RFB) conditions ranging from 0.5 to 0.9 retained volume fractions. The concentration profile and the performance for a RFB at a retained volume fraction of 0.5 and a 1000 mM of feed glycerol concentration is presented in Figure 4. The reaction ran for 600 hours for a total of seven cycles. Figure 4a shows decrease in PDO concentration initially, due to the dilution of reactor content as fresh medium is fed into the bioreactor. However, around 6th cycle, rate of dilution was balanced by rate of PDO production and the system achieved apparent steady state conditions where the productivity stabilized at 1.55 mmol/L-hr, as shown in Figure 4b. At this point, the PDO concentration was 265.2 mM and the yield was 0.64 mol/mol.



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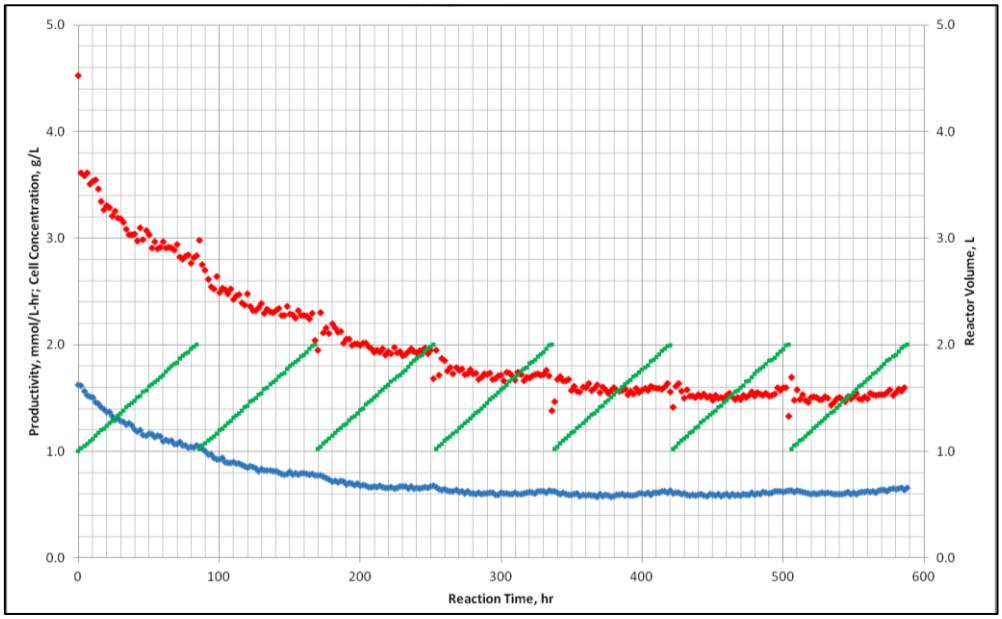

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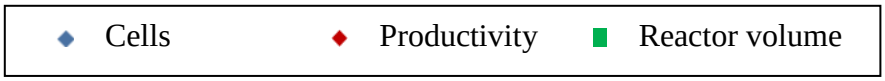


Figure 4. 2-liter RFB reaction at 0.5 retained volume fraction (V_0/V_{max}) and 1000 mM Glycerol (S_{in}) in feed; (a) concentration profiles of metabolite (PDO, acetate and formate) production with glycerol as a carbon source; (b) reactor performance.

The concentration profile and performance for a repeated fed-batch reaction at 0.6 retained volume fraction and a feed glycerol concentration of 1000 mM is given in Figure 5. The test ran for 200 hours and included three cycles. The PDO concentration after 200 hours was 294.1 mM, the yield was 0.67 mol/mol, and the productivity was 1.77 mmol PDO/L-hr.

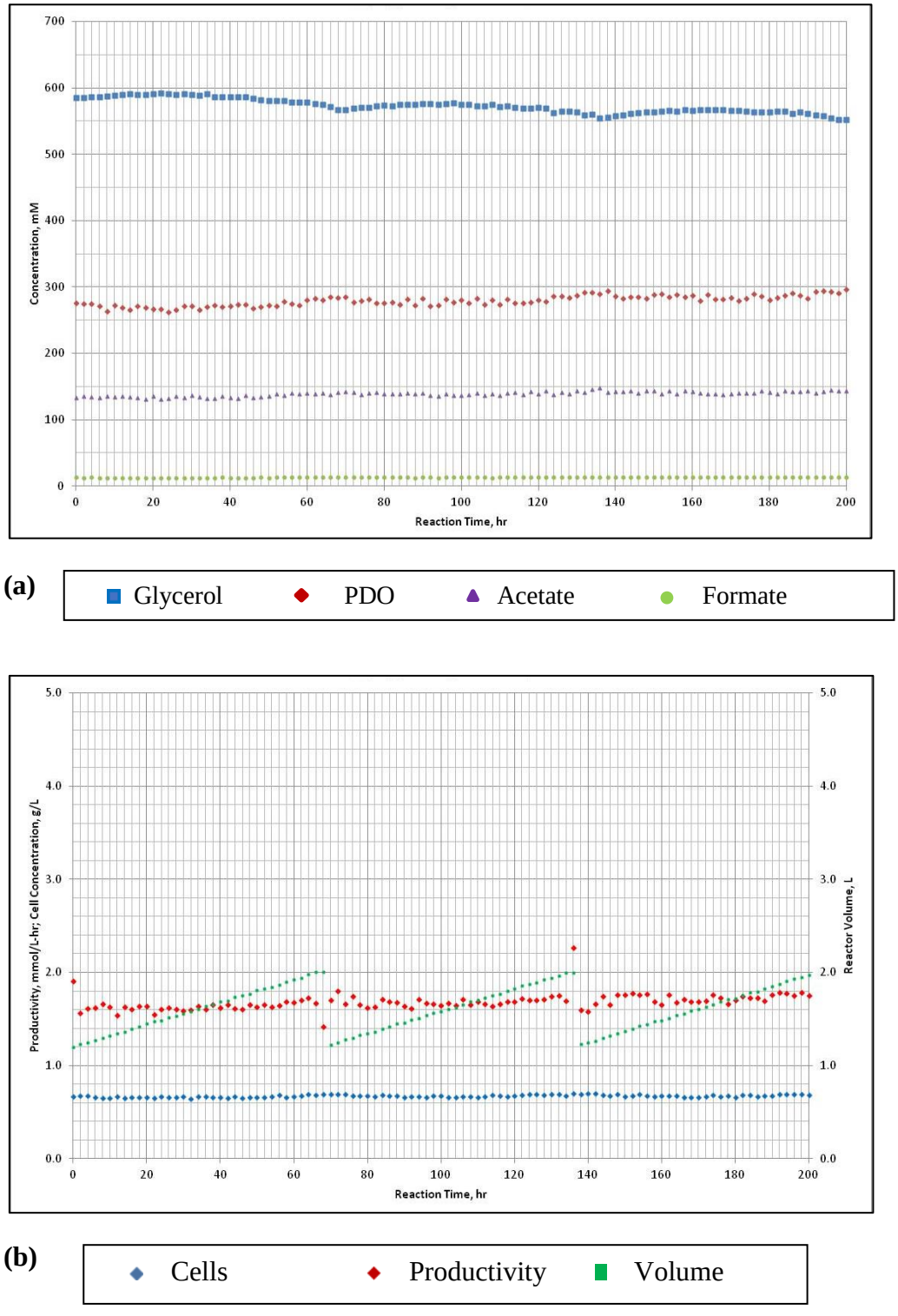


Figure 5. 2-liter RFB reaction at 0.6 retained volume fraction (V_0/V_{max}) and 1000 mM Glycerol (S_{in}) in feed; (a) concentration profiles of metabolite (PDO, acetate and formate) production with glycerol as a carbon source; (b) reactor performance.

Three more tests were performed with different retained volume fractions of 0.7, 0.8, 0.9. Figure 3s, 4s and 5s, supplementary material, provides the concentration profile and the performance of 0.7, 0.8 and 0.9 retained volume reactions. For each of these tests, the feed glycerol concentration was 1000 mM. For the 0.7 retained volume fraction, the reactor achieved apparent steady state conditions after 4 cycles, however, for 0.8 and 0.9 retained volume, apparent steady state conditions were achieved after 3 cycles. Table 2 lists the kinetic parameters obtained under different retained volume fractions. By increasing the retained volume fraction from 0.5 to 0.8, the RFB productivity increased by 26.5 percent and the PDO concentration increased by 22.2 percent. The yield remained nearly constant for all retained volume fractions, varying between 0.63-0.64 mol PDO/mol glycerol.

Table 2. Effect of retained volume fraction on a RFB reactor performance at a glycerol feed concentration of 1000 mM.

Retained Volume Fraction	**0.5**	**0.6**	**0.7**	**0.8**	**0.9**
PDO Concentration (mM)	265.2	294.1	304	324	339
Yield (mol PDO/mol Glycerol)	0.64	0.67	0.62	0.63	0.64
Productivity (mmol PDO/L-hr)	1.55	1.77	1.82	1.96	2.05

An increase in retained volume fraction on the reactor performance can be clearly observed in Figure 6. The productivity was maximum at a retained volume fraction 0.9. However, the excessive cycling at this fraction is not feasible. Therefore, for the remaining tests, 0.8 was used as the retained volume fraction.

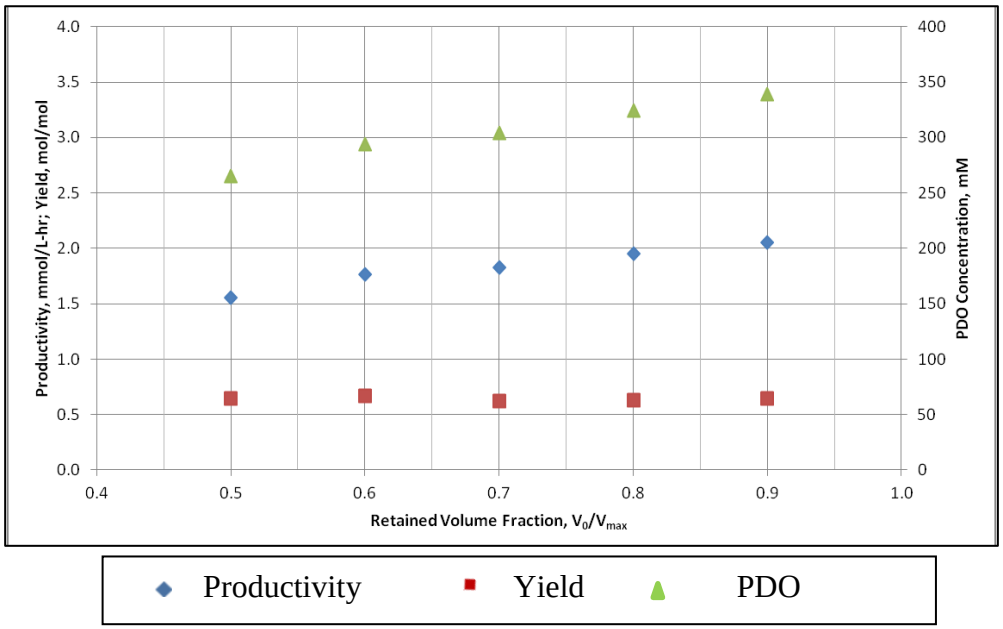


Figure 6. Effect of retained volume fraction on reactor performance.

In the present study, four different reactor tests were performed with four different glycerol feed concentrations of 1500, 2000, 2500, and 3000 mM. Due to the increased viscosity of the feed media, 3000 mM was used as the maximum glycerol concentration. The retained volume fraction in each test was kept same as 0.8. Figure 7 provides the concentration profile and reactor performance of the RFB reactor when glycerol feed concentration was 1500 mM. The reaction ran for 340 hours that included total 10 cycles. The system took approximately 10 cycles to attain apparent steady state conditions. The productivity was also found to be increased by 25.5 percent i.e., from 1.96 to 2.46 mmol/L-hr, with an increase in glycerol feed concentration from 1000 to 1500 mM. The PDO yield remained same while PDO concentration increased by 27%.

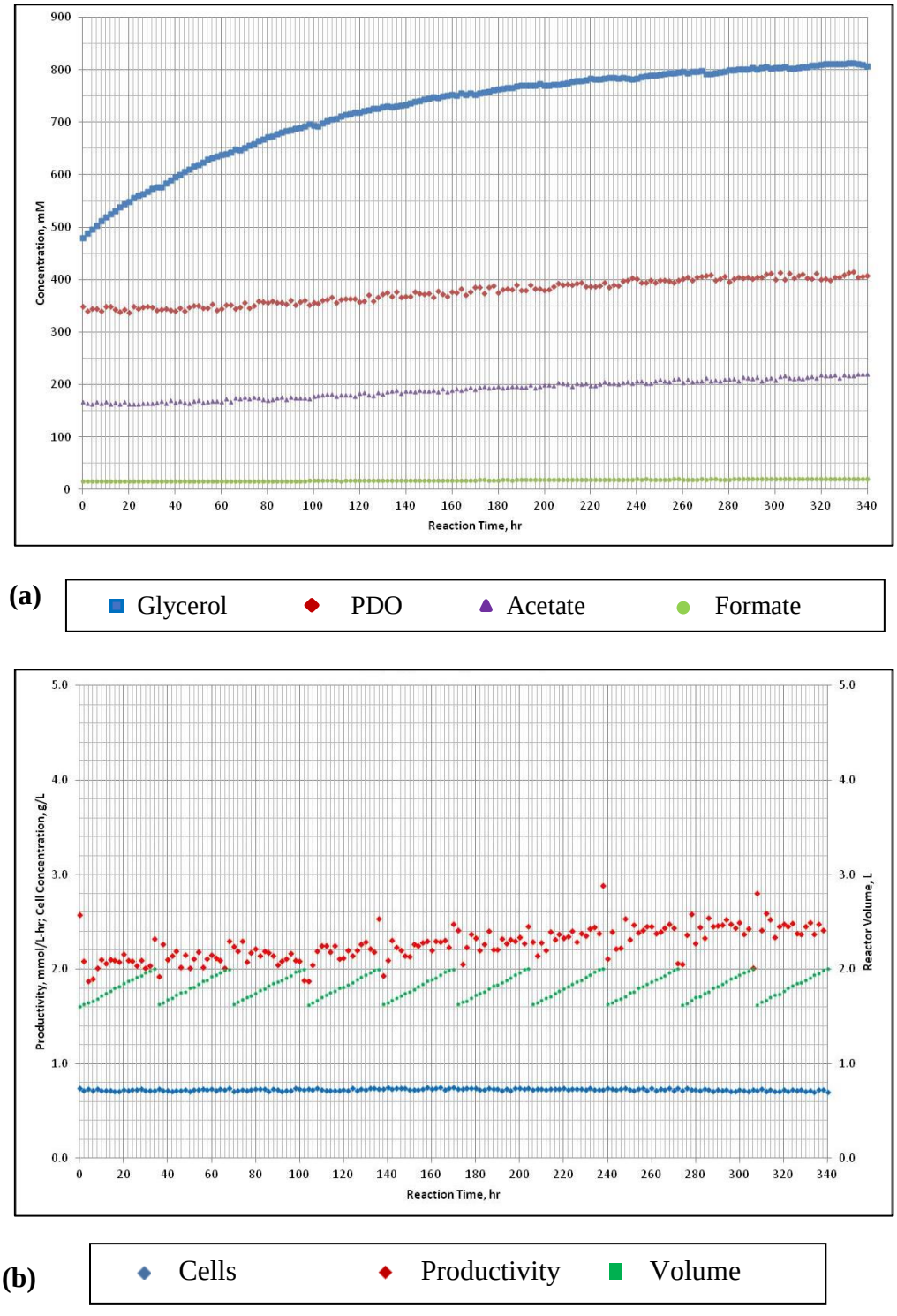
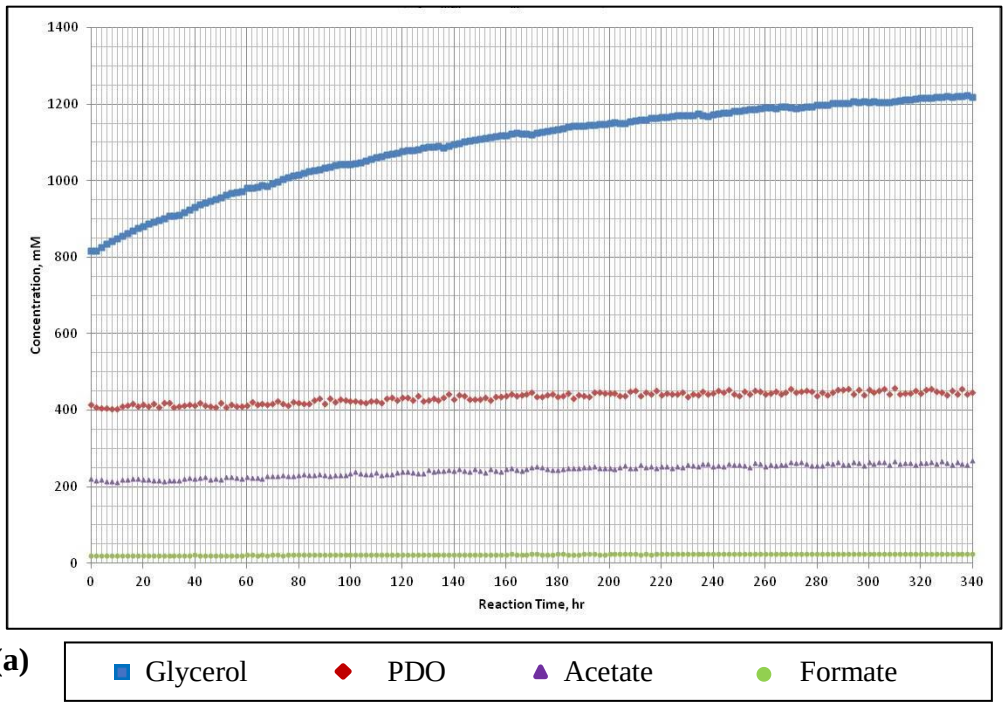


Figure 7. 2-liter RFB reaction at 0.8 retained volume fraction (V_0/V_{max}) and 1500 mM Glycerol (S_{in}) in feed; (a) concentration profiles of metabolite (PDO, acetate and formate) production with glycerol as a carbon source; (b) reactor performance.

Figure 8 shows the concentration profile and the performance of a RFB reactor with a glycerol feed concentration of 2000 mM and retained volume fraction of 0.8. The reaction ran for 340 hours that included 10 cycles. As the feed glycerol concentration increased from 1000 mM to 2000 mM, the PDO concentration increased by 40.8% and productivity increased by 36.7%. Figure 6s and 7s, supplementary materials, provides the concentration profile and the performance of RFB reactor at the glycerol feed concentration of 2500 and 3000 mM. The reaction achieved apparent steady state conditions in 10 cycles. Figure 9 compares the effect of different feed glycerol concentration on the reactor performance. It was observed that the increase in glycerol feed concentration above 2000 mM had a small effect on the RFB reactor performance (Table 3).



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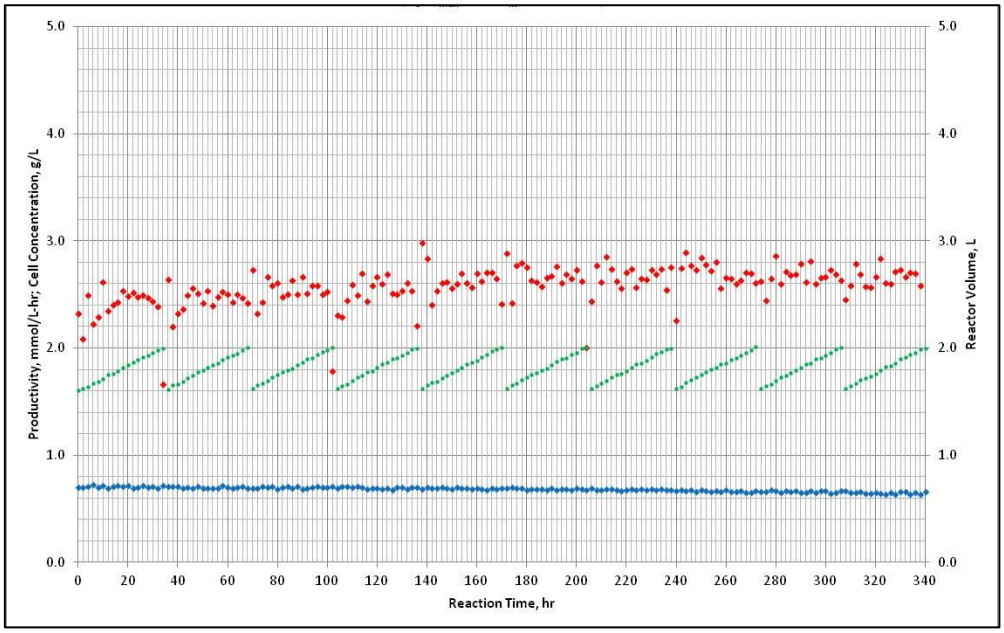

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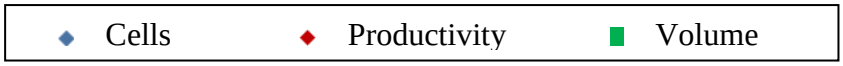
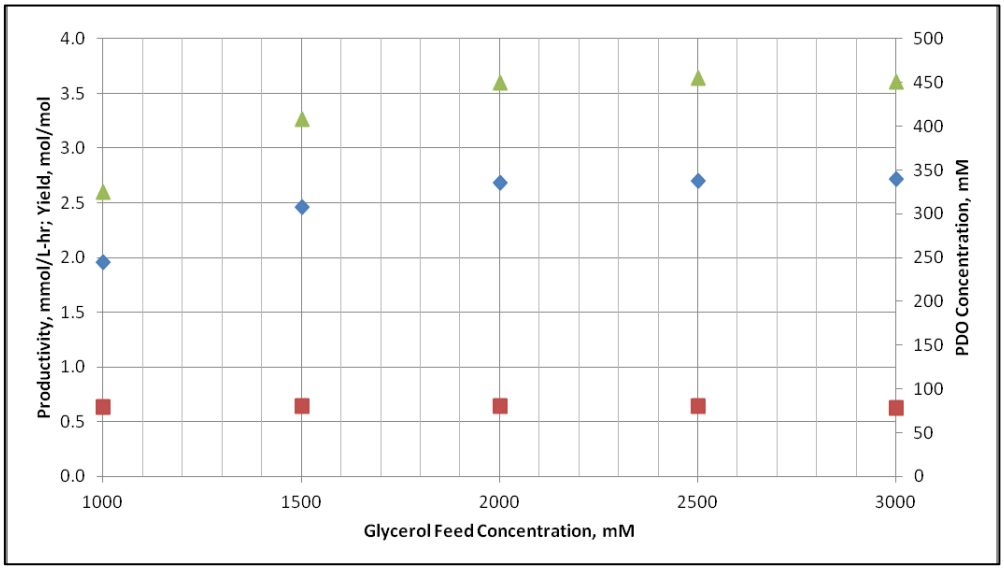


Figure 8. 2-liter RFB reaction at 0.8 retained volume fraction (V_0/V_{max}) and 2000 mM Glycerol (S_{in}) in feed; (a) concentration profiles of metabolite (PDO, acetate and formate) production with glycerol as a carbon source; (b) reactor performance.



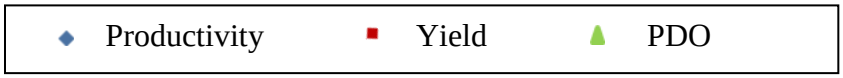


Figure 9. Effect of glycerol feed concentration on the RFB reactor performance.

Table 3. Effect of glycerol feed concentration on a RFB reactor performance at a retained volume fraction of 0.8.

Glycerol Feed Concentration (mM)	1000	1500	2000	2500	3000
PDO Concentration (mM)	324	411.5	456.2	461.8	457.6
Yield (mol PDO/mol Glycerol)	0.63	0.64	0.64	0.64	0.62
Productivity (mmol PDO/L-hr)	1.96	2.46	2.68	2.70	2.71

The maximum PDO concentration of 456.2 and productivity of 2.7 was observed, in the RFB conditions with a retained volume fraction of 0.8 and glycerol feed concentration of 2000 mM,. In all of the above tests, the yield remained constant around 0.64 mol/mol. Moreover, under RFB conditions, a 39.2 percent increase in PDO concentration and 61.7 percent increase in productivity was observed as compared to the simple batch operation.

4. DISCUSSION

1,3-propanediol production has been primarily studied with the bacteria belonging to the species *Clostridium butyricum* and *Klebsiella pneumoniae* [22-25]. There are only a limited number of studies that deal with the haloalkaliphilic bacteria involved in the production of PDO. A similar study by Kivisto et al. showed the production of 1,3-propanediol from glycerol by *Halanaerobium saccharolyticum* [26]. Our prior work performed with *H. hydrogeniformans* revealed its ability to ferment glycerol into PDO. A medium was designed by using statistical approaches in order to identify the factors that have significant impact on this process [18]. The present study was performed by using the optimized medium to analyze the impact of scale up in the reactor volume, retained volume fraction and elevated glycerol concentration on the PDO productivity.

In the current work on *H. hydrogeniformans*, the 2-liter bioreactor resulted in a PDO concentration of 327.8 mM and a productivity of 1.67 mmol/L-hr, under batch fermentation mode. However, there were some differences in the productivity as the reactor volume increased from 2 L to 20 L. Increasing the reactor capacity to 20 L decreased the productivity by nearly 10% to 1.50 mmol/L-hr and PDO concentration by nearly 10% to 300.75 mM. Previous studies have demonstrated that environmental stresses limit the performance of biological processes occurring on large scale [27]. Simple batch operation rarely provides maximum productivity, as the chemical environment changes continuously inside the rector. Therefore, reaction conditions are not optimal for most of the reaction time. Moreover, continuous reactor operation provides better productivity in comparison to the batch operation [28]. In batch reactor, overall productivity is reduced by downtime caused by emptying, cleaning and filling the reactor as compared to the continuous process. Though there are advantages associated with continuous processes, it is seldom used in industries due to the possibility of contamination. However, advantages of a continuous process can be achieved by operating a reactor under repeated fed-batch operation.

Repeated fed-batch (RFB) operation resulted in improved productivity and the PDO concentration as compared to the simple batch operations during our studies. Maximum productivity was found to occur where the reactor is filled but decreases gradually as the reaction enters batch mode. In RFB operations, the system achieved apparent steady state conditions in less than 10 cycles. Repeated fed batch operation with different retained volume fraction were also performed. Retained volume fraction of 0.9 demonstrated the maximum productivity of 2.05 mmol/L-hr. However, due to the infeasibility of excessive cycles at 0.9, retained volume fraction of 0.8 was assessed. As the retained volume fraction increased from 0.5 to 0.8, productivity increased by 26.5% from 1.55 to 1.96 mmol/L-hr. Similarly, the PDO concentration also increased by 22.2% from 265.2 to 324 mM.

It was demonstrated that an increase in glycerol feed concentration resulted in improved reactor performance. However, above 2000 mM glycerol concentration, there

was only a minor effect on the reactor performance. Maximum PDO concentration and productivity were achieved at glycerol feed concentration of 2500 mM. While operated in RFB mode, optimal productivity observed was 2.7 mmol/L-hr with PDO concentration measured at 456 mM when operated at 2000 mM of glycerol feed concentration and 0.8 retained volume fraction. Yield remained near constant at 0.64 mol/mol for all tests. Some studies have shown the negative effect of increased substrate concentration on the biomass yield and PDO formation [29]. This effect has been suggested due to the substrate toxicity on the microorganism and to the increase in osmotic pressure. In a study performed by Metsoviti et al. [30], a similar effect was observed under batch operation, however, in fed-batch operation substrate inhibition was not observed. In the present study, no such inhibitory effect of increased substrate concentration on PDO formation in a repeated fed batch reactor was observed.

1,3-propanediol production at larger scale can be affected by various factors. 1,3-propanediol synthesis by some anaerobic bacteria were reported to be influenced by oxidative stress, osmotic stress and mechanical stress [27]. These stress conditions may explain the decreased PDO concentration as a function of scale up in reactor volume. Moreover, a study performed by Biebl et al [31] noted that by-products of glycerol fermentation, butyric acid and acetic acid, inhibit the production of PDO. Inhibition of PDO production in a fed-batch fermentation by lactic acid, butyric acid and acetic acids have also been reported [23]. I this study, fed-batch fermentation process provided a 39.2 percent increase in PDO concentration and a 61.7 percent increase in the productivity as compared to the batch fermentation process. Metabolically active biomass as inoculum from previous fermentation run was used to begin next fermentation run, thus, reducing the total reaction time. This eliminates the phase of unsteady reactor conditions, microbial adaptation to the media and ultimately improves the PDO production, a result also reported by Szymanowska [32].

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SUPPLEMENTARY MATERIAL

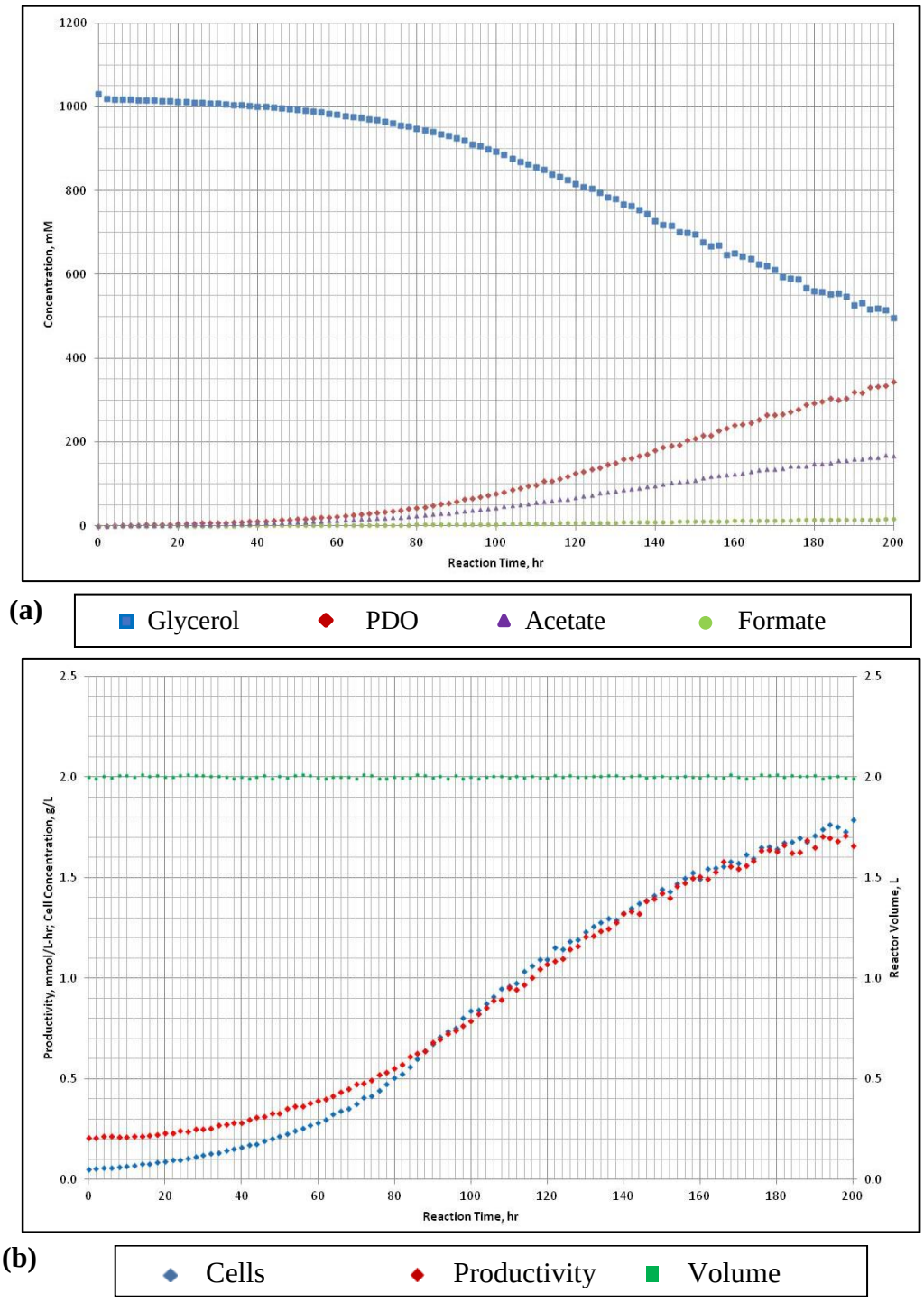
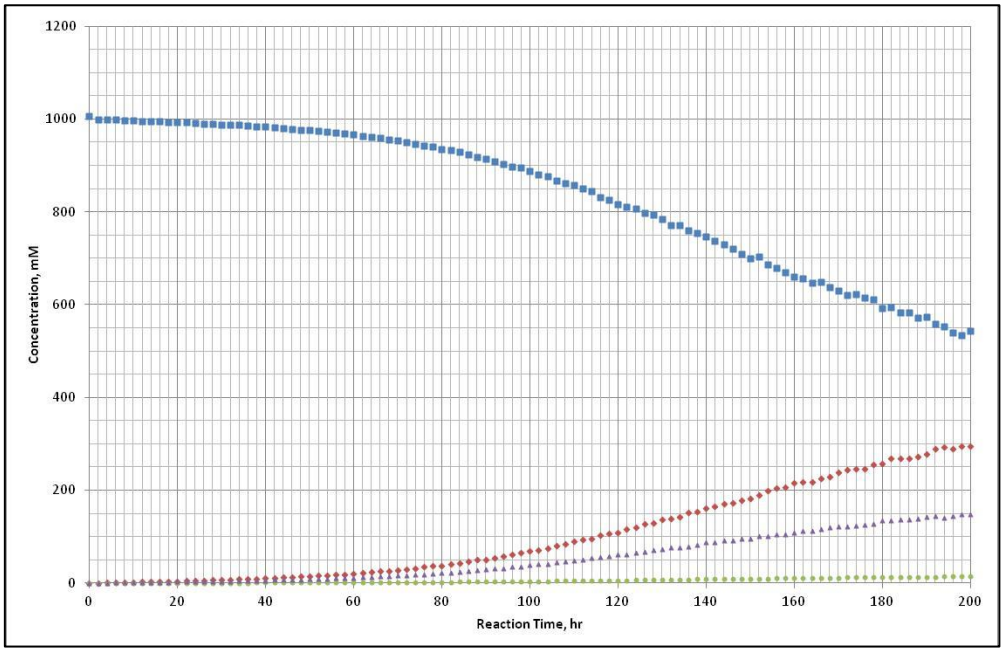


Figure 1s. 2-liter batch reactor at replicated conditions; (a) concentration profiles of metabolite (PDO, acetate and formate) production with glycerol as a carbon source; (b) reactor performance.



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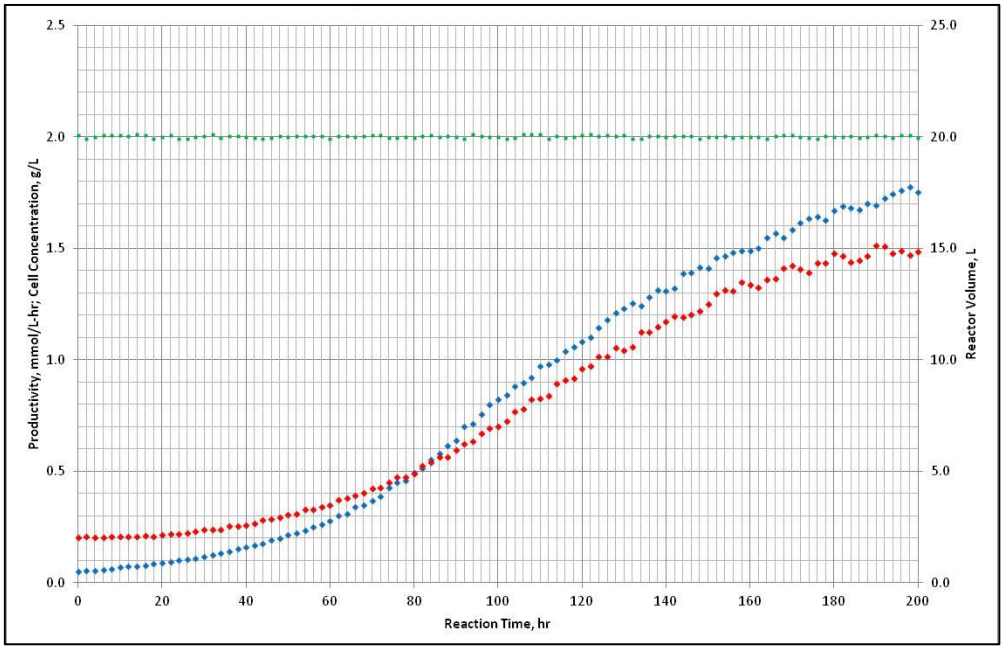

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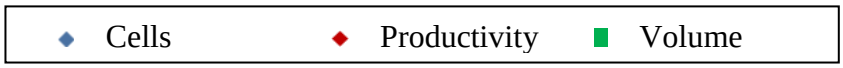


Figure 2s. 20-liter batch reactor at replicated conditions; (a) concentration profiles of metabolite (PDO, acetate and formate) production with glycerol as a carbon source; (b) reactor performance.

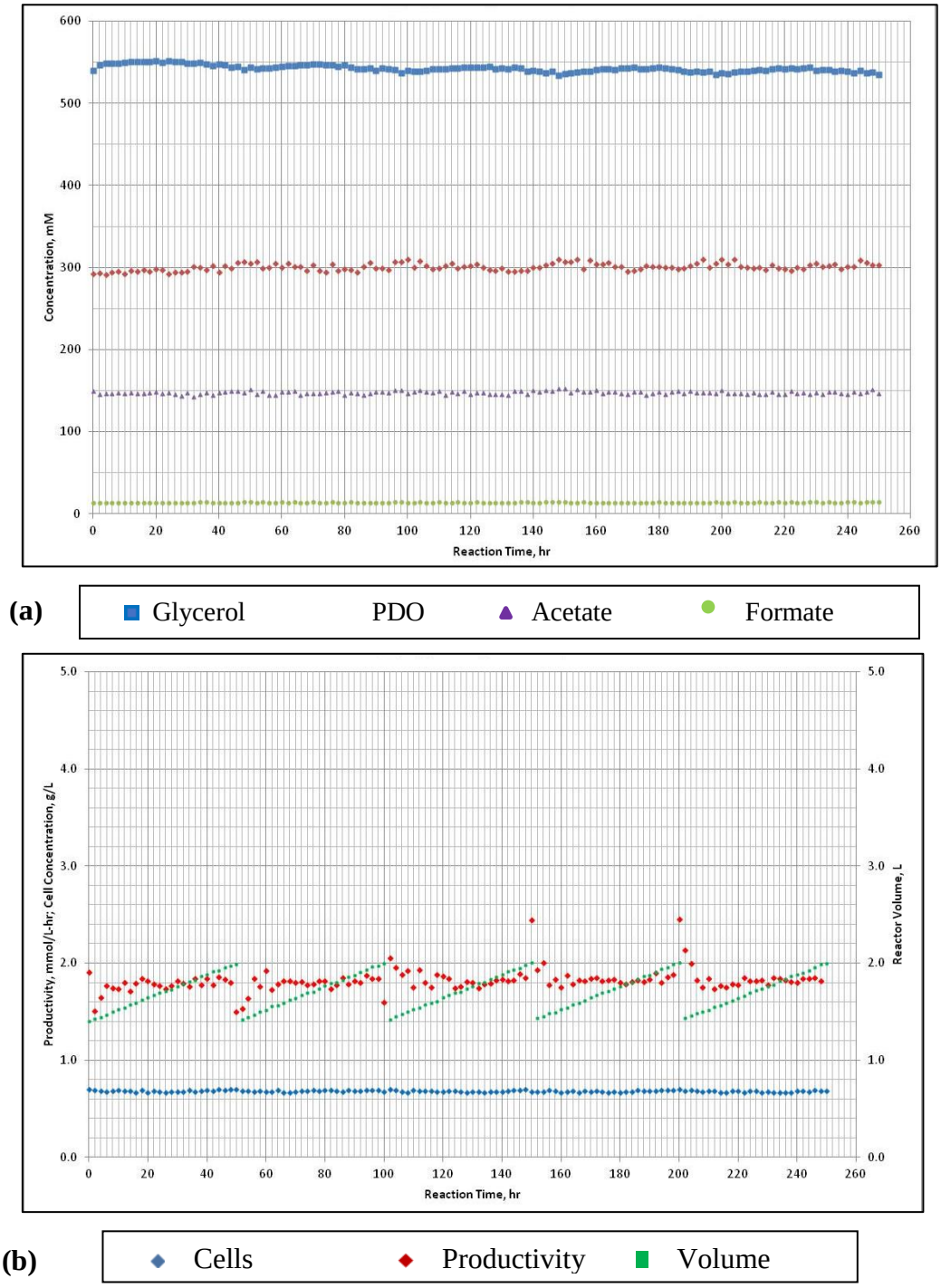
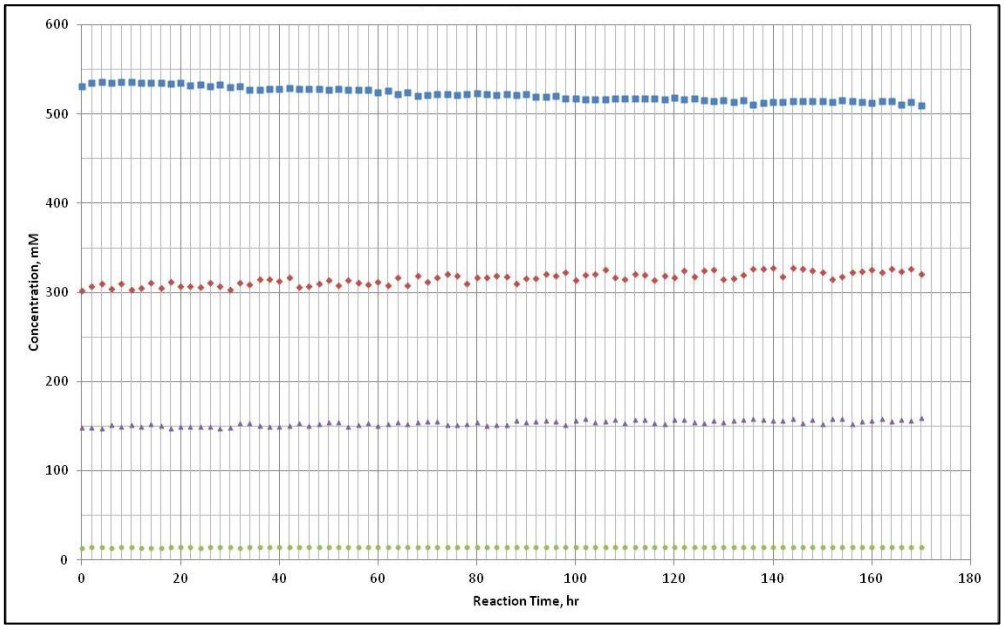


Figure 3s. 2-liter RFB reaction at 0.7 retained volume fraction (V_0/V_{max}) and 1000 mM Glycerol (S_{in}) in feed; (a) concentration profiles of metabolite (PDO, acetate and formate) production with glycerol as a carbon source; (b) reactor performance.



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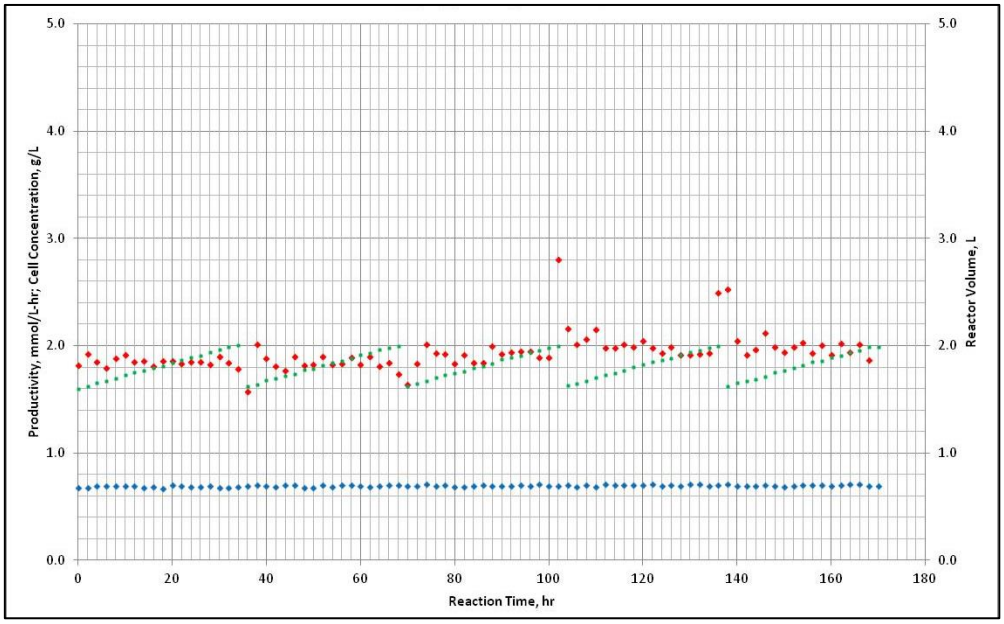

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Figure 4s. 2-liter RFB reaction at 0.8 retained volume fraction (V_0/V_{max}) and 1000 mM Glycerol (S_{in}) in feed; (a) concentration profiles of metabolite (PDO, acetate and formate) production with glycerol as a carbon source; (b) reactor performance.

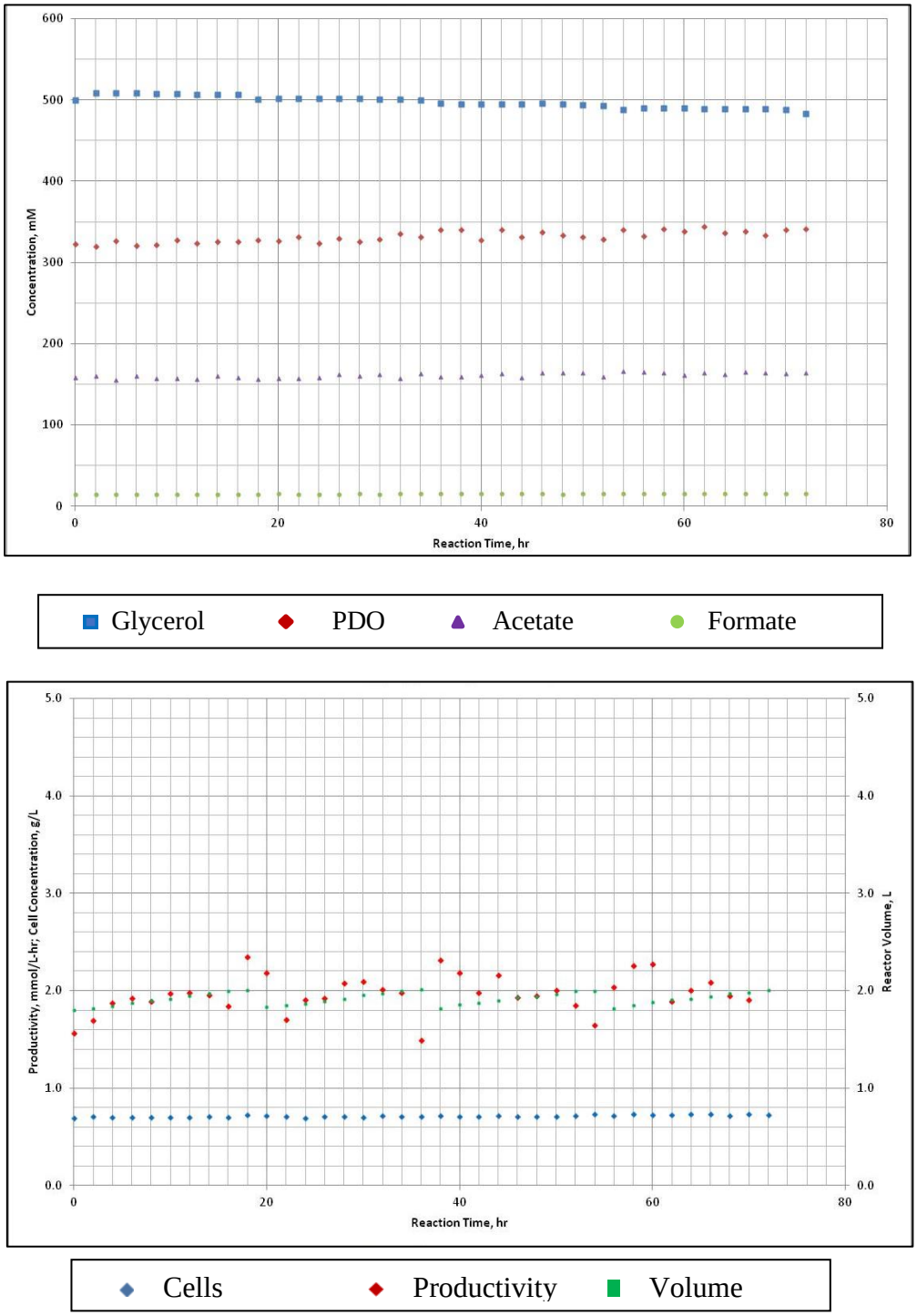
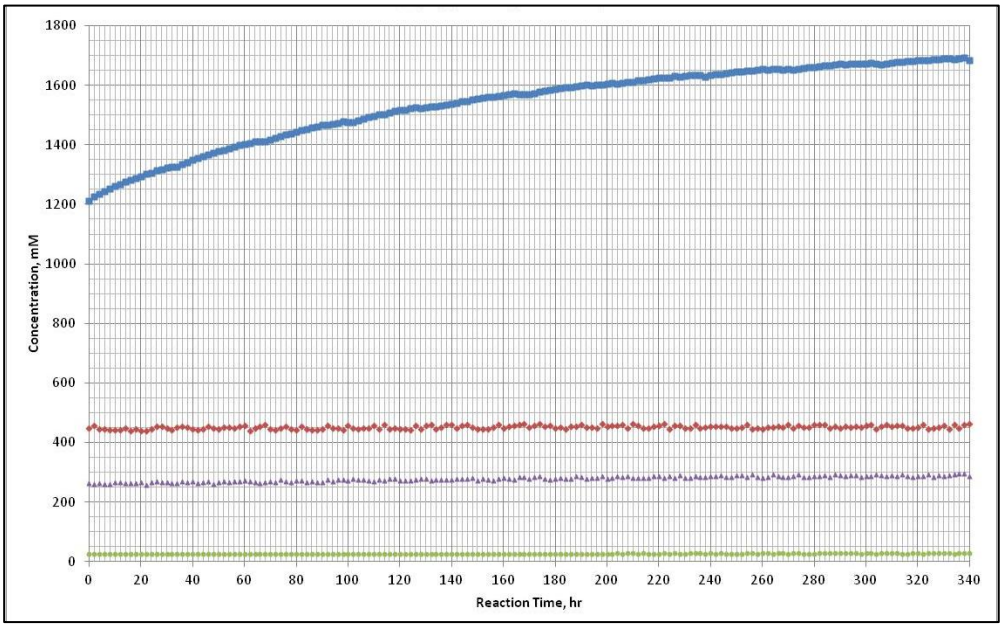
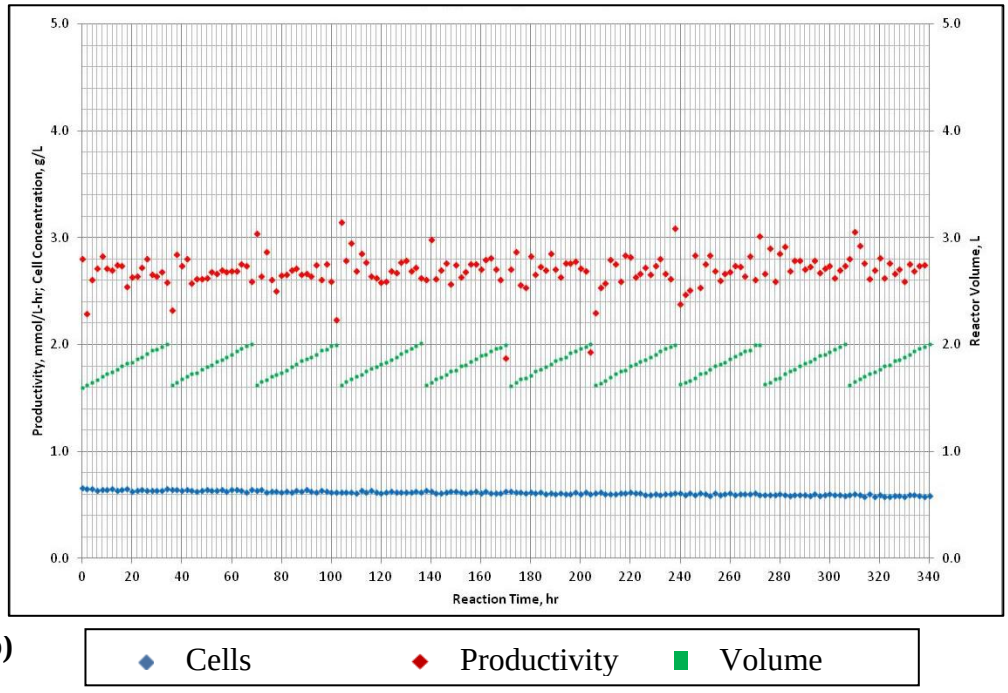


Figure 5s. 2-liter RFB reaction at 0.9 retained volume fraction (V_0/V_{max}) and 1000 mM Glycerol (S_{in}) in feed; (a) concentration profiles of metabolite (PDO, acetate and formate) production with glycerol as a carbon source; (b) reactor performance.

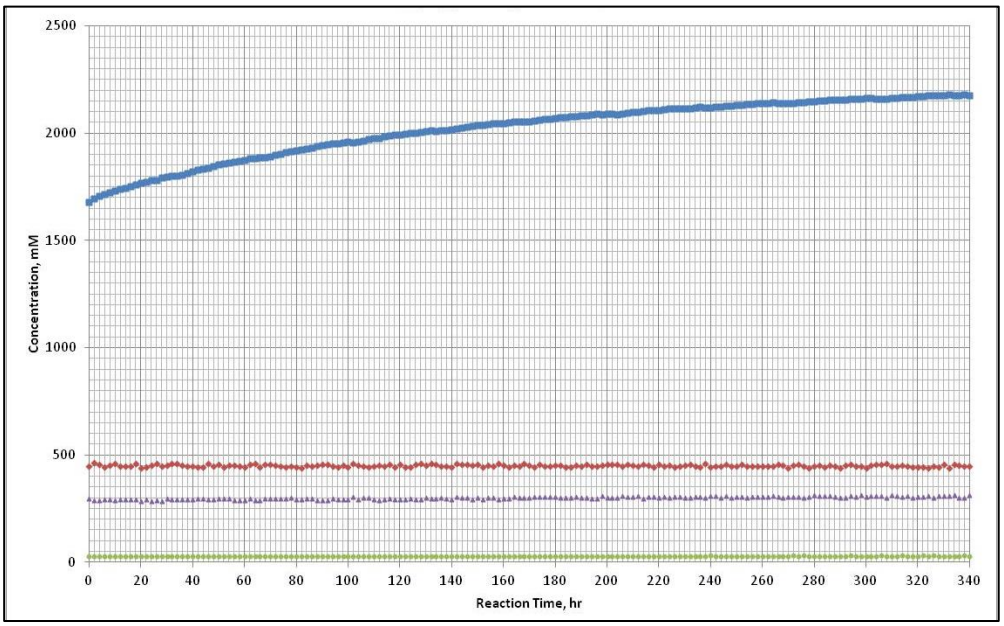


(a)

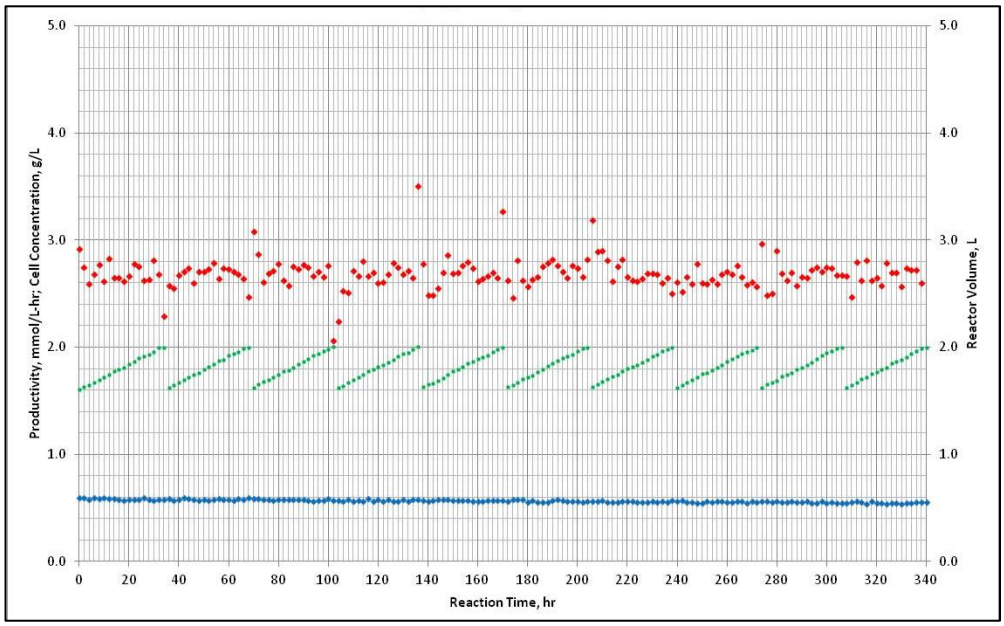


(b)

Figure 6s. 2-liter RFB reaction at 0.8 retained volume fraction (V_0/V_{max}) and 2500 mM Glycerol (S_{in}) in feed; (a) concentration profiles of metabolite (PDO, acetate and formate) production with glycerol as a carbon source; (b) reactor performance.



(a)



(b)

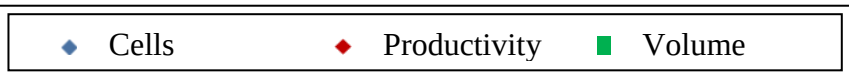


Figure 7s. 2-liter RFB reaction at 0.8 retained volume fraction (V_0/V_{max}) and 3000 mM Glycerol (S_{in}) in feed; (a) concentration profiles of metabolite (PDO, acetate and formate) production with glycerol as a carbon source; (b) reactor performance.

III. ENZYME KINETICS

This manuscript will be prepared for Journal of Biotechnology.

1. INTRODUCTION

Halanaerobium hydrogeniformans, a strict anaerobe and haloalkaliphilic bacterium can metabolize glycerol to produce 1,3-propanediol (PDO). Mainly two enzymes are involved in the PDO production; glycerol dehydratase and 1,3-propanediol dehydrogenase. Glycerol dehydratase converts glycerol to 3-hydroxypropionaldehyde (3-HPA) and 1,3-propanediol dehydrogenase reduces 3-HPA to PDO. Previously performed batch and repeated fed-batch reactor operations confirmed production of PDO by this bacterium. However, other by-products (acetate and formate) were also formed. In order to have better understanding of the glycerol conversion to PDO by the bacterium, it is essential to assess the kinetic activity of the enzymes involved.

This study is aimed at analyzing the activity of both enzymes involved in PDO production by *H. hydrogeniformans*. Two approaches are proposed for performing this study. The first approach is based on performing enzyme assays on enzyme purified from *E. coli* through Histidine tagging by using the C-terminal His_6 tag expression vector, pET29a. Genes of *H. hydrogeniformans* involved in the PDO production were found by using the JGI IMG database [1]. These genes are 1,3-propanediol dehydrogenase (Halsa_0672), iron containing alcohol dehydrogenase (Halsa_2285), glycerol dehydratase cobalamin dependent alpha subunit (Halsa_0984), dehydratase medium subunit (Halsa_0985), dehydratase small subunit (Halsa_0986) and dehydratase reactivase factor large subunit (Halsa_0987). 1,3-propanediol dehydrogenase catalyzes the last step of the PDO pathway. Iron containing alcohol dehydrogenase is a non-specific enzyme that could also catalyze the last step. For glycerol dehydratase, enzyme that catalyzes the initial step in PDO production, *H. hydrogeniformans* potentially possesses four genes. Three of the genes have been annotated for three subunits of the enzyme: alpha, medium and small subunit. A fourth gene is annotated for a reactivase factor, essential to maintain

the activity of glycerol dehydratase enzyme. In order to assess these enzymes, each protein will be purified after ligation of the gene into a C-terminal His-tag expression vector and transformation into *E. coli* cells.

Another approach to perform this study, is to assay enzymes from cell free extract obtained from *H. hydrogeniformans*. Bacteria will be grown in the bioreactor under batch conditions to get high biomass and enzyme assays will be performed on lysed cells. Quantification of enzymatic activity is essential for the enzyme characterization. Furthermore, enzyme activity information determined from assays will be used in modeling the dynamics of culture to maximize the PDO yield and productivity.

2. METHODOLOGY.

2.1. Enzyme assays on purified enzyme obtained through His-tagging. In order to fully understand the biochemical properties of glycerol dehydratase and 1,3-propanediol dehydrogenase enzymes, genes for each enzyme will be amplified and the resulting protein will be characterized. As mentioned above, there are a total of six genes of *H. hydrogeniformans* that are considered to be involved in the PDO production. For this thesis, only one gene, Halsa_2285 was amplified and purified.

2.1.1. Molecular cloning of expression constructs. The coding sequence of Halsa_2285 was cloned into Not1 and Sal1 restriction sites of the C-terminal His_6 tag expression vector, pET29a. For the activity analysis of enzyme, initial step was to isolate the total genomic DNA from the bacterial culture. Halsa_2285 was amplified using PCR performed with designed primers. Primers for each gene were designed by using SeqBuilder feature on DNASTAR navigator software [2]. Restriction sites available on the vector were compared with its presence on the given gene sequence. Restriction sites for Sal1 and Not1 were selected as these enzymes do not cut within the insert. Therefore, unique restriction sites for Sal1 and Not1 were added to the forward and reverse primer sequence, in order to create cohesive ends in the amplified genes in a proper orientation.

Extra base pairs, known as leader sequence were also added to the 5'end of the primer. The designed primers are shown in Table 1

In each primer sequence, GCGT is the leader sequence. On the forward primer, GTCGAC is the flanking restriction sequence for Sal1 and on reverse primer, GCGGCCGC is the restriction sequence for Not1. The melting temperature for each primer sequence was calculated by using the formula:

$$Tm = 2^{o}C \times (nA + nT) + 4^{o}C \times (nG + nC) \quad [3]$$

Table 1: Primer sequences for putative enzymes in PDO-pathway of *H. hydrogeniformans.*

Gene	Primer Sequences	Melting temperature (Tm)
1,3-propanediol dehydrogenase (Halsa_0672)	Forward- 5'GCGT GTCGAC ATG TAT GAT TAT ATG TTA CCA AC 3'	58ºC
	Reverse-- 5'GCGT GCGGCCGC TTA ATA AGC TGC TTT AAA GAT T 3'	60ºC
1,3-propanediol dehydrogenase (Halsa_2285)	Forward- 5' GCGT GTCGAC ATG TCC GAT TAT TAT GAT TAT AT 3'	56ºC
	Reverse- 5' GCGT GCGGCCGC TTA AAA GGC ATC TTC AAA TAT T 3'	54ºC
Glycerol dehydratase alpha subunit (Halsa_0984)	Forward- 5' GCGT GTCGAC ATG GTG AAA AGG TCC AAG CGA TTT 3'	60ºC
	Reverse- 5' GCGT GCGGCCGC	56ºC

	TTA TTC TCC TAT TTC GTC AAT A 3’	
dehydratase medium subunit (Halsa_0985)	Forward- 5’ GCGT GTCGAC ATG CAA TTA AAT GAA GAT GCT ATT 3’	60ºC
	Reverse- 5’ GCGT GCGGCCGC TTA TTT GAA TGT TAC CTC AAC T 3’	56ºC
dehydratase small subunit (Halsa_0986)	Forward- 5’ GCGT GTCGAC ATG GAT AAA GAG TTA GTT GAA TCT 3’	62ºC
	Reverse- 5’ GCGT GCGGCCGC TTA TCT ATC ACC TTT TAG TCT G 3’	58ºC
Glycerol dehydratase reactivating factor large subunit (Halsa_0987)	Forward- 5’ GCGT GTCGAC ATG GAA TAT ATT GTA GGG GTC GA 3’	64ºC
	Reverse- 5’ GCGT GCGGCCGC TTA CTC ATC TAT TAC ACT TCC C 3’	60ºC

Amplifiaction of Halsa_2285 was performed by using the above-mentioned primer sequences. All of the results shown in this thesis are with the same amplified gene. The main idea behind this methodology was to purify enzyme through His-tag, which is added via gene insertion into C-terminal His_6 tag expression vector, pET29a. However, due to the presence of a stop codon on the reverse primer sequence, translation was inadvertently terminated before addition of His-tag at C-terminal of the protein.

Moreover, the sequence was not in-frame with respect to the N and C-tag in the vector. Therefore, new primer sequences, shown in Table 2, were designed to bring sequence in-frame and without a stop codon.

Table 2: Modified primer sequences without stop codons for putative enzymes in PDO-pathway of *H. hydrogeniformans.*

Gene	Primer	Melting temperature (Tm)
1,3-propanediol dehydrogenase (Halsa_0672)	Forward- 5'GCGT GT CGA CAA ATG TAT GAT TAT ATG TTA CCA 3'	56ºC
	Reverse- 5'GCGT GCG GCC GCA ATA AGC TGC TTT AAA GAT TCC 3'	56ºC
1,3-propanediol dehydrogenase (Halsa_2285)	Forward- 5' GCGT GT CGA CAA ATG TCC GAT TAT TAT GAT TAT A 3'	58ºC
	Reverse- 5' GCGT GCG GCC GCA AAA GGC ATC TTC AAA TAT TCT 3'	56ºC
Glycerol dehydratase alpha subunit (Halsa_0984)	Forward- 5' GCGT GT CGA CAA GTG AAA AGG TCC AAG CG 3'	56ºC
	Reverse- 5' GCGT GCG GCC GCA TTC TCC TAT TTC GTC AAT ATC 3'	58ºC
dehydratase medium subunit	Forward- 5' GCGT GT CGA CCA ATG CAA TTA AAT	58ºC

(Halsa_0985)	GAA GAT GC 3’ Reverse- 5’ GCGT GCG GCC GCA TTT GAA TGT TAC CTC AAC TTC 3’	 54ºC
dehydratase small subunit (Halsa_0986)	Forward- 5’ GCGT GT CGA CAA ATG GAT AAA GAG TTA GTT GAA T 3’ Reverse- 5’ GCGT GCG GCC GCA TCT ATC ACC TTT TAG TCT GTT 3’	60ºC 58ºC
Glycerol dehydratase reactivating factor large subunit (Halsa_0987)	Forward- 5’ GCGT GT CGA CAA ATG GAA TAT ATT GTA GGG GTC 3’ Reverse- 5’ GCGT GCG GCC GCA CTC ATC TAT TAC ACT TCC CTC 3’	64ºC 62ºC

PCR for Halsa_2285 using designed primers (Table 1) and Phusion high fidelity PCR kit was performed. The PCR reaction mixture of 50 µl was prepared by adding 10 µl of 5X reaction HF buffer, 1 µl of 10 mM dNTPs, 2.5 µl of forward primers, 2.5 µl of reverse primers, 2 µl of template DNA, 0.5 µl of Phusion DNA polymerase and 31.5 µl nuclease-free water. A positive control, DNA template of 1.35 Kbp and negative control, reaction mixture without DNA, were also prepared by using the same protocol. Amplification of genes of interest were confirmed by performing 1% agarose gel electrophoresis. Subsequently, amplified DNA was purified by using GFX PCR DNA purification kit system.

Purified amplified DNA was digested with Sal1 and Not1 restriction enzymes to create cohesive ends. In addition, a pET29a vector was digested by using the same restriction enzymes, so the gene of interest can be ligated into vector at specific site, in proper orientation.

The restriction sites for Sal1 and Not1 are [4]:

Sal1:	**Not1:**
5’—G TCGAC—3’	5’—GC GGCCGC—3’
3’—CAGCT G—5’	3’—CGCCGG CG—5’

The cohesive ends generation in the gene of interest and vector were confirmed by running samples on 1% agarose gel. Before proceeding with further steps, the cut DNA fragments were purified by using a Qiagen DNA purification kit system. Purified gene segment was ligated into the cut His-tag expression vector pET29a. Thus, the DNA segment was cloned at Sal1 and Not1 restriction site in 5’ to 3’ direction.

pET29a vector was chosen because of the presence of a tag of 6 Histidine residues just after its multiple cloning site. Insertion of the gene into the multiple cloning site of the vector adds a C-terminal tag of Histidine residues. Protein tagging enables the purification of desired recombinant protein from a mixture of proteins by using a His-pur cobalt spin column. pET29a vector contains a gene coding for antibiotic (kanamycin) resistance, used as a selection marker for the transformed clones and lacI gene from lac operon that codes for lac repressor. Moreover, multiple cloning sites arejust after T7 promoter DNA sequence, lac promotor DNA sequence and ribosome binding site. Halsa_2285 was cloned after the T7 promotor DNA sequence which is recognized by T7 RNA polymerase coded by *E. coli* BL21 cells.

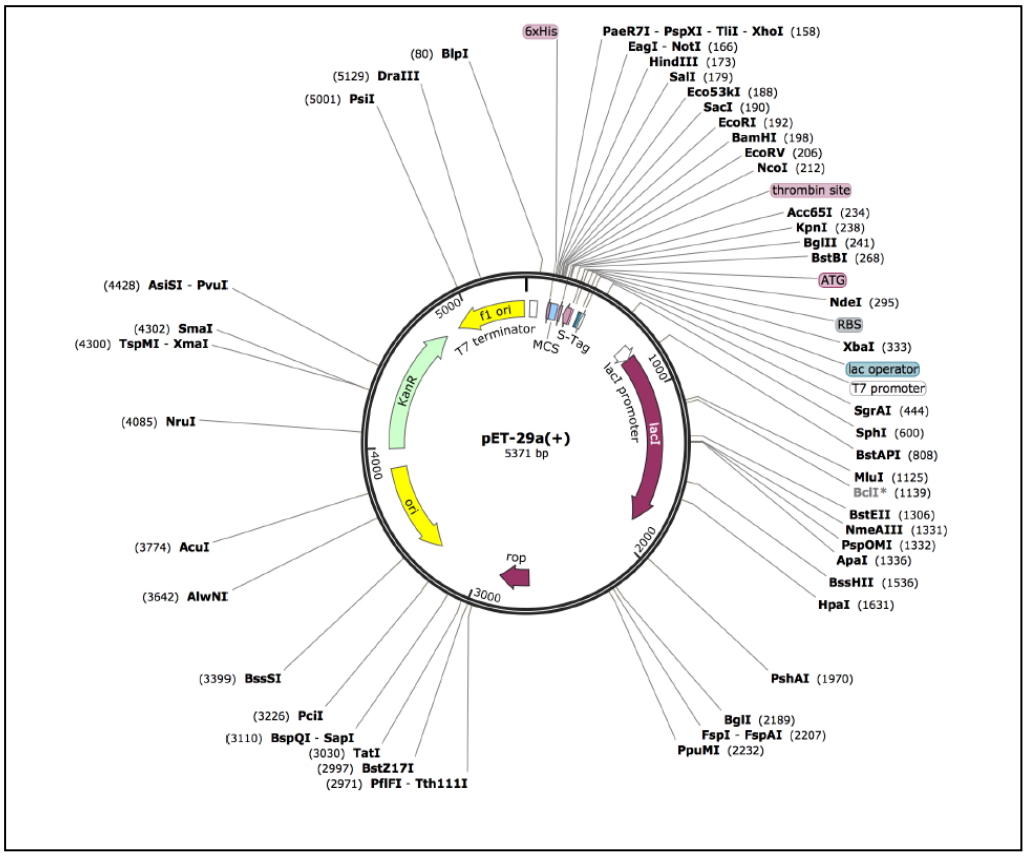


Figure 1. pET29a(+) vector (Novagen) [5]

Subsequently, transformation *E. coli* BL21(DE3) cells with the of pET29a construct was performed by using theheat shock method. *E. coli* BL21(DE3) cells have a gene for T7 RNA polymerase, which is under the control of lac operon [6]. Moreover, *E. coli* BL21(DE3) cells are sensitive towards antibiotic kanamycin. In addition, pET29a vectors have a resistant marker for antibiotic kanamycin. Following transformation when *E. coli* cells were plated onto kanamycin-containing media, only transformed cells should grow. Presence of Halsa_2285 in transformed clones was confirmed by performing plasmid purification. A PCR reaction for purified plasmids with primers designed for the Halsa_2285 (Table1) confirmed the presence of desired gene in the transformed colonies.

2.1.2. Protein expression. In transformed cells, protein expression for genes of interest was induced by adding IPTG (isopropyl ß-D-1-thiogalactopyranoside). IPTG is a non-hydrolyzable and structural analog for lactose. As mentioned earlier, in *E. coli*

BL21(DE3) cells, transformation of T7 RNA polymerase is under the control of the lac operon. Moreover, within the vector, transcription of the gene of interest is under the control of the T7 promotor (Figure 2). Therefore, the addition of IPTG to the media enhances the transcription of T7 RNA polymerase and subsequently will induce the protein expression of the gene of interest.

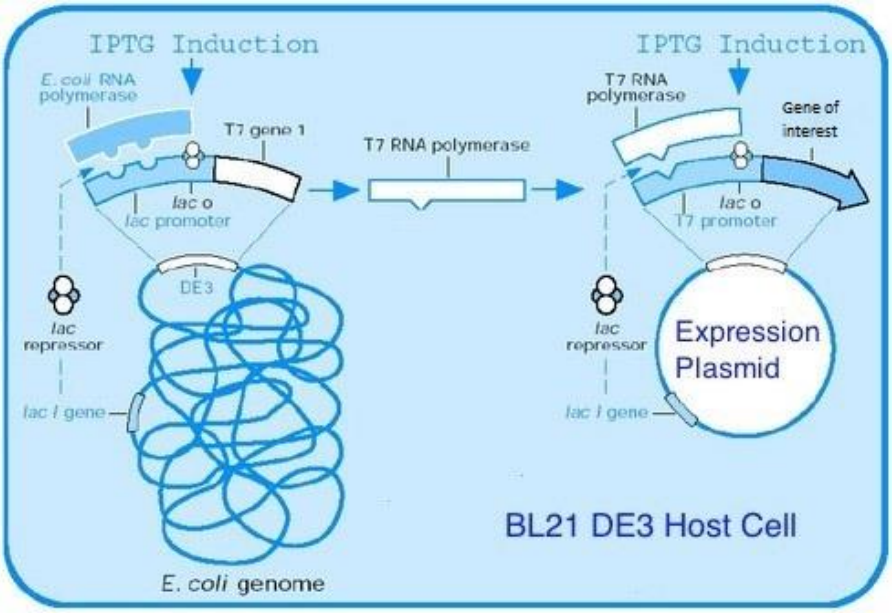


Figure 2. Modified Image for protein expression induction [7]

Bacterial cells were harvested by centrifugation. Cell pellets were suspended in equilibrium/wash solution supplemented with protease inhibitor and phenylmethylsulfonyl fluoride [8]. Cell suspensions were lysed by using the bead beater homogenizer and beads of 0.1 mm diameter. Protein expression post induction was observed by SDS PAGE analysis. SDS PAGE were performed on 8% (v/v) acrylamide by using Bio-Rad Mini protean system.

Once protein expression is confirmed, recombinant proteins from cell lysate will be purified using His-pur cobalt spin columns. Recombinant proteins with a string of poly-histidine tag to their N- or C-terminus can be purified due to the binding of histidine residues to the column. Purified recombinant proteins on the column can be eluted by using imidazole, as it competes with histidine for binding to the column. Eluted proteins

would have some amount of imidazole that can interfere with the enzymatic activity. Therefore, recombinant proteins will be purified and concentrated by using a Slide-A-Lyzer dialysis device kit. Purity of concentrated recombinant proteins or enzymes will be confirmed by using SDS PAGE analysis. Moreover, protein or enzyme concentrations will be determined by using Bradford protein assay.

2.1.3. Enzyme assays. The pH, temperature and salt concentrations critical for the activity of purified enzyme will be optimized. For pH optimization, a pH gradient ranging from 6-12 in the enzyme assay mixture will be created. For temperature optimization, a temperature gradient ranging from 20-40ºC in the enzyme assay mixture will be prepared. For salt optimization, two different salts (NaCl and KCl) will be used, and a gradient of salt concentrations ranging from 1-7% in the enzyme assay mixture will be prepared. For each of the optimization tests, absorbance will be measured by using spectrophotometer.

In addition, enzyme assays to determine the activity of each purified enzyme under optimized conditions will be performed. We will perform glycerol dehydratase enzyme assays for the activity analysis of each of the following subunits: glycerol dehydratase cobalamin dependent alpha subunit (Halsa_0984), glycerol dehydratase medium subunit (Halsa_0985), glycerol dehydratase small subunit (Halsa_0986), and glycerol dehydratase reactivase factor large subunit (Halsa_0987). For glycerol dehydratase enzyme assay, 1,2-propanediol as the substrate will be used. For this study, one activity unit of glycerol dehydratase will be defined as the amount of enzyme catalyzing the formation of 1 µmol of propionaldehyde in a minute [9]. Product formed will be detected by measuring the absorbance at 305 nm by using spectrophotometer. On the basis of this data, *Km, Kcat &* catalytic efficiency values for each subunit will be calculated by using the Michaelis-Menten equation. *Km* measures the affinity of enzyme for the substrate. Turnover number or *Kcat* measures the number of molecules of substrate converted to product per active site per second. Catalytic efficiency is a measurement for the efficiency of an enzyme.

In the case of 1,3-propanediol dehydrogenase, two enzyme assays for subunits 1,3-propanediol dehydrogenase (Halsa_0672) and iron containing alcohol dehydrogenase (Halsa_2285) will be performed. 1,3-propanediol will be used as the substrate due to the instability of 3-hydroxy-propionaldehyde. Henceforth, one activity unit of 1,3-propanediol dehydrogenase will be defined as the amount of enzyme catalyzing the formation of 1 µmol of $NADH_2$ in a minute [10]. Product formed will be detected by measuring the absorbance at 340 nm. On the basis of this data, we will calculate *Km*, *Vmax*, *Kcat &* catalytic efficiency values for 1,3-propanediol dehydrogenase.

2.2 Enzyme assays on cell free extract.

Culture of *H. hydrogeniformans* were grown in a 20-liter reactor under batch culture and anaerobic conditions. Culture conditions were maintained at a temperature of 30ºC, pH 11, and salt concentration of 7%. Anaerobic conditions were maintained by sparging media with argon at an agitator speed of 250 rpm. Culture were grown for 7 days and collected anaerobically. Collected cells were centrifuged at 6500 rpm for 30 min at 1ºC to collect pellets [11]. Pellets were washed with Tris buffer (1 liter; 50 mM Tris-HCl, 2 mM DTT and 1 mM $MnCl_2$; pH 7.4) and resuspended in the same buffer up to an O.D. of 10 [11, 12]. Cells were lysed using a French press anaerobically. Cell lysate were collected in glass vials and stored at -20ºC. Bicinchoninic acid (BCA) protein assay was performed to measure the protein concentration in cell lysate. Protein expression was visualized using SDS PAGE analysis. Optimum pH, temperature and salt conditions for the enzymes will be determined. Enzyme assays will be performed to determine the activity of enzyme under standard optimized conditions. Anaerobic conditions will be maintained anaerobic for each step.

3. RESULTS

3.1. Enzyme assays on purified enzyme obtained through His-tagging.

It is suspected that a total of six genes of *Halanaerobium hydrogeniformans* are involved in the PDO production. The first amplified gene was Halsa_2285 (iron-

containing alcohol dehydrogenase). Halsa_2285 has a molecular size of 1.29 kbp and its amplification was confirmed by agarose gel electrophoresis, Figure 4. For PCR, a positive control, molecular size 1.35 kbp and a negative control were also ran as shown in the Figure 3. Five samples out of six showed gene amplification with a molecular size of 1.29 kbp.

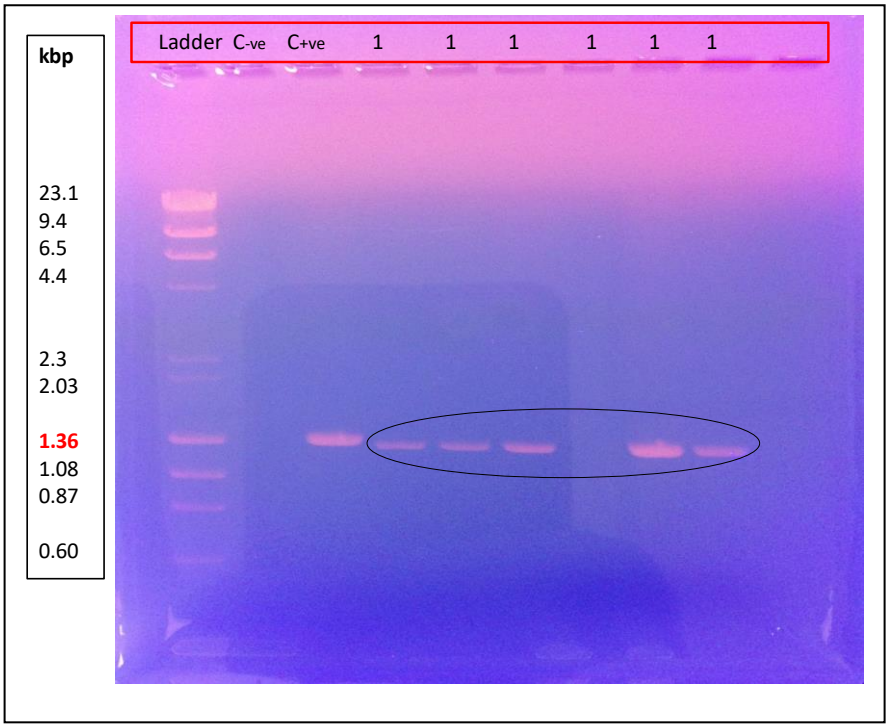


Figure 3. Agarose gel image for PCR. Lanes labeled as 1 represents the amplification of Halsa_2285.

The amplified DNA fragments were purified and digested by Sal1 and Not1 restriction enzymes to create cohesive ends. The restricted DNA was ligated into cut His-tag expression vector pET29a and transformed into *E. coli* BL21(DE3) cells by using heat shock method. Growth of transformed clones, plated onto agar plates containing antibiotic kanamycin after overnight incubation, confirmed the successful transformation of E. coli cells. The presence of Halsa_2285 in transformed clones was confirmed by plasmid purification. Plasmids were purified from transformed clones by using a plasmid DNA purification kit from Qiagen. A PCR reaction was performed on the purified

plasmids by using primers designed for Halsa_2285. In order to confirm amplification or presence of Halsa_2285, agarose gel electrophoresis was performed. Figure 4 confirms the presence of the gene of interest (shows a band size of 1.29 kbp) in two of the transformed clones.

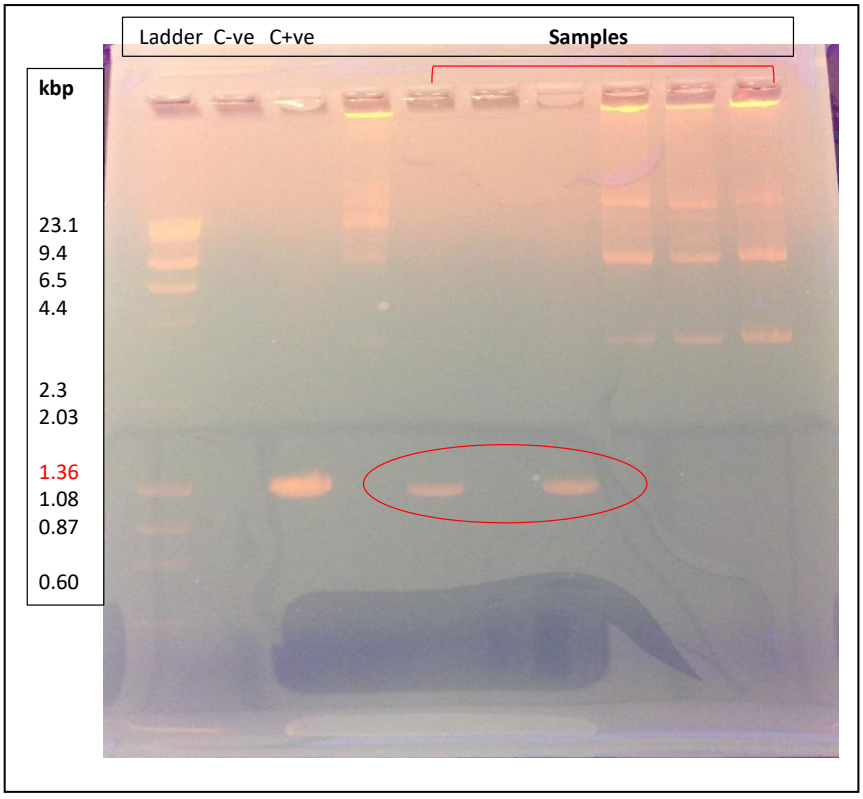


Figure 4. Agarose gel electrophoresis for PCR performed on purified plasmid.

Transformed clones that showed the presence of Halsa_2285, were utilized further to induce the protein expression. Along with transformed clones, a control (*E. coli* cells containing vector only) was also kept, in order to compare the results. Protein expression in induced and uninduced cultures was visualized by SDS PAGE analysis on 8% separating gel, Figure 5. Samples were collected after 4 hours of adding IPTG and overnight incubation. Halsa_2285 protein has a molecular weight of 105 kDa. Protein expression induction was observed in both induced sample (1') and induced control (C'), however, for a protein with molecular weight of approximately125 kDa. There was no induction observed for protein of interest.

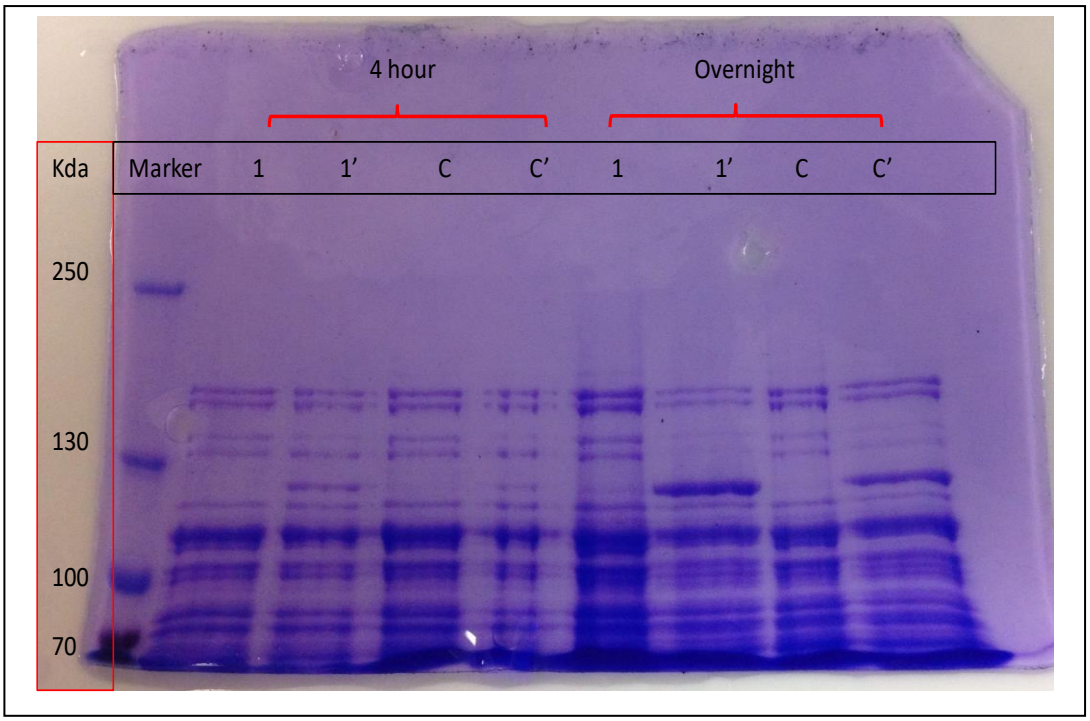


Figure 5. SDS PAGE pattern of proteins in lysed cells. Lanes labeled as 1 are for the uninduced samples. Lanes labeled as 1' are for the induced samples. Lanes labeled as C are for induced control. Lanes labeled as C' are for induced control.

Halsa_2285 protein is an insoluble and extracellular protein. Insolubility of protein was determined using a sequence based protein solubility evaluator software, PROSO II [13] and extracellular using PrediSi software [14]. Initially it was thought that the protein either due to its insolubility was being lost or it had a toxic effect on *E. coli* cells due to which its production was being hindered. A solubility protocol for the recovery of insoluble proteins was utilized, but still expression of desired protein was not observed by using SDS PAGE. However, final analysis revealed that gene was not in-frame with the N- and C-terminal of the vector, due to which desired protein was not being formed. This problem was associated with the designed primer sequences (Table 1). Therefore, modified primer sequences were designed to bring gene in-frame (Table 2). The genes will be re-amplified with modified primers and analyzed for protein expression.

3.2. Enzyme assays on cell free extract.

A culture of *Halanaerobium hydrogeniformans* has been grown in 20-liter bioreactor under anaerobic conditions. Further steps on the grown culture are being performed.

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SECTION

2. CONCLUSIONS

Preliminary experiments showed the ability of *Halanaerobium hydrogeniformans* to convert glycerol into PDO. Media supplementation with vitamin B_{12} almost doubled the production of PDO [17]. In this work, media screening tests showed that out of 24 factors tested, eight factors, glycerol, vitamin B_{12}, pH, ammonium ion, sodium sulfide, cysteine, iron, and cobalt, had a significant impact on PDO production. Glycerol is the initial substrate and carbon source in the medium, therefore, its high concentration would lead to high PDO production. Glycerol dehydratase, enzyme involved in PDO production is a vitamin B_{12} dependent enzyme, which indicates the significance of vitamin B_{12}. Moreover, cobalt is a part of vitamin B_{12} structure, making it essential for PDO production. Ammonium ions are the source of nitrogen in the medium. Sodium sulfide and cysteine are the reducing agents that reduce the oxidation-reduction potential of the medium. *H. hydrogeniformans* grows at high pH and hence, more PDO is produced under alkaline conditions. Moreover, iron is essential for the activity of many alcohol dehydrogenases [18]. Therefore, the resulting medium of glycerol (1000 µM), vitamin B_{12} (500 µg/L)), ammonium ion (3000 µM)), cysteine (300 µM), sodium sulfide (200 µM), cobalt (100 µM), iron (50 µM) and pH (11) was used for the remaining experiments.

The impact of scale up in reactor volume, retained volume fraction, and elevated glycerol concentration on the reactor productivity was analyzed. Scale up studies were performed under batch mode using 2 liter and 20 liter reactor volumes. A 2-liter bioreactor operation resulted in a PDO concentration of 327.8 mM and a productivity of 1.67 mmol/L-hr. As reactor volume increased from 2 L to 20 L, productivity decreased by 10% to 1.50 mmol/L-hr and PDO concentration also by 10% to 300.75 mM. However, both productivity and PDO concentration were enhanced with repeated fed-batch operation as compared to batch operation.

Environmental stresses have been reported to be associated with limiting performances of the biological processes at large scale [18]. Moreover, simple batch operation rarely provides maximum productivity, as the reaction conditions are not optimal for most of the reaction time. Moreover, continuous reactor operation provides better productivity in comparison to the batch operation [19]. Though there are advantages associated with continuous processes, it is seldom used in industries due to the possibility of contamination. However, advantages of a continuous process can be achieved by operating a reactor under repeated fed-batch operation.

In repeated fed batch operations, apparent steady state conditions were achieved within 10 cycles. Repeated fed batch operation with different retained volume fraction showed increase in reactor productivity with increase in retained volume fraction. As the retained volume fraction increased from 0.5 to 0.8, the PDO concentration increased by 22.2% from 265.2 mM to 324 mM, similarly, productivity increased by 26.5% from 1.55 to 1.96 mmol/L-hr. Furthermore, increase in glycerol concentration in the feed media, enhanced the reactor productivity. As glycerol feed concentration increased from 1000 mM to 2000 mM at a retained volume fraction of 0.8, the productivity increased by 38.3% and the PDO concentration increased by 42.8%. On comparing results obtained from repeated fed batch operation with simple batch operation, a 61.7 % increase in productivity and a 39.2 % increase in PDO concentration was observed.

Glycerol to PDO conversion is accomplished by two enzymes: glycerol dehydratase and 1.3-propanediol dehydrogenase. In order to study the kinetics of enzymes of *H. hydrogeniformans* involved in PDO production, the gene for 1,3-propanediol dehydrogenase (Halsa_2285) enzyme was ligated into the C-terminal His_6 tag expression vector pET29a and transformed into *E. coli* BL21 (DE3) cells. Presence of the gene in the transformed clones was confirmed through PCR performed on plasmids purified from the transformed clones. Protein expression in the transformed clones was induced by the addition of 1 mM IPTG. However, SDS PAGE analysis did not show the expression for Halsa_2285 (molecular weight 105 KDa). It was determined that gene was not in-frame with the N- and C-terminal of the vector, ergo the desired protein was not

being formed. Therefore, modified primer sequences were designed to bring the gene in-frame. There is another ongoing approach to analyze the activity of these two enzymes, which is to perform enzyme assays on cell free extract.

Since enzymes will be obtained from a haloalkaliphile bacterium (optimum growth conditions; pH 11 and salt concentration 7%), optimum pH and salt concentration for the enzymes are expected to be high. However, pH and salt concentration inside the cell would be less as compared to the media pH and salt level. Thus, optimum pH is expected between 9-10 and salt concentration between 2-4%. Moreover, optimum temperature for the enzyme activity should be around 30ºC, similar to the optimum growing temperature of the bacterium. The stability of these enzymes at extreme pH, temperature and salt conditions could provide unique features for the application of enzymes in crude glycerol fermentation processes at an industrial scale. These findings would also facilitate the understanding of structural and biochemical basis of the stability of molecules under extreme conditions.

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