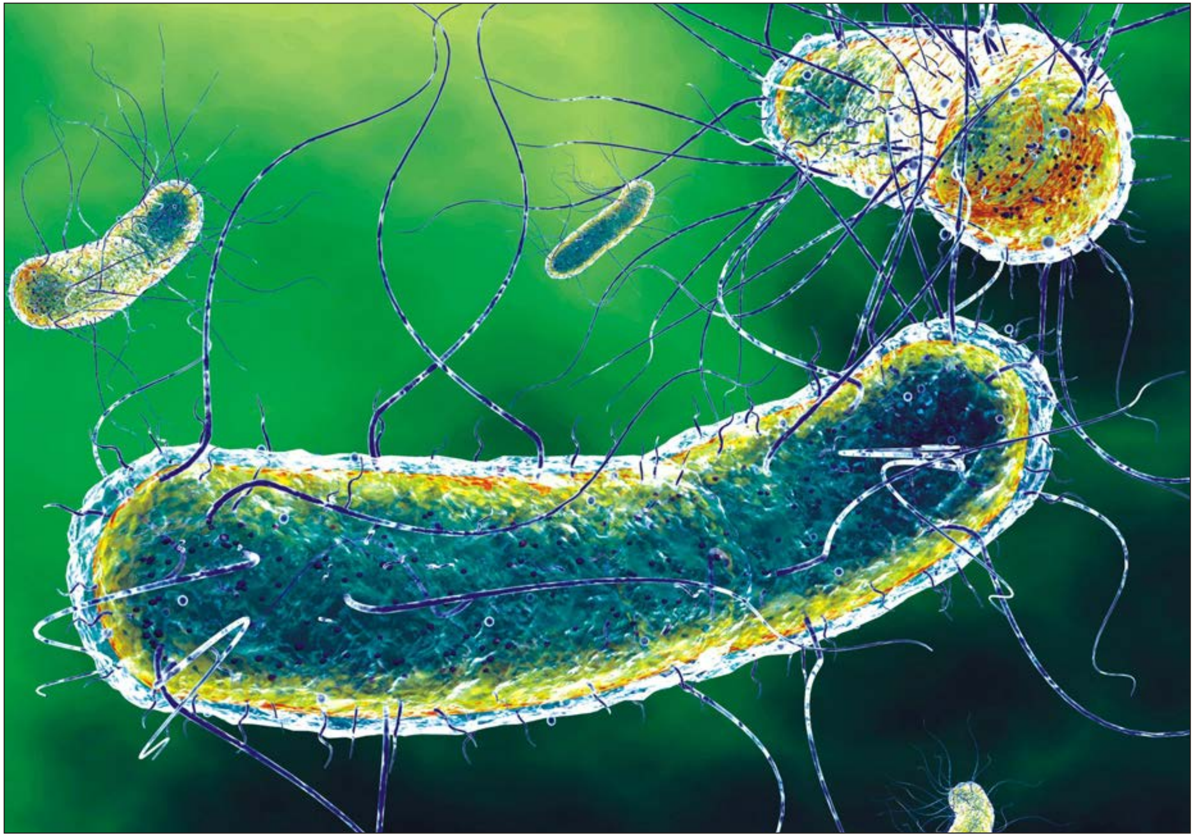

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Growth and 1, 3-propanediol Production on Pre-Treated Sunflower Oil Bio-Diesel Raw Glycerol Using a Strict Anaerobe-*Clostridium butyricum*

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Abstract: The objectives of the present investigation were to evaluate the pre-treated sunflower oil biodiesel raw glycerol for the growth and 1,3-propanediol (1,3-PD) production by *Clostridium butyricum* DSM 5431. The growth inhibition percentages of *Clostridium butyricum* DSM 5431 on grade A (pH, 4.0) and grade B (pH, 5.0) pre-treated sunflower oil biodiesel raw glycerol (SOB-RG) were almost similar to those of pure glycerol at 20 g glycerol L^{-1} i.e., 18.5±0.707 to 20.5±0.7% inhibition. In grade A raw glycerol, the growth inhibition was reduced from 85.25±0.35 to 32±1.4% (about 53.2% reduction) at 40 g glycerol L^{-1} by giving a two times washing to grade A raw glycerol with n-hexanol (grade A-2). When two times n-hexane washed grade AH-2 raw glycerol was employed a reduction of 42.9% was observed which is significantly lower as compared to that of grade A-2 raw glycerol. In anaerobic fed-batch cultures, 42 and 45 g L^{-1} of 1, 3-PD was accumulated in the fermented broth at the expense of 85 and 87.8 g L^{-1}, giving almost similar yields, i.e., 0.51 and 0.49 g 1,3-propanediol per g of pure and grade A-2 raw glycerol, respectively. The kinetic parameters for 1,3-propanediol formation and glycerol consumption in anaerobic fed-batch cultures did not differ significantly, thus suggesting the feasibility of pre-treated sunflower oil bio-diesel raw glycerol fermentation to 1,3-propanediol.

Key words: Bio-diesel, free fatty acids, heavy metals, fed-batch culture, glycerin

INTRODUCTION

Glycerol (glycerin) is usually released as a by-product of oil and fat saponification. However, nowadays, bio-diesel production on a large commercial scale has led to an accumulation of surplus raw glycerol in the world markets. With the production of 10 kg of bio-diesel, 1 kg of glycerol is produced (Meesters *et al*., 1996). This resulted in a very sharp decline in the raw glycerol prices over the past two years i.e., from about 25 cents lb^{-1} to about 2.5 cents lb^{-1}, a 10 times reduction and causing Dow Chemicals and Procter and Gamble Chemicals to shut down their glycerol production plants (Yazdani and Gonzalez, 2007), thus attracting researchers attention to convert this feed stock to high-value added commodity chemicals. Glycerol bio-conversion to other commodity and specialty chemicals is also essential to increase and maintain the economic viability of the bio-diesel production process using vegetable oils.

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Glycerol has been subjected to its bioconversion into propionic acid, succinic acid, butanol, ethanol, formate, hydrogen as well as 1.3-propanediol (1,3-PD) (Yazdani and Gonzalez, 2007). However, special attention has been paid to the microbial production of 1,3-PD from glycerol as this diol finds its applications in the production of new polymers, especially PTT, with enhanced biodegradable properties (Zeng and Biebl, 2002). Certain bacterial groups ferment glycerol to 1,3-PD. These include species of *Klebsiella*, *Citrobacter*, *Enterobacter* (Cheng *et al*., 2007; Barbirato *et al*., 1998), *Lactobacillus* (Shutz and Radler, 1984) and *Clostridium* (Gonzalez-Pajuelo *et al*., 2005).

Although the utilization of raw glycerol in fermentation offers a remarkable advantage against the use of pure glycerol, majority of the studies for microbial 1,3-PD production have been conducted using pure glycerol (Biebl *et al*., 1999). In fact, only a very few reports have recently investigated the potentials of raw glycerol, issued from biodiesel production, for 1,3-PD biosynthesis (Papanikolaou *et al*., 2007; Mu *et al*., 2006).

Clostridium butyricum DSM 5431 could not grow at all in the presence of raw glycerol (Petitdemange *et al*., 1995). Due to the presence of certain impurities such as soap, methanol and free fatty acids (FFAs) in considerable amounts; raw glycerol issued from the biodiesel production using sunflower oil goes unfit for microbial growth and fermentation. We could successfully develop a pre-treatment process in our laboratories, recently, to get rid of most of the inhibitory substances present in the raw glycerol. It is, thus, the purpose of this paper to report the utilization of this pre-treated raw glycerol for growth and 1,3-PD production by *C. butyricum* DSM 5431. Growth inhibition experiments using various grades of pre-treated raw glycerol were conducted and the data was compared to that of pure glycerol and discussed. To our knowledge, there is no report on the utilization of pre-treated sunflower oil biodiesel raw glycerol for the growth and 1,3-PD formation by *C. butyricum* DSM 5431.

MATERIALS AND METHODS

The present study was conducted in the premises of Life Sciences and Bioengineering laboratories, Graduate School of Life and Environmental Sciences, University of Tsukuba, Japan during the year 2007.

Biodiesel Preparation

A methanol; sunflower oil molar ratio of 10:1 was mixed together at 400 rpm along with 0.5% (w/w) of NaOH, at 60°C, for 1 h. These conditions were already optimized in our laboratories (Bambase *et al*., 2007) and the process for efficient biodiesel production from sunflower oil has recently been scaled up to a pilot plant.

Solvent Washing Process for Pre-Treated Raw Glycerol

A pre-treatment process for raw glycerol from bio-diesel production using sunflower oil has recently been developed in our laboratories, successfully. The characterization of the various grades of raw glycerol, developed in consequence of the pre-treatment process, is shown in Table 1. The grade

Table 1: Characterization of various grades of pre-treated sunflower oil bio-diesel raw glycerol (SOB-RG)

SOB-RG grades	pH[a]	Soap[a] % (w/w)	Glycerol[b] % (w/w)	Methanol[b] % (w/w)
A[d]	4.0	n.d[c]	90	<1.0
B[d]	5.0	1.0	88	1.0
C[d]	6.0	3.8	85	1.5
D[d]	7.0	9.3	75	1.0

[a]Before methanol distillation, [b]After methanol distillation, [c]Not detected, [d]Raw glycerol after pre-treatment

A raw glycerol was washed three times with either n-hexanol or n-hexane at 300 rpm for 15 min at room temperature and, consequently, three further grades of n-hexanol or n-hexane washed grade A raw glycerol, depending upon the ratio (w/w) of n-hexanol or n-hexane: grade A raw glycerol, were obtained; when grade A raw glycerol was washed one time with n-hexanol (n-hexanol: grade A raw glycerol, 1:1), it was called grade A-1 raw glycerol. This grade A-1 raw glycerol was again washed with n-hexanol (n-hexanol: grade A-1 raw glycerol, 1:2) and called grade A-2 raw glycerol. When grade A-2 raw glycerol was given a third washing with n-hexanol (n-hexanol: grade A-2 raw glycerol, 1:4), grade A-3 raw glycerol was obtained. The n-hexanol in each washing was recovered by vacuum distillation and used in subsequent washings. Likewise, when grade A raw glycerol was washed one, two and three times with n-hexane, it was called grade AH-1, AH-2 and AH-3, respectively.

Microorganism and Culture Maintenance

Clostridium butyricum DSM 5431, purchased from the German collection of microorganisms (DSMZ) Germany, was used in the present investigation. The spores of the strain were obtained by growing vegetative cells on milk agar slants (Biebl, 1991). Spore suspensions of the strain were maintained on Reinforced Clostridial Medium (RCM, Difco) in Hungate tubes, at 4°C. The spores were heat shocked at 80°C for 10 min, prior to inoculation.

Growth Inhibition Experiments

Growth inhibition experiments were conducted in Hungate tubes, with butyl rubber septa for syringe operation. All Hungate tubes were flushed with 99.99995% pure nitrogen for anaerobiosis, prior to autoclaving at 121°C for 15 min. All tubes were filled with 9 mL RCM. The experimental tubes were supplemented with either pure or various grades of pre-treated sunflower oil biodiesel raw glycerol (SOB-RG) at 20, 40, 60, 80 and 100 g glycerol L^{-1}. The control tubes were devoid of glycerol. The initial pH in all tubes was 7.0. The pre-culture for growth inhibition experiments was also grown in Hungate tubes containing RCM with out glycerol. All experimental and control tubes were inoculated with 1 mL exponential phase pre-culture under anaerobic conditions and incubated at 32°C, without agitation. About 200 μL samples were withdrawn out of each tube after 6 h interval, with the aid of a syringe, for optical density measurements. The growth inhibition percentage was determined from the following formula:

$$\frac{(OD_{c\,(t=6h)} - OD_{c\,(t=0)}) - (OD_{Ei\,(t=6h)} - OD_{Ei\,(t=0)})}{(OD_{c\,(t=6h)} - OD_{c\,(t=0)})} \times 100$$

Where:

OD = Optical density

c = Control

Ei = Experimental (i = 1, 2…., x.).

Fermentation Experiments

The pre-cultures for fermentation experiments were grown in 100 mL screw capped bottles with rubber septa for syringe operation at 32°C and without agitation, overnight. The bottles were filled with 50 mL pre-boiled medium and sealed under nitrogen (99.99995% pure) followed by autoclaving at 121°C for 15 min. The medium contained (per litre deionized water): pure glycerol (99% w/w) 20 g, K_2HPO_4 3.4 g, KH_2PO_4 1.3 g, $(NH_4)_2SO_4$ 2 g, $MgSO_4.7H_2O$ 0.2 g, $CaCl_2.2H_2O$ 0.02 g, $FeSO_4.7H_2O$ 5 mg, $CaCO_3$ 2 g, yeast extract 1 g, trace element solution (Biebl and Pfenning, 1981) SL_7 2 mL. When this medium was used for pH controlled fed-batch cultures, the phosphate concentration was reduced to K_2HPO_4 1 g, KH_2PO_4 0.5 g and $CaCO_3$ was omitted. All chemicals (excluding yeast extract, Difco) were purchased from Wako Chemicals, Japan. A pre-culture at an

exponential phase was used as an inoculum. The initial concentration of pre-treated SOB-RG or pure glycerol for fed-batch cultures was 50 g L^{-1}. A 1.5 L magnetically stirred bioreactor (Biochemical Engineering Marubishi, B.E.M, Japan) with a working volume of 1 L was employed. The reactor was filled with the medium, autoclaved at 121°C for 15 min and sparged with a 99.9995% pure nitrogen until the oxidation-reduction potential (ORP) of the medium was declined to -150 mv. The pH was maintained at 7.0 through out the course of fermentation by automatic addition of 4 mol L^{-1} KOH solution. The incubation temperature was kept at 32°C. About 5 mL samples were withdrawn periodically and analyzed for growth and 1,3-PD production, as well as glycerol consumption.

Analytical Techniques

The optical density of both Hungate tube and batch culture was measured at 650 nm using a Uv/Vis spectrophotometer (Amersham Bioscience, England). The vacuum distillation of methanol was carried out using an Eyela (Tokyo, Japan) NE-2001 rotary evaporator operated at 165 hPa. Methanol, 1,3-PD and glycerol were analyzed by a gas chromatograph GC 14A (Shimadzu, Japan) installed with a 2.1 m long glass column (i.d, 3.2 mm) packed with chromosorb 101 (80-100 mesh) and flame ionization detector (FID). The injector and detector temperatures were 200 and 240°C, respectively. The column temperature was 200°C. Glycerol appeared at the end as a broad peak allowing a rough estimation of this compound. Therefore, glycerol was further analyzed by the enzymatic test kit (Boehringer Mannheim, Germany) according to the instructions of the test kit manufacturer. Soap contents of various grades of raw glycerol were analyzed by colorimetric titration using bromophenol blue as an indicator and 0.1 mol L^{-1} HCl as a titrant. For FFAs analysis, the samples were submitted to Environmental Research Center (http: www.erc-net.com), Tsukuba science city, Japan. Metals and heavy metals were analyzed by inductively coupled argon plasma atomic emission spectrophotometer (ICAP-757v, Nippon Jarrell-Ash, Japan).

Statistical Analysis

The statistical analysis was conducted by MSTAT-C software (version 1.3). The comparison was done at 0.01 level.

RESULTS AND DISCUSSION

Growth Inhibition of *Clostridium butyricum* DSM 5431

The growth inhibition results of *C. butyricum* DSM 5431 at various concentrations of pure and grade A and B raw glycerol were almost similar i.e., 18.5±0.707 to 20.5±0.7% inhibition, at 20 g glycerol L^{-1} (Fig. 1). This is in consistent with the results of Gonzalez-Pajuelo *et al*. (2004). However, growth inhibition was very profound (85.25±0.35 to 112.5±3.5% inhibition) when the glycerol concentration was between 40 and 100 g L^{-1} in all grades of pre-treated SOB-RG. These results deviated from those of the previous workers (Mu *et al*., 2006). Raw glycerol often contains sodium and heavy metals ions which may interfere with cell division, consequently reducing the cell viability (Gonzalez-Pajuelo *et al*., 2004). Growth of *C. butyricum* was inhibited at 12 g sodium ions L^{-1} (Homann *et al*., 1990). Lead (Pb) interfered with cell growth and glucose fermentation by *C. butyricum* at 25 mg L^{-1} (Francis and Dodge, 1987). Nickel also inhibited the cell growth (Keeling and Cater, 1998). It is worth pointing out, however, that the increasing levels of percentage inhibitions at increasing concentrations of various grades of pre-treated SOB-RG should not be attributed to the metal and heavy metal ions, such as sodium, lead and nickel, as the concentration of these ions never exceeded 0.068, 1.50 and 3.0 mg L^{-1}, respectively in all experiments. The other heavy metal ions (Cd, Fe, Al, Cr, Cu etc.) were either absent or present below the detection limits of ICAP.

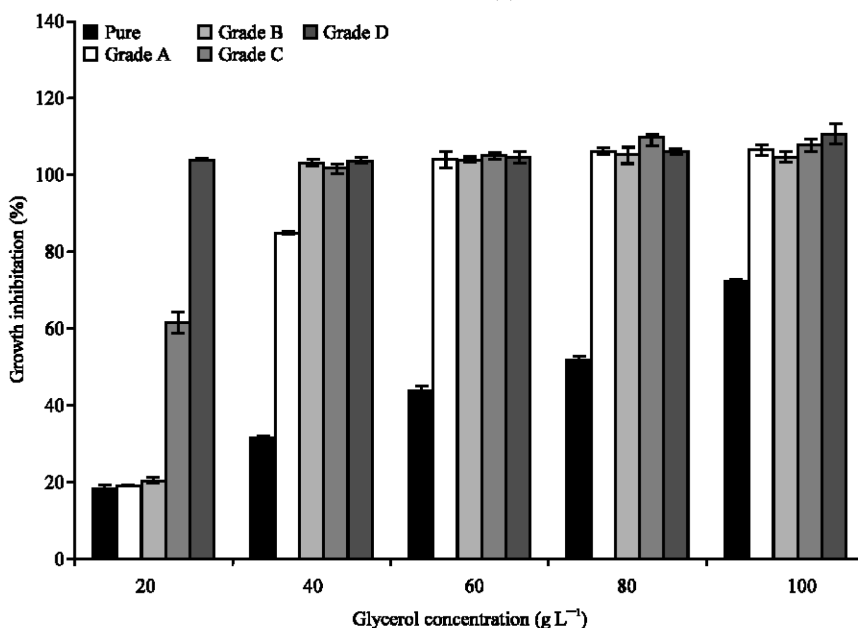

Fig. 1: The effect of different grades of pre-treated raw glycerol on the growth inhibition of *Clostridium butyricum* DSM 5431 at various concentrations. Culture conditions: incubation temperature 32°C; initial pH 7.0. Determinations were carried out in duplicates

At low pH (grade A and B), the increased inhibitory effect might be due to the presence of FFAs, mainly released during soap splitting, in concentration high enough to inhibit bacterial growth. The various FFAs and their relative percentages (w/w) in the raw glycerol were; linolenic acid, 0.14% < stearic acid, 3.85% < palmitic acid, 5.46% < oleic acid, 39.1% < linoleic acid, 51.44%. The saturated FFAs (palmitic acid and stearic acid) and linolenic acid could be removed from raw glycerol by simple decantation; however, oleic acid and linoleic acid could not be recovered completely during decantation and certain proportions of these unsaturated FFAs remained in the acid-treated raw glycerol. Saturated FFAs do not usually interfere with bacterial growth, however, unsaturated FFAs (particularly oleic acid and linoleic acid) have profound influence on the viability of bacterial cells (Furusawa and Koyama, 2004). Remarkable decreases of viability and morphological changes such as loss of cell shape and disruption of cell membrane were observed in bacteria exposed to unsaturated FFAs (Hazell and Graham, 1990; Khulusi *et al*., 1995; Knapp and Melly, 1986). The growth of bacteria used to assimilate FFAs from vegetable oils was also restricted by unsaturated fatty acids, particularly linoleic acid (Rodrigues *et al*., 2007). At pH 6.0 (grade C) and 7.0 (grade D), the inhibitory effect might be due to the presence of high concentrations of soap, 3.8 and 9.3% (w/w), respectively and FFAs, acting simultaneously. Furusawa and Koyama (2004) reported that the viability in bacteria was completely lost on exposure to oleic acid and linoleic acid at about 8.5 mg L^{-1}, due to the instantaneous depolarization of cell membrane potential. In the present investigation, however, it was observed that *C. butyricum* DSM 5431 could tolerate oleic acid at 18.4 mg L^{-1} and linoleic acid at 32.2 mg L^{-1} and did not completely loose the cell viability i.e., when the grade A acid-treated raw glycerol was used at 40 g glycerol L^{-1}, presenting 85.25±0.35% growth inhibition which is still less pronounced as compared to 103.5±0.70%, 102±1.14% and 104.5±0.70% growth inhibition on grade B, C and D raw glycerol, respectively. The mechanism operating behind the ability of the present strain to retain cell viability at such a high concentration of oleic acid and linoleic acid is unknown.

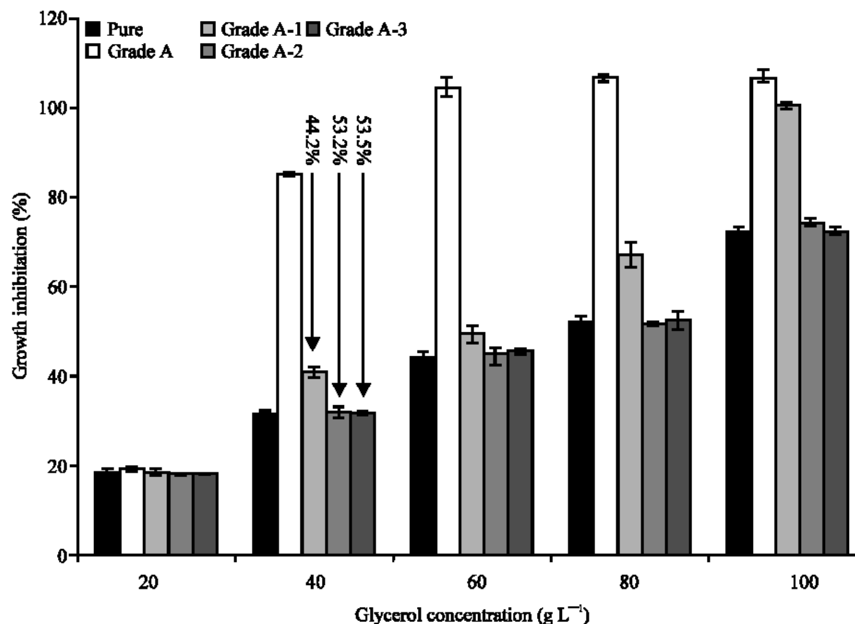

Fig. 2: The effect of subsequent washings of grade A raw glycerol with n-hexanol on the growth inhibition of *Clostridium butyricum* DSM 5431 at various concentrations. The n-hexanol: grade A raw glycerol (w/w) ratio was; (i) First washing, 1:1 (Grade A-1 raw glycerol); (ii) Second washing, 1:2 (Grade A-2 raw glycerol); (iii) Third washing, 1:4 (Grade A-3 raw glycerol). Culture conditions as in Fig. 1. Determinations were carried out in duplicates

The grade A raw glycerol, being most tolerated by the bacterium, was given subsequent washings with n-hexanol and the results of the effects of various further grades of n-hexanol washed grade A raw glycerol on the bacterial growth are presented in Fig. 2. When grade A-1 and A-2 raw glycerols were employed, a remarkable reduction in percentage inhibition was observed between the glycerol concentrations of 40 to 100 g L^{-1} e.g., at 40 g glycerol L^{-1}, a 44.2 and 53.2% reduction in the growth inhibition was observed when grade A-1 and grade A-2 raw glycerols were used, respectively. The dramatic reduction in growth inhibition percentage of the bacterium might be attributed to the mitigation of the inhibitory effects pertaining to a reduction in the concentrations of oleic acid and linoleic acid from 46 mg and 80.5 mg per 100 g of glycerol, respectively, to non-detectable levels, hence alleviating the growth inhibition. However, employing grade A-3 raw glycerol did not improve the growth inhibition percentage, significantly (53.5% reduction in growth inhibition).

When n-hexane washed raw glycerol was employed, a 35.1, 42.9 and 43.7% reductions in the growth inhibition of *C. butyricum* DSM 5431 were observed at grade AH-1, AH-2 and AH-3 raw glycerols, respectively, when the glycerol concentration was 40 g L^{-1} (Fig. 3). These reductions were significantly lower as compared to n-hexanol washed raw glycerols, as described, hence suggesting the optimization of n-hexanol washed i.e., grade A-2, raw glycerol for bioreactor studies.

A Comparative Kinetic Study in Anaerobic Fed-batch Cultures of *Clostridium butyricum* DSM 5431

In order to extend the fermentation knowledge of *C. butyricum* DSM 5431 grown on pre-treated SOB-RG, anaerobic fed-batch cultures were conducted employing pure and grade A-2 raw glycerols

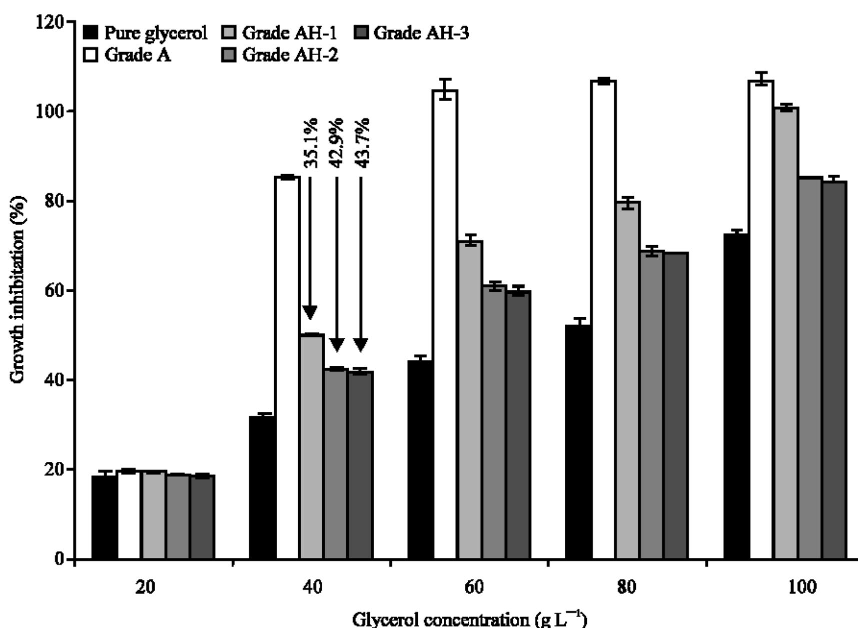

Fig. 3: The effect of subsequent washings of grade A raw glycerol with n-hexane on the growth inhibition of *Clostridium butyricum* DSM 5431 at various glycerol concentrations. The n-hexane: grade A raw glycerol (w/w) ratio was; (i) First washing, 1:1 (Grade AH-1 raw glycerol); (ii) Second washing, 1:2 (Grade AH-2 raw glycerol); (iii) Third washing, 1:4 (Grade AH-3 raw glycerol). Culture conditions as in Fig. 1. Determinations were carried out in duplicates

and the data was analyzed and compared, kinetically. Fed-batch cultures were started as batch cultures with either pure or grade A-2 raw glycerol at an initial concentration of 50 g glycerol L^{-1}. The time of complete glycerol consumption was accompanied by a sudden decline in the ORP (oxidation reduction potential) of the culture i.e., from -620 mv to -550 mv in 15 min. This was used as a signal to start the glycerol feeding so that the glycerol concentration in the culture becomes 20 g L^{-1}. In this way a total of 110 g glycerol L^{-1} was supplied in three consecutive intermittent (pulse) additions. The glycerol consumption was incomplete and 42 and 45 g L^{-1} of 1, 3-PDO was accumulated in the fermented broth at the expense of 85 and 87.8 g L^{-1} of pure and grade A-2 raw glycerol, respectively. This incomplete glycerol utilization might be attributed to a change in the C/N ratio due to exhaustion of the nutrients before the complete utilization of glycerol, given that the concentrations of product (1,3-PDO) and by-products such as acetic acid and butyric acid remained below the inhibitory level. The inhibitory levels for 1, 3-PDO, acetic acid and butyric acid for clostridia are 64, 27 and 19 g L^{-1}, respectively, at pH 6.5 (Biebl, 1991). The complete consumption of glycerol achieved by Gunzel *et al.* (1991) in a fed batch culture could be attributed to using 5 g $(NH_4)_2SO_4$/100 g of glycerol, however, in our case it was 2 g/100 g glycerol.

Table 2 presents the comparison of various kinetic parameters for 1,3-PD formation and glycerol consumption. No significant differences were observed between pure and grade A-2 raw glycerols in terms of volumetric rate ($Q_{1,3\text{-PD}}$), yield ($Y_{p/s}$ and $Y_{p/x}$) and specific rate of formation ($q_{1,3\text{-PD}}$) of 1,3-PDO. Similar was the case with kinetic parameters for glycerol consumption, hence suggesting

Table 2: Kinetic parameters for 1,3-PD formation and consumption of pure and grade A-2 raw glycerol in anaerobic fed-batch cultures of *Clostridium butyricum* DSM 5431

	Glycerol types	
Kinetic parameters	Pure	Grade A-2
Specific growth rate		
μ (h^{-1})	0.158	0.155
1, 3-propanediol formation parameters		
Q_p (g Lh^{-1})	2.14	2.00
$Y_{p/s}$ (g g^{-1})	0.51	0.49
$Y_{p/x}$ (g g^{-1})	15.00	15.00
q_p (g gh^{-1})	2.37	2.32
Glycerol consumption parameters		
Q_s (g Lh^{-1})	0.03	0.03
$Y_{x/s}$ (g g^{-1})	4.17	4.04
q_s (g gh^{-1})	4.62	4.70

Culture conditions: incubation temperature 32°C; pH 7.0. Determinations were carried out in duplicates

the suitability of the pre-treated sunflower oil biodiesel raw glycerol (SOB-RG) for bioconversion to a specialty chemical i.e., 1, 3-PDO, by *Clostridium butyricum* DSM 5431.

CONCLUSION

The production of 1,3-PD by *C. butyricum* DSM 5431 using pre-treated SOB-RG as a sole carbon and energy source was feasible. The critical problem in the pre-treatment of SOB-RG i.e., the inability of *C. butyricum* DSM 5431 to withstand the inhibitory effects of high concentrations of oleic acid and linoleic acid was lucratively trounced by two washings of grade A raw glycerol with a suitable alcohol i.e., n-hexanol (grade A-2 raw glycerol). Although the use of n-hexanol during solvent washing process could be feasible on a semi-pilot scale due to its recovery by vacuum distillation, it should be pointed out, however, that the economic viability of the pre-treated SOB-RG fermentation to 1,3-PD on an industrial scale will rely mainly upon the costs of n-hexanol and its distillation, which are relatively higher. Therefore, we propose that some potential alternative methods, to purge SOB-RG of its FFAs, such as in-situ assimilation of FFAs by other microorganisms, e.g. *Pseudomonas* sp. or enzymatic de-acidification, as used for vegetable oils (Cho *et al*., 1990; Bhosle and Subramanian, 2005), should be investigated to envisage an industrial process for the utilization of pre-treated SOB-RG for efficient 1,3-propanediol production.

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REFERENCES

Bambase, M.E., N. Nakamura, J. Tanaka and M. Matsumura, 2007. Kinetics of hydroxide-catalyzed methanolysis of crude sunflower oil for the production of fuel-grade methyl esters. J. Chem. Technol. Biotechnol., 82: (3): 273-280.

Barbirato, F., E.H. Himmi, T. Conte and A. Bories, 1998. 1,3-propanediol production by fermentation: An interesting way to valorize glycerin from the ester and ethanol industries. Ind. Crop. Prod., 7 (2-3): 281-289.

Bhosle, B.M. and R. Subramanian, 2005. New approaches in de-acidification of edible oils- a review. J. Food. Eng., 69 (4): 481-494.

Biebl, H. and N. Pfenning, 1981. Isolation of Members of the Family Rhodospirillaceae. In: The Prokaryotes: A Handbook on Habitats, Isolation and Identification of Bacteria, Starr, M.P., H. Stolp, H.G. Trüper, A. Balows and H.G. Schlegel (Eds.). Springer, Berlin Heidelberg New York, pp: 267-273.

Biebl, H., 1991. Glycerol fermentation to 1,3-propanediol by *Clostridium butyricum*: Measurement of product inhibition by use of a pH auxostat. Applied Microbiol. Biotechnol., 35 (6): 701-705.

Biebl, H., K. Menzel., A.P. Zeng and W.D. Deckwer, 1999. Microbial production of 1,3-propanediol. Applied Microbiol. Biotechnol., 52 (3): 289-297.

Cheng, K.K., J.A. Zhang, D.H. Liu, Y. Sun, H.J. Liu, M.D. Yang and J.M. Xu, 2007. Pilot-scale production of 1,3-propanediol using *Klebsiella pneumoniae*. Process Biochem., 42 (4): 740-744.

Cho, S.Y., T.W. Kwon and S.H. Yoon, 1990. Selective removal of free fatty acids in oils using a microorganism. J. Am. Oil. Chem. Soc., 67 (9): 558-560.

Francis, A.J and C.J. Dodge, 1987. Effects of lead oxide and iron on glucose fermentation by *Clostridium* sp. Arch. Environ. Contam. Toxicol., 16 (4): 491-497.

Furusawa, H. and N. Koyama, 2004. Effect of fatty acids on the membrane potential of an alkaliphilic *Bacillus*. Curr. Microbiol., 48 (3): 196-198.

Gonzalez-Pajuelo, M., J.C. Andrade and I. Vasconcelos, 2004. Production of 1,3-propanediol by *Clostridium butyricum* VPI 3266 using a synthetic medium and raw glycerol. J. Ind. Microbiol. Biotechnol., 31 (9): 442-446.

Gonzalez-Pajuelo, M., J.C. Andrade and I. Vasconcelos, 2005. Production of 1,3-propanediol by *Clostridium butyricum* VPI 3266 in continuous cultures with high yield and productivity. J. Ind. Microbiol. Biotechnol., 32 (9): 391-396.

Gunzel, B., S. Yonsel and W.D. Deckwer, 1991. Fermentative production of 1,3-propanediol by *Clostridium butyricum* up to a scale of 2 m^3. Applied Microbiol. Biotechnol., 36 (3): 289-294.

Hazell, S.L. and D.Y. Graham, 1990. Unsaturated fatty acids and viability of *Helicobacter* (*Campylobacter*) *pylori*. J. Clin. Microbiol., 28 (5): 1060-1061.

Homann, T., C. Tag, H. Biebl, W.D. Deckwer and B. Schink, 1990. Fermentation of glycerol to 1,3-propanediol by *Klebsiella* and *Citrobacter* strains. Applied Microbiol. Biotechnol., 33 (2): 121-126.

Keeling, A.A. and G.L.F. Cater, 1998. Toxicity of copper, lead nickel and zinc in agar culture to aerobic diazotrophic bacteria extracted from waste derived compost. Chemosphere, 37 (6): 1073-1077.

Khulusi, S., H.A. Ahmed, P. Patel, M.A. Mendall and T.C. Northfield, 1995. The effect of unsaturated fatty acids on *Helicobacter pylori in vitro*. J. Med. Microbiol., 42 (4): 276-282.

Knapp, H.R. and H.A. Melly, 1986. Bactericidal effects of polyunsaturated fatty acids. J. Infect. Dis., 154 (1): 84-94.

Meesters, P.A.E.P., G.N.M. Huijberts and G. Eggink, 1996. High cell density cultivation of the lipid accumulating yeast *Cryptococcus curvatus* using glycerol as a carbon source. Applied Microbiol. Biotechnol., 45 (5): 575-579.

Mu, Y., H. Teng, D.J. Zhang, W. Wang and Z.L. Xiu, 2006. Microbial production of 1,3-propanediol by *Klebsiella pneumoniae* using crude glycerol from biodiesel preparations. Biotechnol. Lett., 28 (21): 1755-1759.

Papanikolaou, S., S. Fakas, M. Fick, I. Chevalot, M. Goliotou-Panayotou, M. Komaitis, I. Marc and G. Aggelis, 2007. Biotechnological valorization of raw glycerol discharged after bio-diesel (fatty acid methyl esters) manufacturing process: Production of 1,3-propanediol, citric acid and single cell oil. Biomass. Bioenergy., doi:10.1016/j.biombioe.2007.06.007.

Petitdemange, E., C. Durr, S. Abbad-Andaloussi and G. Raval, 1995. Fermentation of raw glycerol to 1,3-propanediol by new strains of *Clostridium butyricum*. J. Ind. Microbiol. Biotechnol., 15 (6): 498-502.

Rodrigues, C.E.C., C.B. Goncalves, E. Batista and A.J.A. Meirelles, 2007. Deacidification of vegetable oils by solvent extraction. Rec. Patents. Eng., 1 (1): 95-102.

Shutz, H. and F. Radler, 1984. Anaerobic reduction of glycerol to 1,3-propanediol by *Lactobacillus brevis* and *Lactobacillus buchneri*. Syst. Applied Microbiol., 5 (2): 169-178.

Yazdani, S.S. and R. Gonzalez, 2007. Anaerobic fermentation of glycerol: A path to economic viability for the biofuels industry. Curr. Opin. Biotechnol., 18 (3): 213-219.

Zeng, A.P. and H. Biebl, 2002. Bulk chemicals from biotechnology: the case of 1,3-propanediol production and the new trends. Adv. Biochem. Eng. Biotechnol., 74: 239-259.