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Unveiling the potential of a novel alkalophilic *Citrobacter freundii* IIPDR3 strain in valorizing glycerol to 1,3-propanediol

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Abstract

Glycerol-based biotechnological production of 1, 3-propanediol (PDO) has taken center stage after its recent commercialization by Metabolic Explorer. The present study unveils the potential of a novel alkalophilic, BSL-1 bacterium *Citrobacter freundii* IIPDR3 to produce 1,3-PDO from glycerol. The culture conditions were optimized at shake flask level using one variable at a time (OVAT) approach and statistically significant parameters were identified. The optimal conditions for batch production of 1,3-PDO were: phosphate buffered glycerol-based medium essentially containing 1.5 gL^{-1} ammonium sulfate, 3 gL^{-1} yeast extract, and 0.5 gL^{-1} magnesium sulphate; temperature: 36°C; pH-stat of 8.0 and inoculum size being ≥ 35 $\mu g_{bacterial\ cells}$/ml on dry cell weight (DCW) basis. Incorporation of 0.2% Tween-80 in the fermentation medium, improved glycerol uptake rate and prevented self-flocculation of bacterial cells. Using liquor NH_3 as a neutralizing agent, *C. freundii* IIPDR3 produced a maximum of 18.04 ± 0.76 gL^{-1} 1, 3-PDO from pure glycerol, with productivity and yield being 1.72 $gL^{-1}h^{-1}$ and 0.53gg^{-1}, respectively. When the robustness of the strain was tested on crude glycerol (CG), it accumulated 9.65 and 11.11gL^{-1} 1,3-PDO in 7h and 10.5h with initial glycerol concentrations of 20 and 40gL^{-1}, respectively. The study confirms that *C. freundii* IIPDR3 can be a promising candidate to tackle CG, a prominent waste derived from biodiesel industry, and simultaneously upgrade it to valorized products such as 1,3-PDO.

Key words: *Citrobacter freundii* IIPDR3; pure glycerol; 1,3-Propanediol; Crude glycerol; Batch-fermentation

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1. Introduction

Globally, the need for sustainable and carbon-neutral manufacturing has been realized. As a result, unprecedented growth of the bio-based chemicals market is anticipated. Worldwide, governments are promoting and incentivizing technical innovations that get translated into green products, paving the way towards creating a circular economy. Furthermore, the processes become more lucrative, when they use inexpensive, abundant, and renewable carbon feedstock, and a value chain is created by producing "platform" chemicals, which ensure persistent high market demand. Crude glycerol (CG) represents one such industrial feedstock that has revived research interests in its valorization, owing to the increasing market for biodiesel [1]. As per the report of Oils and Fats International (OFI), the global biodiesel production in 2023 was 70.96 million tons, with the US, Indonesia, and the European Union being the major producers [2]. Generally, the transesterification reaction yields glycerol and biodiesel in a volumetric ratio of 10:1, implying that for every 1 m^3 of biodiesel, 0.1 $m^3$of crude glycerol (CG) is generated [3]. As a result, 7.09 million tons of CG was generated globally in 2023. Considering this data, shortly alternate strategies for the simultaneous management of CG and its valorization would become indispensable, besides its conventional use. CG upgradation to value-added products can be done through various technological platforms: thermochemical, biochemical, physic-chemical, and electrochemical [1, 4-6].

In general, microbial transformations are poised as green and environmentally benign processes with selective product formation under ambient conditions of temperature and pressure, offering distinct advantages over other technological platforms. Positive market sentiments towards sustainable alternatives have fueled the growth of biotechnological interventions to produce renewable fuels and chemicals. Through the microbial route, glycerol can be valorized into an array of value-added products including1,3-propanediol (1,3-PDO) as reviewed extensively [4].1,3-PDO also known as trimethylene glycol, is versatile platform chemical, important organic solvent, and a chemical building block. It is used as an intermediate in the manufacturing of polyesters, polyether, and polyurethanes and has wide applications in the food, cosmetics, and pharmaceutical industries. It acts as a monomer in polytrimethylene terephthalate (PTT) synthesis, whose global market in 2022 was USD 948.44 million, a key driver responsible for the sudden upsurge in the 1,3-PDO demand [7]. Moreover, 1,3-PDO-derived polyesters have better stretching and stretch-recovering, lower dyeing temperature, higher UV resistance, and better wash-fastness than other commercially available polyesters [8].

Fermentative production of glycerol-based 1,3-PDO is reigned by bacteria, which can efficiently metabolize it under oxygen-limited/anaerobic conditions. In a two-step reductive pathway, glycerol is first dehydrated to 3-hydroxy propionaldehyde (3-HPA) in the presence of glycerol dehydratase (vitamin B_{12} dependent or independent). Later, NAD(P)H dependent 1,3-PDO reductase, catalyzes the reduction of 3-HPA to1,3-PDO. This pathway has a physiological role of regenerating NAD^+, especially under oxygen-limited/anaerobic conditions and 1,3-PDO is obtained as the end product of the fermentative pathway. Presently, France-based Metabolic Explorer is the only commercial player that produces glycerol-based 1,3-PDO [9]. Cradle-to-gate analysis reveals that the greenhouse gas (GHG) emissions (kg CO_2 eq. /kg) of 100% bio-sourced 1,3-PDO(Brand Name: TILAMAR® PDO) from non-GMO rapeseeds is 27% less than synthetic 1,3-PDO [10]. DSM further claims that 1.7 kg CO_2 eq/kg biogenic carbon uptake credit is associated with this product, suggesting low-carbon manufacturing of TILAMAR® PDO. In light of this evidence, glycerol-derived 1,3-PDO production via microbial route has once again seen an upsurge. Therefore, various biotechnological approaches such as microbial bioprospecting and process intensification (PI) during fermentation have gained attention [3].

In the present study, the potential of a novel glycerol assimilating bacterium *Citrobacter freundii* IIPDR3 strain was explored for the production of 1,3-PDO, as described previously [11]. Batch studies were conducted at shake flask level

with this Biosafety level 1 (BSL-1) bacterium, using one variable at a time (OVAT) approach, to understand the metabolic behavior of *C. freundii* IIPDR3. Various media components (such as organic and inorganic nitrogen source, magnesium salt, and use of co-substrates) and physicochemical parameters (such as pH and temperature) were optimized along with the selection of the best neutralizing agent. After complete optimization, a paired t-test was performed to identify parameters that exhibited a statistically significant correlation with glycerol consumption and 1,3-PDO production. In the concluding part, the robustness of the strain was tested for CG valorization. This study becomes more pertinent as the *C. freundii* IIPDR3 grows well under alkaline conditions and CG generally displays a pH above 7.0, owing to the usage of alkaline catalysts during transesterification reaction [12].

2. Materials and Methods

2.1. Chemicals

All the chemicals and media components used in this study were of analytical or laboratory grade either procured from Sigma Aldrich, USA, or Hi-Media Laboratories (Mumbai, India). Corn steep powder (CSP) was obtained from two different sources (a) Roquette Limited, France, and (b) Hexon Industries, Nashik, India. They were referred to as CSP-R and CSP-H, respectively. CG was gifted by the Chhattisgarh Biofuel Development Authority (CBDA) from their Jatropha oil-based biodiesel plant, with glycerol content being 42.14 ± 1.2%.

2.2. Bacterial strain

C. freundii IIPDR3 was obtained from the soil samples of the solvent storage site and fuel-contaminated area of CSIR-Indian Institute of Petroleum, Dehradun (India) while exploring glycerol assimilating soil microbiome [11]. The bacterium was preserved as glycerol stocks in a -80°C deep freezer (Thermoscientific ULT-2586) for long-term preservation. It was routinely revived in nutrient broth and maintained on nutrient agar plates as well.

2.3. Glycerol fermentation

The media used for glycerol fermentation in the present study contained (gL^{-1}): KH_2PO_4-0.26, Na_2HPO_4-2.17, $MgCl_2$-0.2, $CaCl_2$-0.025, $(NH_4)_2SO_4$-2.0, yeast extract (YE)- 2.0, Bromothymol blue-0.01 and glycerol -20.0, which was fortified with 2mlmineral salt (MS) solution. One liter of MS solution contained: 0.4 g Na_2EDTA, 0.34 g $MnSO_4.4H_2O$, 0.40 g $(NH_4)_6 Mo_7O_{24}.4H_2O$, 0.8 g $FeSO_4.7H_2O$, 0.04 g $CoCl_2.6H_2O$, 0.02 g $NiCl_2.6H_2O$, 0.02 g $ZnSO_47H_2O$. Bromothymol blue is a pH indicator dye which acted as a visual aid to observe drop in pH and helped in controlling pH during glycerol fermentation. Based on the earlier study carried out by Garg et al [11], the fermentation trials were conducted at 32°C, 160 rpm with pH maintained at 8.3 ± 0.2. When the dye color changed from blue-green (alkaline pH) to deep yellow (acidic pH), the pH was restored to optimum pH using 8N NaOH. Nutrient broth and CM-5 media served as pre-seed and seed cultures, respectively. The fermentation in the shake flask was performed by inoculating bacterial cells having an OD_{600nm} of 7.5 in 100 ml media which accounted for 35 ± 5 µg/ml cells on a dry weight basis.

2.4. Analytical methods

The cell-free supernatant obtained post-fermentation was analyzed for residual glycerol and metabolites including 1,3-PDO, lactate, and acetate. These metabolites were quantitatively analyzed using HPLC (Shimadzu Corporation, Japan) fitted with Aminex HPX-87H ion exclusion column (300 X 7.8 mm; 9-µm; Make-Biorad). The instrument was equipped with a refractive index detector (RID-10A; Shimadzu Corporation Japan) and an auto-injector facility (SIL-20AC HT, Shimadzu Corporation Japan). The column was isocratically eluted with 5.0 mM H_2SO_4, with the flow rate being 0.55 ml/min, while the column temperature was maintained at 55 °C (Wang et al.,2023). Samples were filtered through a 0.22 µm Millipore membrane before analysis. Calibration curves were drawn using pure glycerol, 1,3-PDO, acetic acid, lactic acid, ethanol, succinic acid, methanol, and sugars as standards (0.2–1.0 mg /ml). For determining dry cell weight (DCW) at the end of the fermentation (EOF), the fermentation medium was pelleted down (Dynamica

Velocity 18R; 6500 rpm; 5 min; 18°C) in a pre-weighed micro-centrifuge tube and dried at 60°C in a hot air oven till a constant weight was achieved.

2.5. Parameters considered for the OVAT approach

Optimization of all the parameters that could critically influence the biological valorization of glycerol to 1,3-PDO was done using the OVAT (One Variable at a Time) approach, by changing one factor at a time while keeping others constant. Some of these variables included: incubation temperature, surfactants, nitrogen salts, doping with co-substrate, influence of pH, yeast extract, magnesium salts, use of neutralizing agents, etc. All the experiments were performed in duplicates and the presented values are the average ± standard deviation. After optimizing all the variables using the OVAT approach, the strain was evaluated at three glycerol concentrations namely 20, 40, and 60 g L^{-1}. The study concluded by assessing the robustness of the strain by replacing PG with CG.

2.6. Statistical analysis

The correlation (whether positive or negative) between various factors and 1,3-PDO production including glycerol consumption was identified by performing paired T-test with α being 0.05. Generally, the term "α" refers to the significance level used in the hypothesis tests and the lower the value, the more statistically significant the correlation.

3. Results and discussion

3.1. Effect of incubation temperature on 1,3-PDO production

Incubation temperature is one of the most crucial operating parameters which not only governs bacterial growth but also facilitates substrate uptake and metabolite production. During glycerol fermentation, when the temperature studies were conducted with *C. freundii* IIPDR3 in the range of 28 to 40°C for a period of 10h, the following results were obtained as shown in Figure 1.

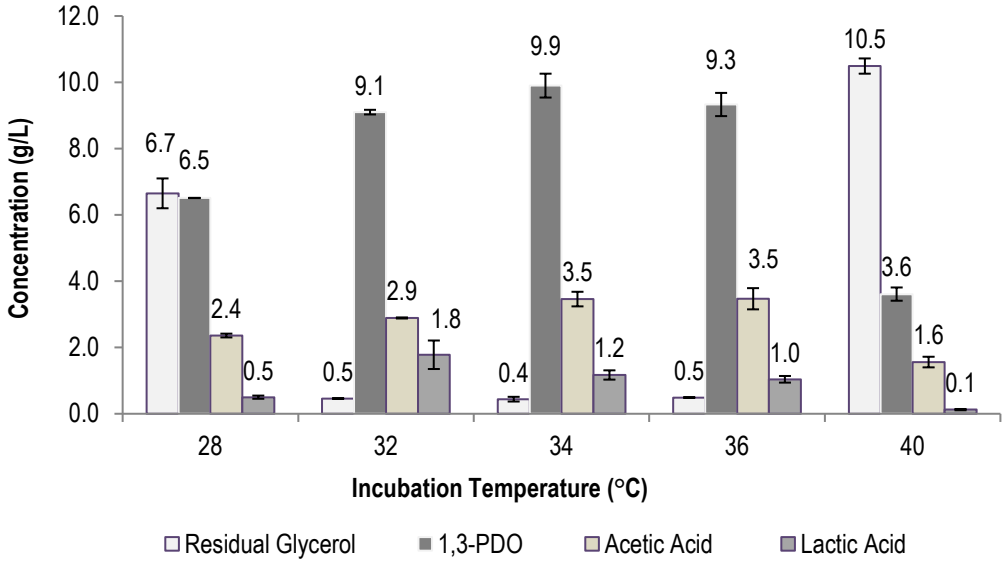


Figure 1: Effect of incubation temperature 1,3-PDO production from glycerol by *C. freundii* IIPDR3 after 10h fermentation

As observed in Figure 1, 32-36ºC was favorable, as it not only facilitated enhanced glycerol uptake but also resulted in maximum accumulation of 1,3 PDO. The 1,3-PDO and acetate concentrations were almost similar with a concomitant decrease in lactic acid titers. However, when the incubation temperature was raised from 36ºC to 40ºC, it adversely affected the glycerol consumption rate (GCR) indicating poor cell survival and resulting in poor product yields. Since the residual glycerol concentrations at 32, 34, and 36°C were <1gL^{-1}, it became difficult to adjudge the optimal temperature which resulted in maximum GCR and simultaneously displaying the highest 1,3-PDO productivity.

Hence, in the next experiment, fermentation was restricted to 8h only (data not shown) where 36ºC emerged as the best incubation temperature for *C. freundii* IIPDR3 with GCR and 1,3-PDO productivity being 2.17 and 1.05 $gL^{-1}h^{-1}$, respectively. Compared to 32°C and 34°C, both the values were 28% and 11-13% higher at 36°C. The present results are agreeable to the earlier study of Maervoet et al., [13] wherein they evaluated seven different strains of *Citrobacter* for 1,3-PDO production at 37°C, and *Citrobacter werkmanii* emerged as a potential candidate. Earlier, even Homann et al. [14] and Ainala et al., [15] have used 37°C for cultivating *Citrobacter freundii* and *Citrobacter amalonaticus* Y19 for biotransformation of glycerol to 1,3-PDO.

However, lately the performance of a few *Citrobacter* strains such as *C. freundii* VK19, *C. freundii* FMCC8, and *C. freundii* AD119, has been assessed on CG at a temperature of 30°C for 1,3-PDO production [16-19]. Thus, the current state of the art reveals that most of the glycerol assimilating and 1,3-PDO-producing strains belonging to genus *Citrobacter* display a temperature optimum in the range of 30-37°C for maximum growth and product formation.

3.2. Screening of surfactants

It was observed that as the cell density increased during glycerol fermentation, the self-flocculating properties of the bacterium led to cell settling. Hence, it became inevitable to screen various non-ionic surfactants that could inhibit cell agglomeration, promote cell growth, and simultaneously enhance GCR with increased product titers. When Tween 80, Tween 20, Polyethylene glycol 6000 (PEG), and Triton X-100 were screened at a concentration of 0.1%, the following results were obtained as demonstrated in Table 1.

Table 1: Effect of surfactant addition on the growth of *C. freundii* IIPDR3 and glycerol fermentation to 1,3-PDO (Initial glycerol: 20.65 ± 1.29 g L^{-1}; Temp: 36°C; Initial DCW: 34.82 ± 0.95 µg/ml; Duration: 9h)

Surfactant (0.1%)	Concentration (gL^{-1})			Dry cell weight (DCW µg/ml)	1,3-PDO productivity ($gL^{-1}h^{-1}$)
	Residual glycerol	1,3-PDO	Acetic Acid		
No surfactant	4.57 ± 0.15	7.49 ± 0.21	2.20 ± 0.07	1215 ± 35.36	0.83
Tween 80	3.78 ±0.19	8.60 ± 0.24	2.67 ± 0.12	1375 ± 21.21	0.96
Tween 20	4.20 ± 0.27	8.53 ± 0.68	2.78 ± 0.14	1350 ± 113.14	0.95
Triton X-100	4.88 ± 0.02	7.25 ± 0.26	2.59 ± 0.01	1350 ± 113.14	0.81
PEG 6000	5.81 ± 0.03	6.68 ± 0.27	2.10 ± 0.09	1240 ± 14.14	0.74

The visual observation during the said experiment confirmed that surfactant addition was effective in reducing the self-flocculating properties of the bacterial cells in the fermentation medium. However, the performance of Tween 80 was most notable as it not only facilitated enhanced glycerol uptake but also promoted high cell growth and 1,3-PDO accumulation in comparison to control where no surfactant was added (Table 1). Earlier, Pachapur et al., [20] carried out a detailed analysis to study the effect of Tween 80 addition on GCR by a co-culture of *Enterobacter aerogenes* and *Clostridium butyricum*. Their results proved that doping 5 mg L^{-1} Tween 80 in a fermentation medium concurrently increased glycerol bioavailability and enhanced H_2 production by 25% when biodiesel-derived CG was used as the substrate. Further, to decipher the optimal concentration, fermentation trials were conducted with varying Tween-80 concentrations (0-0.5%) for a period of 9h, as depicted in Figure 2.

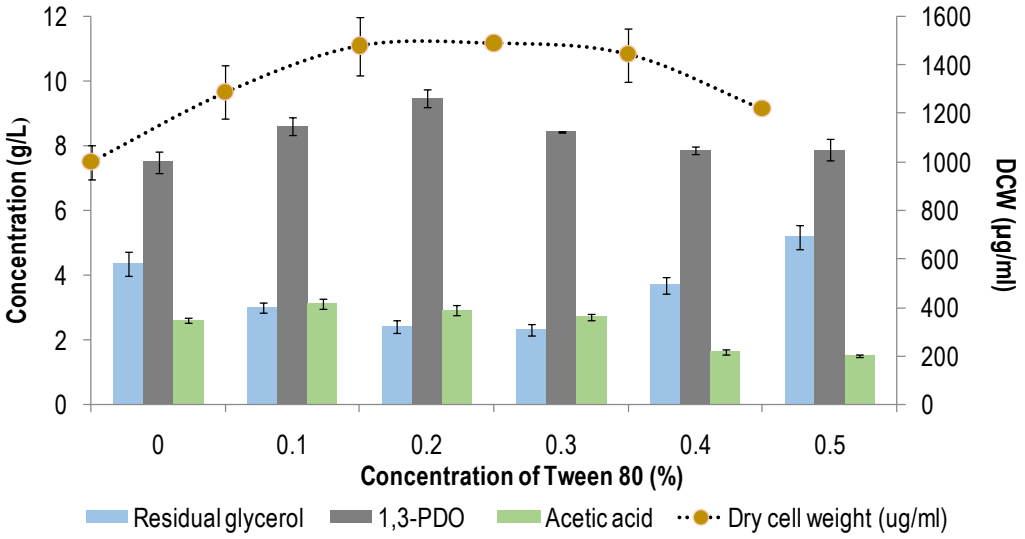


Figure 2: Effect of increasing Tween-80 on the glycerol fermentation to 1, 3-PDO by C. *freundii* IIPDR3 after 9h

It clearly shows that 0.2% was the most preferred concentration of Tween-80, as it led to maximum cell growth, glycerol uptake, and 1,3-PDO accumulation. At the said concentration, the 1,3-PDO production of *Citrobacter freundii* IIPDR3 peaked and attained a titer of 9.48 ± 0.29 g L^{-1} within 9h, with GCR being 1.82 g $L^{-1}h^{-1}$. Saturation was observed in cell growth beyond 0.2% Tween-80 doping. Moreover, when the Tween 80 conc. was increased from 0.2% to 0.5%, the cell growth declined by 18% showing its inhibitory effect which was reflected in glycerol assimilation as well. Interestingly, at both 0.2% and 0.5% Tween 80 concentration, a maximum molar yield of 0.7 was attained, indicating that a minimum threshold concentration of cells is required for biotransformation of glycerol to 1,3-PDO and hence, this metabolite is not an outcome of growth associated fermentation.

This is the first study wherein we report enhanced 1, 3-PDO production by the addition of 0.2% Tween-80 with *C. freundii*. However, 0.1% Tween-80 is often incorporated in De Mann, Robosa, Sharpe (MRS) medium, popular for the cultivation of *Lactobacillus* sp. that can convert glycerol to 1,3 PDO [21-23].

3.3. Screening of nitrogen sources

Different nitrogen salts were screened for 1,3-PDO production keeping nitrogen doping fixed (0.42gL^{-1}), besides yeast extract (0.2%) as organic nitrogen source. Following results were obtained as mentioned in Table 2.

Table 2: Effect of nitrogen salts on the growth of *C. freundii* IIPDR3 and glycerol fermentation to 1,3-PDO (Initial glycerol: 15.66 ± 0.73 gL^{-1}; initial DCW: 49.81 ± 2.32 µg/ml; Incubation temperature: 36°C; Duration:9h)

Nitrogen salt	Salt conc.(gL^{-1})	Concentration (gL^{-1})			DCW (µg/ml)
		Residual glycerol	1,3-PDO	Acetic Acid	
$(NH_4)_2SO_4$	2.0	BQL	8.76 ± 0.12	2.88 ± 0.09	1850 ±139.41
Urea	0.91	2.68± 0.77	6.84 ± 0.36	2.30 ± 0.07	1470 ± 84.85
$(NH_4)_2HPO_4$	1.99	BQL	8.28 ± 0.05	2.59 ± 0.70	1685 ±106.07
NH_4Cl	1.62	BQL	8.09 ± 0.05	2.68 ± 0.11	1790 ± 197.77
$NaNO_3$	2.57	4.59± 0.24	4.66 ± 0.29	3.52 ± 0.38	1488 ± 85.56
KNO_3	3.06	5.08 ± 0.92	4.37 ± 0.32	3.48 ± 0.23	1425 ± 76.78
$NH_4H_2PO_4$	3.475	2.10 ± 0.10	6.22 ± 0.13	2.20 ± 0.23	1525 ± 119.20

Note: BQL-below quantifiable limits

Considering cell growth based on DCW, the performance of various nitrogen salts was: $(NH_4)_2SO_4$> NH_4Cl > $(NH_4)_2HPO_4$> $NH_4H_2PO_4$> $NaNO_3$> Urea > KNO_3. Earlier, in the case *of Klebsiella pneumoniae,* glycerol consumption is directly related to cell growth, which was not the case in the present study [24]. This experiment suggested that though nitrate supported the growth of *Citrobacter*, but interfered with the glycerol uptake by the microbe (Table 2).

Authors believe that when nitrate was used as the nitrogen source, instead of following the traditional pathway of nitrate assimilation where nitrate is converted to ammonia involving nitrate and nitrite reductase enzymes, the bacteria exhibited denitrifying activity as reported earlier in the case of *Citrobacter diversus*[25]. Owing to the denitrifying activity of an organism in the current study, the usage of nitrate salt changed the entire dynamics of glycerol metabolism and it was mainly diverted to more acetate formation, where acetate met the carbon demand of denitrification (10 $CH_3COOH + 4NO_3^{-1} \rightarrow 2N_2 + 5CO_2 + 3H_2O + 4OH^-$)as suggested earlier (Huang and Tseng, 2001). Furthermore, high acetate levels in the case of nitrate doping as observed in the present study agree with earlier findings of Keevil et al. [26], where they cultivated *Citrobacter freundii*, in a batch mode with nitrate as a terminal electron acceptor. The following could be the plausible reason for the reduced 1,3-PDO formation, despite high acetate levels in the case of nitrate: The presence of nitrate salts induced the oxidative pathway of glycerol leading to acetate formation which in turn supplied reducing equivalents (NAD(P)H) to nitrate as a terminal electron acceptor, thereby facilitating reduction of nitrate→nitrite→N_2. This rapid oxidation of (NAD(P)H), can result in reduced availability of reducing equivalents for 1,3 propanediol oxidoreductase (PDOR) activity, resulting in low 1,3-PDO titers and high acetate accumulation (Table 2). These high acetate levels indicate high activity of acetate kinase (*Ack*) in the presence of nitrate salts. Since *AcK* catalyzes the conversion of acetyl phosphate to acetate with the formation of ATP, it positively impacted cell growth despite the low titers of the desired product (Table 2).

Considering low residual glycerol, high cell accumulation, and 1,3-PDO production, 0.2% $(NH_4)_2SO_4$ emerged as the best nitrogen source. Strikingly similar results were obtained with glycerol assimilating *Klebsiella pneumoniae,* wherein ammonium salts proved to be a better nitrogen source than nitrates for 1, 3-PDO production [27]. Direct doping of nitrogen in the form of ammonium salts likely led to its easy uptake and assimilation resulting in better cell growth, substrate utilization, and product formation. To decipher which concentration of $(NH_4)_2SO_4$ was the best; experiments were performed by varying its concentration from 0-3.5gL^{-1}, with results depicted in Figure 3.

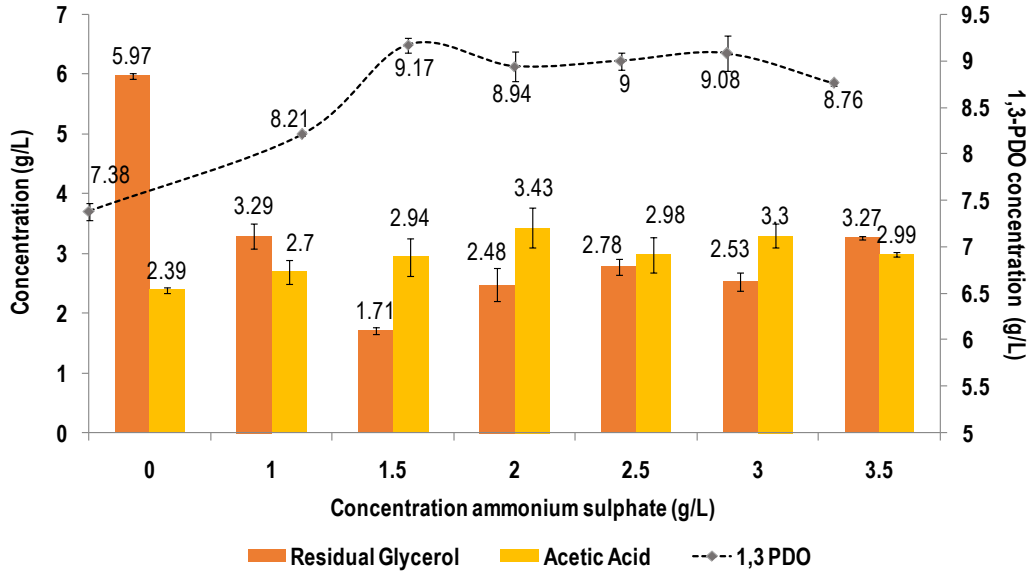


Figure 3: Effect of increasing ammonium sulphate concentration on the glycerol biotransformation to 1,3-PDO by *C.freundii*IIPDR3 after 9h fermentation

The authors anticipated that when no $(NH_4)_2SO_4$ was supplemented, it would halt glycerol bioconversion to 1,3-PDO. However, fortification of media with 0.2% YE supported the cell growth leading to the formation of ~7.4 gL^{-1} of 1, 3-PDO. The addition of 0.15% $(NH_4)_2SO_4$ was sufficient or more precisely optimum for 1,3-PDO accumulation with

productivity being 1.02 $gL^{-1}h^{-1}$. The DCW obtained at this $(NH_4)_2SO_4$ concentration was 40% higher than the fermentation media where no salt was added. Earlier, in the case of *Clostridium butyricum* strain L4, 15 $gL^{-1}(NH_4)_2SO_4$ was reported as the best nitrogen source for 1,3-PDO production, which is ten times higher than the amount reported in the present study [28].

3.4. Effect of different sugars and sugar alcohols as co-substrate to glycerol

In prokaryotic systems, glycerol metabolism occurs essentially via an oxidoreductive pathway for producing 1,3-PDO. The oxidative pathway furnishes the energy/ reducing equivalents requirements for cell growth, maintenance, and 1,3-PDO formation. Earlier, Biebl and Marten [29], reported that in *K. pneumoniae* DSM 4270 and *Citrobacter freundii* DSM 30040, glycerol was completely converted to PDO if a portion of it was substituted with another H-donor substrate. Hence in the present study, different simple sugars and sugar alcohols namely glucose, sucrose, mannitol, sorbitol, lactose, and galactose were tested for being effective co-substrates aiming to divert maximum glycerol flux towards 1,3-PDO biosynthesis. Following results were obtained when different co-substrates were doped at a concentration of 0.2%, as observed in Figure 4.

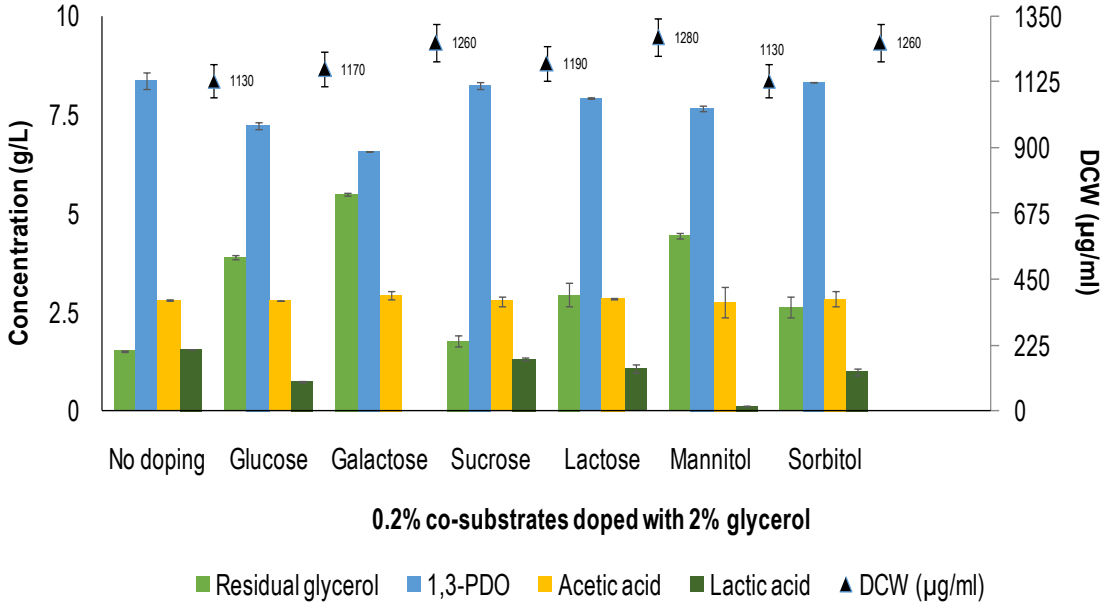


Figure 4: Effect of doping co-substrates on the glycerol fermentation to 1,3-PDO by *C.freundii* IIPDR3 after 9h

The final DCW obtained at the end of the fermentation reaffirmed that all the co-substrates supported cell growth with the highest biomass accumulation in the case of lactose and galactose and the lowest being in mannitol. Doping of sugars and sugar alcohols invariably impeded the GCR (galactose > mannitol > glucose > lactose > sorbitol), with sucrose being the exceptional case (Fig 4). It was anticipated that co-substrate would be preferably used for biomass formation, whereas the entire glycerol flux would be diverted towards 1,3-PDO production. Rather, the addition of co-substrates led to the formation of ethanol and succinate which were detected qualitatively but could not be quantified. Formation of lactic acid was prominently seen in all the cases except when galactose and mannitol were doped as co-substrates (Fig 4). Galactose followed by glucose exerted the most negative impact on 1, 3-PDO formation. The metabolic behavior of *C. freundii* IIPDR3 especially with respect to glycerol consumption in the presence of glucose was radically different from *Citrobacter werkmanii* DSM 17579 [30]. If the present strain showed 13.4% reduced glycerol uptake, in the latter strain presence of glucose significantly enhanced glycerol consumption by 142.2% under

anaerobic conditions. However, the presence of glucose reduced 1,3-PDO production by 13.5% and 2.76%, when the organism was *C. freundii* IIPDR3 and *C. werkmanii* DSM 17579 respectively.

3.5. Effect of pH on 1,3-PDO production

Among several physiochemical parameters, the pH of the fermentation medium can be a decisive factor for microbial growth and product accumulation, especially where the process leads to inevitable acid production. Though our initial studies showed that an alkaline pH was favorable for 1,3-PDO production by *C. freundii* IIPDR3 [11], the present experiment was conducted to reaffirm whether the said strain was alkalophilic or alkali-tolerant. Two sets of initial pH were chosen 7 and 8, whereas in the case of control, the initial pH of the medium was kept at 8.5. The results of the said experiment are mentioned in Table 3.

Table 3: Effect of initial and final pH of the medium on the growth of *C. freundii* IIPDR3 and glycerol fermentation to 1,3-PDO (Initial glycerol: 19.23 ± 1.03 gL^{-1}; initial DCW: 31 ±1.5 µg/ml; Incubation temperature: 36°C; Duration: 9h)

pH of the medium		Concentration (gL^{-1})			DCW (µg/ml)
Initial	Final	Residual glycerol	1,3-PDO	Acetic Acid	
8.5	8.5	3.80 ± 0.06	9.12 ± 0.03	3.04 ± 0.04	2006 ± 88.11
7.0	7.0	7.03 ± 0.15	7.02 ± 0.05	2.59 ± 0.14	1414 ± 79.30
7.0	8.0	2.24 ± 0.10	9.66 ± 0.01	3.29 ± 0.10	1919 ±17.62
7.0	9.0	2.39 ± 0.11	9.61 ± 0.13	3.23 ± 0.36	2000 ± 61.67
8.0	7.0	5.68 ± 0.41	7.65 ± 0.16	2.67 ± 0.17	1588 ± 26.43
8.0	8.0	2.13 ± 0.14	9.99 ± 0.27	2.95 ± 0.26	1950 ± 114.5
8.0	9.0	4.61± 0.15	8.64 ± 0.46	2.79 ± 0.07	1919 ± 17.62

Table 3 shows that pH stat at 7 was less favorable for 1, 3-PDO accumulation, and cell growth compared to pH stat wherein pH was ≥8.0. Final DCW reaffirmed that *C. freundii* IIPDR3 was alkalophilic, as the biomass accumulation was maximum when the pH-stat conditions were ≥8.0. However, a pH stat of 8.0 turned out to be best, resulting in maximum 1, 3-PDO production (9.99 ± 0.27 gL^{-1}). The GCR, 1,3-PDO productivity, and molar yields in this condition were 1.98 $gL^{-1}h^{-1}$, 1.11 gL^{-1} h^{-1}, and 0.67, respectively. The present results (Table 3) further suggest that the acetic acid formation at any pH stat conditions (whether it was 7,8 or 9) was meagerly affected, but the biosynthesis of 1,3-PDO preferred alkaline conditions.

Earlier, 1,3-propanediol oxidoreductase (PDOR) of *Clostridium butyricum*, which catalyzes the rate-limiting step, has shown maximum activity at pH 8.0 [31]. Likewise, Yun et al., have also reported that the PDOR which catalyzes a reversible reaction, showed maximum reduction activity at pH 9.0 in the case of *C. butyricum* YJH-09 [32]. Besides *Clostridium*, PDOR of even Gram-negative bacterium *Enterobacter aerogenes* CNCM 1210 has shown an optimal pH of 7.8. In their review, Jiang et al., have outlined the importance of pH conditions as it is capable of changing the structural and binding properties of catalytic site within PDOR enzyme. Molecular dynamic simulation studies have revealed that alkaline pH leads to constriction of the cleft in PDOR favoring the better binding of NADPH molecule while acidic pH results in the widening of the cleft reducing the binding of this cofactor [33]. As a result, PDOR showed a maximum reducing activity at alkaline pH only. Besides the aforementioned state of the art, even the kinetic studies conducted for the glycerol dehydratase (GDHt) and PDOR (reducing) activity of *Citrobacter freundii*, whose genes were co-expressed in *E. coli*, reveal that the said enzymes displayed optimal activity at 8.5 and 8, respectively [34]. It is supporting evidence for results obtained in the present study as well. Henceforth, all the studies were conducted at an initial pH being 8.0 and maintaining the same pH stat.

This revelation would help in minimal pretreatment of CG when studies would be initiated with CG. In 2019, the market price of CG with 80% purity was about USD 170 per ton, and with its purification done, the price increased by almost seven times [35]. CG has a pH of about 9.6 which makes our strain very relevant due to its alkalophilic nature,

as it will lower the overall production cost. Moreover, no state-of-the-art until now has mentioned an alkalophilic glycerol assimilating *Citrobacter* strain.

Hitherto, most of the researchers thrust upon pH stat conditions that promote uninterrupted glycerol uptake and its subsequent biotransformation to 1,3-PDO. However, they fail to disclose that what is the threshold value till which the pH of the medium should be allowed to drop and then should be restored, to maximize 1,3-PDO titers. So far, in all the studies undertaken, a pH-stat of 8.3 ± 0.2 was maintained, wherein the pH was allowed to drop to 6.3 ± 0.2 before its restoration. However, a systematic study was conducted wherein in independent experiments the pH of the medium was allowed to drop to 5.5, 6.0, 6.5, and 7.0 and the pH was then restored to 8.0. Authors anticipated that it is one of the most crucial aspects, as it could cause a change in metabolic pattern and even accumulation of acetic acid titers, thereby affecting 1,3-PDO production. The results as displayed in Table 4, clearly indicate that if the pH of the medium was allowed to drop to 6.5 and was restored to 8.0, it favored maximum accumulation of 1,3-PDO.

Table 4: Effect of different pH-stat conditions on the growth of *C.freundii* IIPDR3 and glycerol fermentation to 1,3-PDO (Initial glycerol: 20.84 ± 0.16 gL^{-1}; initial DCW: 33.07 ±0.57µg/ml; Incubation temperature: 36°C; Duration: 9h)

initial-drop-final (pH)	Concentration (gL^{-1})			DCW (µg/ml)	1,3-PDO productivity ($gL^{-1}h^{-1}$)
	Residual glycerol	1,3-PDO	Acetic Acid		
8.0 - 5.5 - 8.0	6.76 ± 0.04	7.17 ± 0.16	2.62 ± 0.11	1745 ± 35.36	0.8
8.0 - 6.0 - 8.0	6.1 ± 0.14	7.06 ± 0.06	2.47 ± 0.08	1565 ± 35.36	0.78
8.0 - 6.5 - 8.0	2.9 ± 0.29	9.22 ± 0.39	3.15 ± 0.06	1925 ± 35.36	1.02
8.0 - 7.0 - 8.0	2.72 ± 0.07	8.78 ± 0.00	3.45 ± 0.07	2110 ± 70.71	0.98

It was also conducive to both acetate formation and biomass production. This experiment further confirmed that high acetate formation resulted in better biomass accumulation. The acetate kinase (*Ack*) from *E. coli* is known to display better stability in the range of 6.5-7.8 [36]. It is likely that in the present case also, a pH drop of 1-1.5 provided a conducive environment for *Ack* enzyme, which catalyzes the conversion of acetyl phosphate to acetate and ATP. ATP, the energy currency in turn promoted bacterial growth, as discussed in subsection 3.3.

There is one isolated report by Petrov and Stoyanov [37] wherein they implemented a similar kind of strategy and termed it forced pH fluctuations to enhance 1,3-PDO titers in the case of *Klebsiella pneumoniae*. They kept the initial pH of medium 7.0 and then after every 3h forced the pH to reach 8.0 using NaOH. Compared to pH stat conditions of 7.0, the strain's 1,3-PDO productivity increased from 0.94 to 1.15 $gL^{-1}h^{-1}$ and the major by-product changed from lactic acid to 2,3-BDO. Their strategy reduced the overall acid (acetic acid, lactic acid, and succinic acid) production as by-products, while ethanol production remained unaffected. In the current study, upon data analysis on both the pH-based experiments, authors believe that a pH drop in the range of 6.5-7.0 and thereby its restoration back to 8.0-9.0 provided an amicable environment for both the oxidative and reductive branches of glycerol metabolic pathway to work optimally.

3.6. Effect of size of the inoculum on 1,3-PDO production

It was anticipated that if a higher size of inoculum was added to the fermentation media, the cells would rapidly multiply and reach a threshold concentration within a shorter incubation time wherein maximum glycerol bioconversion to 1,3-PDO occurs. Table 5 illustrates the effect of increasing the inoculum size on 1,3-PDO production after 9h of fermentation. The results indicated that an inoculum having an OD_{600nm} range of 10-15.0 was the best for 1,3-PDO production. However, higher OD_{600nm} of 15.0 favored the maximum accumulation of 1,3-PDO.Near-to-complete consumption of glycerol implies that this bacterial cell population in the 9^{th} hour was already under stress due to substrate limitation, some cells probably entering their death phase too, causing maximum excretion of 1,3-PDO

Table 5: Effect of size of the inoculum on the growth of *C. freundii* IIPDR3 and 1,3-PDO production from glycerol (Initial glycerol: 19.62 ± 0.62 gL^{-1}; Incubation temperature: 36°C; Fermentation time: 9h)

Optical density at 0h (OD_{600nm})	Initial DCW (µg/ml)	Dry cell weight (µg/ml)		Conc. (gL^{-1}) after 9h fermentation		
		After 6h	After 9h	Residual glycerol	1,3-PDO	Acetic acid
2.5	7.8	660 ± 28.3	1555 ± 7.1	8.83 ± 0.31	5.17 ± 0.06	2.23 ± 0.03
5.0	15.5	810 ± 28.3	1655 ± 35.4	7.24 ± 0.33	5.95 ± 0.31	2.17 ± 0.25
7.5	23.3	1165 ± 63.6	1655 ± 49.5	5.49 ± 0.31	7.03 ± 0.44	2.60 ± 0.12
10*	31.1	1180 ± 28.3	1680 ± 28.3	4.25 ± 0.24	8.49 ± 0.17	2.71 ± 0.05
12.5	38.8	1400 ± 42.3	1765 ± 35.4	2.15 ± 0.22	8.78 ± 0.40	2.66 ± 0.07
15.0	46.5	1370 ± 14.1	1805 ± 35.4	BQL	9.59 ± 0.20	2.84 ± 0.05

Note: * denotes Control; BQL denotes below quantifiable limits

. Hence, it may become necessary to restrict the fermentation time to 8h, if a higher inoculum of OD_{600nm}:15 is added. On the other hand, when the inoculum with OD 2.5-5.0 was added to the medium, even in the 9th hour the bacterial cells were still in their logarithmic phase and bacterial cells did not reach a threshold concentration to voraciously uptake glycerol and transform it to 1,3-PDO. This experiment confirmed that an initial cell concentration ≥ 50µg/ml (on a dry cell weight basis) was favorable for promoting high glycerol consumption and attaining high 1,3-PDO productivity. Rajagopalan et al., also found that higher inoculum size (10 to 12.5% v/v) of *Clostridium* strain BOH3 resulted in higher cell density which in turn facilitated higher 1,3-PDO production [38]. Based on the results (Table 5), in all the further experiments, fermentation was terminated in 8h only.

3.7. Need of MS solution and YE for 1,3-PDO production

If YE is a rich source of water-soluble vitamins, especially Vitamin B_{12} other than organic nitrogen source, MS solution contains several mineral salts as micronutrients one being $CoCl_2$. During glycerol metabolism, the glycerol dehydratase (GDHt) enzyme in *Citrobacter* species has been reported to be Vitamin B_{12} dependent as reviewed extensively [39]. Hence doping of YE becomes mandatory for the proper functioning of GDHt in *Citrobacter* and it can be a rate-limiting component in the fermentation medium. Likewise, $CoCl_2$ helps in the reactivation of the GDHt [40]. Hence it was important to assess their need and vitality in the fermentation media, during the evaluation of 1,3-PDO production by glycerol assimilating *C. freundii* IIPDR3. In this study, the effects of MS solution and YE were simultaneously assessed to understand their role in 1,3-PDO production. Figure 5 depicts the effect of YE on glycerol assimilation and 1,3-PDO production by *C. freundii* IIPDR3 after 8h of fermentation with initial DCW and initial glycerol concentration being 53.3 ± 0.25 µg/ml and 20.41 ± 0.67gL^{-1}, respectively.

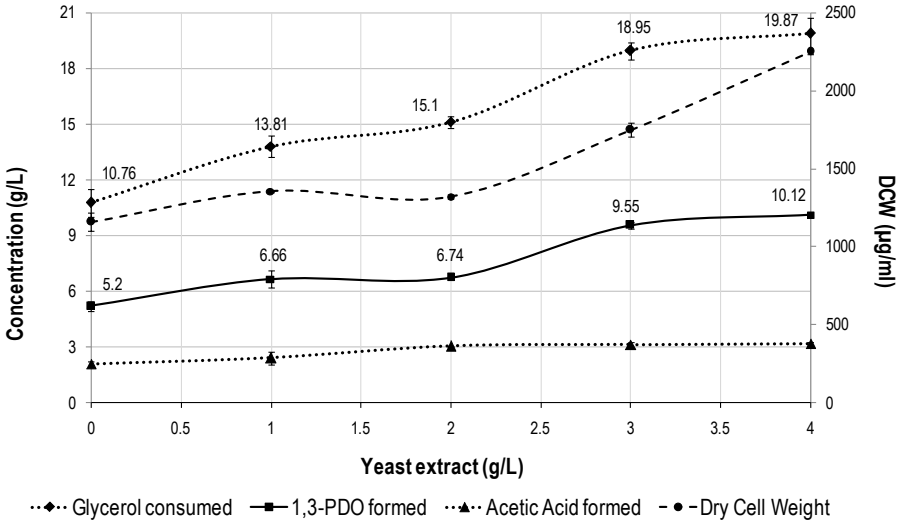


Figure 5: Effect of yeast extract on the glycerol biotransformation to 1,3-PDO by *C. freundii* IIPDR3 after 8h

As observed in Fig 5, increasing the concentration of YE facilitated glycerol uptake and promoted cell growth, which eventually resulted in high 1,3-PDO titers. When the YE concentration was increased from $2gL^{-1}$ to $3gL^{-1}$, there was a significant rise in 1,3-PDO titers from 6.74 ± 0.03 to 9.55 ± 0.18 gL^{-1}. However, 1, 3-PDO titer (10.12 ±0.06 gL-1) saturated at 4 gL^{-1}YE concentration. At both 3 and 4 gL^{-1}YE, the 1,3-PDO productivity was >1 $gL^{-1}h^{-1}$ and residual glycerol was < 1.5 gL^{-1}, respectively. However, increasing the concentration of YE from 3 to 4 gL^{-1} led to a significant rise of 28.5% final DCW indicating that the glycerol flux was diverted more towards biomass formation at this concentration. In all the cases succinate and lactate formation was observed but it could not be quantified. Considering the high cost of the YE and merely 5.9% enhancement in 1,3-PDO levels, 3 gL^{-1}YE was shortlisted for all future studies. Our results agree with the study by Drożdżyńska et al.[19], where they optimized the YE requirement of *C. freundii* AD119 for 1,3-PDO formation and found that 5 gL^{-1} YE was essential to attain high 1,3-PDO (19.47 gL^{-1}) titers which flattened when its concentration was further raised to 7.5 and 10 gL^{-1}.

Surprisingly, when no YE was doped to the medium and only MS solution was added, the organism accumulated 5.2 ± 0.25 gL^{-1} of 1,3-PDO by consuming 10.76 gL^{-1} glycerol (Table 6). On the contrary, when neither MS nor YE was added, the organism produced basal levels of 1,3-PDO and acetic acid with very low glycerol consumption. There was a notable rise of 73.2% and 95.84% in the case of glycerol consumption and 1,3-PDO production, respectively by merely adding MS solution (2 ml/L) in the media devoid of YE (Table 6).

Table 6: Effect of adding YE and MS solution on 1,3-PDO production from glycerol by *C. freundii* IIPDR3 Initial glycerol: 20.41 ± 0.67gL^{-1}; Incubation temperature: 36°C; Initial DCW: 53.3 ± 0.25 µg/ml; Fermentation time: 8h)

Yeast Extract ($2gL^{-1}$)	MS solution (2 ml/L)	Conc. (gL^{-1}) after 8h fermentation		
		Residual glycerol	1,3-PDO	Acetic acid
NO	NO	14.2 ± 0.14	2.66 ± 0.08	1.24 ± 0.04
NO	YES	9.65 ± 0.05	5.20 ± 0.25	2.09 ± 0.16
YES	NO	5.31 ± 0.07	6.79 ± 0.09	3.02 ± 0.26
YES	YES	6.71 ± 0.03	6.74 ± 0.03	3.04 ± 0.21

There is a plausibility that our alkalophilic *Citrobacter* strain harbored the pathway for Vitamin B_{12} biosynthesis and MS solution provided the necessary cobalt salt for activating "*cob*" genes required for cobalamin synthesis when no YE was added externally under microaerobic conditions. Earlier also, *Citrobacter freundii* strain has been reported to synthesize Vitamin B_{12} during soybean fermentation to temph [41]. Likewise, 1,3 PDO-producing *C. amalonaticus* Y19 has been reported to harbor "*cob*" genes as discussed earlier [15]. This experiment reaffirmed that the present strain under study contained coenzyme B_{12}-dependent "GDHt" which catalyzes the dehydration of glycerol to 3-HPA.However, doping of YE masked the importance of the MS solution (Table 6).

3.8. Screening of various magnesium salts for enhanced 1,3-PDO production

Magnesium ions are known to play a critical role in the functioning of many enzymes and some prior art studies reveal that its salts exert a stimulatory impact on 1,3-PDO production [19]. Hence, a systematic study was conducted wherein various magnesium salts were screened for 1,3-PDO production with an Mg^{+2} concentration being 50 ppm. Table 7 depicts the effect of various magnesium salts on 1,3 PDO production by *C. freundii* IIPDR3.As observed, the addition of magnesium salt was mandatory and Mg^{+2} concentration at merely 50 ppm increased the 1,3-PDO titers in the range of 50.1-68.1% depending on the salts used. Compared to 1,3-PDO, the addition of magnesium salt affected the glycerol uptake rate more significantly, reflecting its importance during glycerol fermentation. Magnesium hydroxycarbonate emerged as the best salt followed by magnesium sulphate. However, when magnesium hydroxycarbonate was used as a magnesium source, 2.42± 0.04 gL^{-1} of lactic acid was detected whereas in the case of other magnesium salts, the lactic acid formation was below quantifiable limits.

Table 7: Effect of various magnesium salts (Mg^{+2} concentration: 50ppm) on 1,3-PDO production from glycerol by *C. freundii* IIPDR3 (Initial glycerol: 21.96 ± 0.63 gL^{-1}; Temp: 36°C; Initial DCW: 80.68 ± 0.40 µg/ml; Fermentation time: 8h)

Magnesium Salts	Mg^{2+}salt added to medium (gL^{-1})	Conc. (gL^{-1}) after 8h fermentation		
		Residual glycerol	1,3-PDO	Acetic acid
No	NIL	10.93 ± 0.19	5.84 ± 0.05	2.37 ± 0.07
$MgCl_2$	0.2	2.89 ± 0.07	8.77 ± 0.03	2.77 ± 0.17
$MgSO_4.7H_2O$	0.51	3.13 ± 0.001	9.49 ± 0.13	3.02 ± 0.16
$Mg(CH_3COO)_2.4H_2O$	0.44	3.52 ± 0.11	9.16 ± 0.20	2.89 ± 0.10
MgO	0.08	2.28 ± 0.10	9.23 ± 0.07	2.96 ± 0.05
$Mg(OH)_2$	0.12	2.79 ± 0.26	8.89 ± 0.07	2.75 ± 0.02
CH_2MgO_4	0.37	1.86 ± 0.02	9.82 ± 0.12	2.93 ± 0.03

Hence, 0.05% of $MgSO_4.7H_2O$ was shortlisted for all the studies. Earlier, this magnesium salt has been used for the cultivation of several glycerol assimilating and 1,3-PDO-producing bacteria such as *Citrobacter, Clostridium*, *Klebsiella,* etc. [18, 42, 43]. After shortlisting $MgSO_4.7H_2O$, it became necessary to identify its optimum concentration, which not only promoted glycerol consumption but led to enhanced 1,3-PDO production. Hence various levels (0.025% to 0.15%) of $MgSO_4.7H_2O$ were tested for 1,3-PDO production. The following results were obtained after 8h fermentation with initial glycerol and DCW being 21.28 ± 1.07 gL^{-1} and 86.65 ± 0.58 µg/ml, respectively (Figure 6).

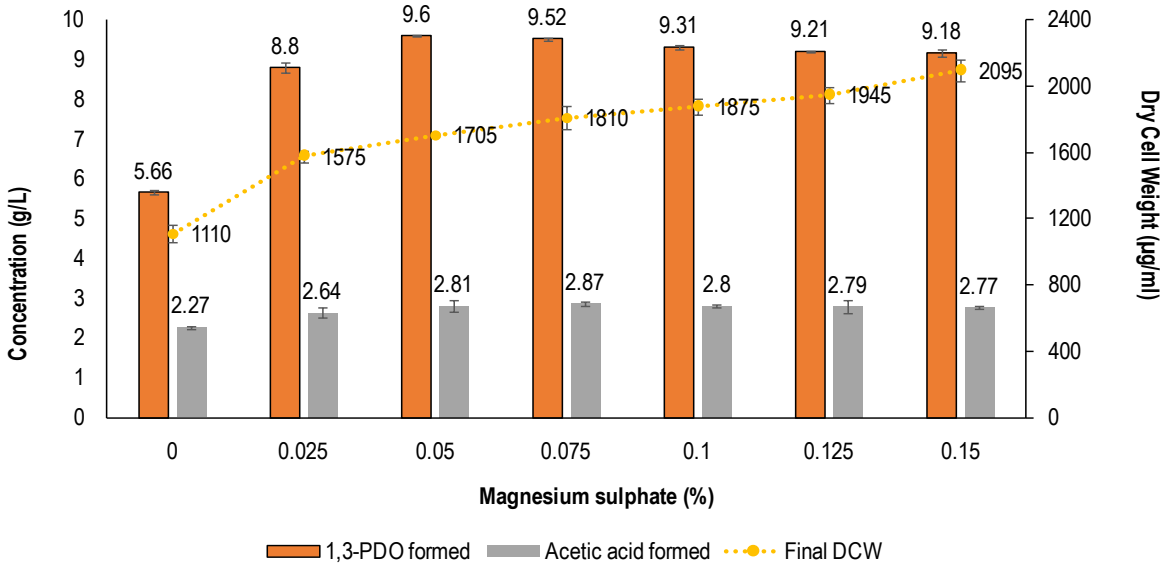


Figure 6: Effect of increasing $MgSO_4$ concentration on glycerol bioconversion to 1,3-PDO by *C. freundii* IIPDR3

In the present experiment, no quantifiable amount of residual glycerol was detected after 8h of fermentation except in a medium doped with 0.025% $MgSO_4.7H_2O$, which contained 2.18 ± 0.3 gL^{-1} of glycerol. Figure 6 clearly shows that after 0.05% $MgSO_4.7H_2O$, both the products namely 1,3-PDO and acetic acid, attained saturation. However, its higher concentration was favorable for biomass accumulation, as discussed earlier [19]. Therefore 0.05% of $MgSO_4.7H_2O$ was chosen for all future studies.

3.9. Replacement of YE with corn steep powder (CSP)

CSP, the spray-dried form of corn steep liquor (CSL) is a by-product of the corn milling industry and can be an excellent low-cost substitute for YE. Wang et al., [44] have reported that the bulk price of CSP is $0.286/kg which is 32.2-fold less compared to YE ($9.2/kg) [45]. Therefore, it became essential to study whether CSP could replace YE

during 1,3-PDO production or not. CSP from two different makes were simultaneously evaluated for 1,3-PDO production with YE being control. The following results were obtained as shown in Table 8.

Table 8: Effect of replacing YE with CSP on 1,3-PDO production from glycerol by *C. freundii* IIPDR3 (Initial glycerol: 20.71 ± 0.7gL^{-1}; Incubation temperature: 36°C; Initial DCW: 62.54 ± 1.68 µg/ml; Fermentation time: 8h)

Organic N_2 source	Concentration (gL^{-1})	Conc. (gL^{-1})			Final DCW (µg/ml)
		Residual glycerol	1,3-PDO	Acetic acid	
YE	3	BQL	9.02 ± 0.14	2.85 ± 0.03	2390 ± 14.14
CSP-H	1	8.7 ± 0.13	6.02 ± 0.08	2.28 ± 0.16	1445 ± 7.07
	3	6.25 ± 0.11	7.04 ± 0.08	2.34 ± 0.03	1540 ± 14.14
	5	5.62 ± 0.17	7.50 ± 0.12	3.23 ± 0.02	1850 ± 14.14
	7	3.55 ± 0.03	8.18 ± 0.01	2.48 ± 0.08	2300 ± 14.14
CSP-R	1	8.02 ± 0.08	6.07 ± 0.04	2.46 ± 0.14	1770 ± 28.28
	3	6.46 ± 0.23	6.22 ± 0.10	2.31 ± 0.02	2055 ± 49.50
	5	3.65 ± 0.12	8.60 ± 0.11	2.91 ± 0.15	3035 ± 91.92
	7	BQL	9.65 ± 0.01	3.04 ± 0.03	3900 ± 70.71

Note: BQL means below quantifiable limits

Among the CSP, procured from two different sources, the performance of CSP-R was better than CSP-H. The quantity of CSP-R was required 2.3 times more than YE, to produce the same amount of 1,3 PDO. However, the replacement of YE with CSP-R was challenging. When CSP-R was used as an organic nitrogen source, a lot of precipitation was observed in the medium, especially at higher concentrations which led to obtaining erroneous dry cell weight (Table 7) at the end of the fermentation. Moreover, precipitation owing to high CSP-R concentration (7gL^{-1}) in the seed medium could interfere while measuring the optical density at 600 nm, a primary route (OD_{600nm}: ≥15.0) to add inoculum to the fermentation. The present experiment confirmed that CSP-R could be a replacement for YE. Yet for all future experiments, we continued using YE as an organic nitrogen source.

3.10. Parameters exhibiting significant correlation with glycerol utilization and 1,3-PDO production

Analysis using paired T-test gave a fair idea of which variables displayed a significant correlation (whether negative or positive) with 1, 3-PDO production and glycerol consumption in the case of *C.freundii* IIPDR3. Table 9 provides statistical evidence that in the case of both glycerol consumption and 1,3-PDO production, the size of the inoculum in terms of dry cell weight (µg/ml), yeast extract, ammonium sulphate, and magnesium sulphate showed a strong positive correlation. For 1,3-PDO production, the positive impact of various parameters was in the order of the size of inoculum >YE >$(NH_4)_2SO_4$>$MgSO_4.7H_2O$. However, in the case of glycerol consumption, there was one more parameter that exhibited a strong positive correlation. When the initial pH of the medium was kept at 7.0, restoration at higher pH favored better glycerol uptake. Even, when *C. freundii* IIPDR3 was grown at an initial pH being 8.0, though restoration at a higher pH favored better glycerol uptake, the value of both, ρ (0.856 to 0.263) and α (0.02 to 0.03) reduced. These statistical correlations reaffirmed that the *C. freundii* strain was alkalophilic and showed higher glycerol consumption at alkaline pH. Unlike 1,3-PDO production, in the case of glycerol consumption, the impact was in the order of size of inoculum >YE>Restoration at higher pH with initial pH being 7.0> $MgSO_4.7H_2O$>$(NH_4)_2SO_4$. Higher ΔpH (or pH drop) during glycerol fermentation adversely affected glycerol consumption and 1,3-PDO production in a significant manner, as shown in Table 9.

Table 9: Variable exhibiting strong and significant correlation with 1,3-PDO production and glycerol consumption

Variables that exhibited strong and significant correlation with 1,3 PDO production				
Factors	**Range**	**Optimized value chosen**	**(ρ)**	**(α)**
Inoculum Size as DCW (µg/ml)	7.8, 15.5, 23.3, 31.1, 38.8, 46.5 µg/ml	≥50 µg/ml	0.988	0.01
Yeast Extract	0, 1, 2, 3, 4 (gL^{-1})	3 gL^{-1}	0.961	$6.10611E^{-05}$
Ammonium sulphate	0, 1, 1.5, 2, 2.5, 3 and 3.5 (gL^{-1})	1.5 gL^{-1}	0.750	$7.16138E^{-07}$
Magnesium sulphate	0, 0.025, 0.05, 0.075, 0.1, 0.125, 0.15 (wt%)	0.05(wt%)	0.616	$2.71527E^{-06}$
pH drop with initial pH and final pH being 8.0	Δ pH: 1,1.5,2, 2.5	Δ pH: 1.5 (6.5)	-0.817	0.004
Variables that exhibited strong and significant correlation with glycerol consumption				
Factors	**Range chosen for the study**	**Optimized value chosen**	**(ρ)**	**(α)**
Inoculum size as DCW (µg/ml)	7.8, 15.5, 23.3, 31.1, 38.8, 46.5 µg/ml	≥50 µg/ml	0.996	0.006
Yeast Extract	0, 1, 2, 3, 4 (gL^{-1})	3 gL^{-1}	0.984	0.00015
Restoration at higher pH with initial pH being 7.0	Restoration pH: 7, 8 and 9	pH: 8.0	0.856	0.02
Magnesium sulphate	0, 0.025, 0.05, 0.075, 0.1, 0.125, 0.15 (wt%)	0.05(wt%)	0.707	$2.71527E^{-06}$
Ammonium sulphate	0, 1, 1.5, 2, 2.5, 3 and 3.5 (gL^{-1})	1.5 gL^{-1}	0.588	$6.77749E^{-08}$
pH drop with initial pH and final pH being 8.0	Δ pH: 1,1.5,2, 2.5	Δ pH: 1.5 (6.5)	-0.937	0.001

Note: ρ represents Pearson correlation coefficient and α represents the level of significance. While performing paired T-test the value of α was chosen to 0.05 and only those factors were chosen whose Pearson correlation coefficient was in the range of 0.5-1.0 as it represents the strong correlation

. In the case of both glycerol consumption and 1,3-PDO production, the size of the inoculum in terms of DCW and YE were the two utmost important parameters that displayed a strong positive correlation. Hence, one can observe that when the size of the inoculum was relatively high (>50 µg/ml), it resulted in high 1,3-PDO productivity and residual glycerol was also below quantifiable limits. Statistical analysis reveals that during designing the experiment, it would be essential that pH should not be allowed to drop below 6.5 as it could negatively affect 1,3 PDO titers and prominently reduce glycerol consumption.

3.11. Assessment of neutralizing agents toward enhanced 1,3-PDO production

Generally, researchers use alkali metal hydroxides as neutralizing agents for carrying out pH stat fermentation. In the present study three different neutralizing agents namely NaOH, Na_2CO_3, and liquor ammonia were assessed to see their effect on the growth of *C. freundii* IIPDR3, glycerol consumption, and its subsequent valorization to 1,3-PDO. Liquor ammonia offered the additional advantage of being a nitrogen source whereas Na_2CO_3 was chosen owing to its higher buffering capacity in the aqueous medium. The following results were obtained after 7h fermentation, when the initial glycerol concentration was 22.23 ± 1.80 g L^{-1} and the initial DCW was 59.36 ±0.86 µg/ml (Figure 7).

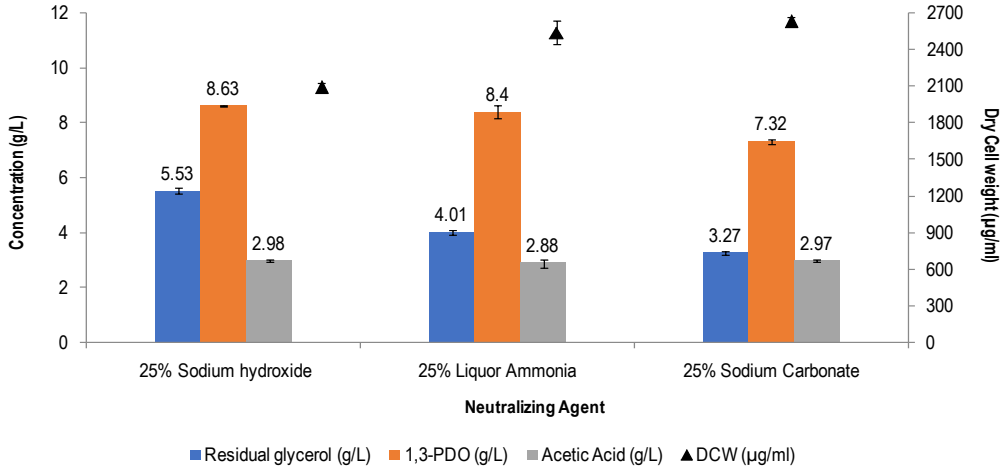


Figure 7: Effect of various neutralizing agents on 1,3-PDO production from glycerol by *C. freundii* IIPDR3

In the case of Na_2CO_3, despite higher glycerol uptake, owing to the buffering effect of the carbonates, the pH drop was slow and it resulted in maximum biomass formation and appreciable accumulation of by-products namely lactic and succinic acid, besides acetic acid (data not shown). Both NaOH and liquor NH_3 performed equally well in terms of 1, 3-PDO production. However, improved glycerol uptake and higher final DCW in the case of liquor NH_3 might play a crucial role when higher glycerol concentrations are used. To prove this hypothesis, it became inevitable to adjudge the performance of both the neutralizing agents at higher glycerol concentration, as active cell concentration could be a limiting and decisive factor for 1,3-PDO production. The following results were obtained as shown in Table 10.

Table 10: Effect of neutralizing agents on 1,3-PDO production by *C. freundii* IIPDR3 grown at varying glycerol concentrations (Temp: 36°C; Initial DCW: 37.89 ± 0.40 µg/ml)

Fermentation time (h)	Neutralizing agent	Glycerol (gL^{-1})		1,3-PDO			Final DCW (µg/ml)
		Initial	Residual	T (gL^{-1})	Y (gg^{-1})	P ($gL^{-1}h^{-1}$)	
7	NaOH	19.10 ± 0.6	3.10 ± 0.18	8.58 ± 0.01	0.54	1.23	1755 ± 7.07
	Liquor NH_3		1.09 ± 0.19	7.93 ± 0.01	0.44	1.13	1960 ± 84.8
10.5	NaOH	39.57 ± 0.7	21.89 ± 0.51	9.68 ± 0.6	0.55	1.38	1950 ± 14.1
	Liquor NH_3		5.31 ± 0.01	18.04 ± 0.76	0.53	1.72	2235 ± 63.6
13.5	NaOH	57.94 ± 0.1	36.16 ± 0.7	12.58 ± 0.7	0.50	0.93	2515 ± 77.78
	Liquor NH_3		29.19 ± 0.7	14.04 ± 0.7	0.49	1.04	3015 ± 106.0

Note: TYP represents Titer, yield, and productivity

The study indicated that at higher glycerol concentrations (≥40 gL^{-1}), NaOH addition elevated the osmotic stress of the organism, resulting in significantly reduced glycerol uptake and low 1,3-PDO titers. Interestingly, on the other hand, neutralizing with liquor NH_3 promoted higher glycerol uptake. As quoted earlier, besides neutralizing agent, liquor NH_3 being a nitrogen source improved biomass accumulation and relieved the strain's performance from nitrogen limitation. As a result, a maximum of 18.04 gL^{-1} 1,3-PDO was accumulated with yield and productivity being 0.53 gg^{-1} and 1.72 g $L^{-1}h^{-1}$, when the initial glycerol concentration was 39.57 ± 0.7 gL^{-1}. However, at an initial glycerol concentration of 57.94 ± 0.1 gL^{-1}, the pH drop slowed after 10.5h suggesting that some nutrients became limiting which were vital to boost glycerol bioconversion to 1,3-PDO. As a result, the 1,3-PDO production remained confined to 12.58 and 14.04 gL^{-1}titers, when the neutralizing agent was NaOH and liquor NH_3, respectively. The probability of substrate inhibition looks dicey as biomass accumulation was good even at ~60 gL^{-1}initial glycerol. Moreover, as the fermentation proceeded, microscopic observation confirmed heterogeneous cell population with significant reduction in cell motility, which in turn could negatively impact glycerol fermentation. Liquor NH_3 has been earlier used for pH restoration during fermentative upgradation of glycerol to 1,3-PDO with microbes like *Clostridium beijerinckii* DSM 791 and *Clostridium pasteurianum* [46,47].

3.12. Assessment of *C. freundii* IIPDR3 on CG

When the strain was assessed on CG with optimized parameters, the following results were obtained as shown in Table 11.

Table 11: Assessment of *C. freundii* IIPDR3 on varying concentrations of CG (Temp: 36°C; Initial DCW: 64.11 ± 3.88 µg/ml)

Fermentation time (h)	Methanol conc. in CG (gL^{-1})	Glycerol conc. (gL^{-1})		1,3-PDO			Acetic acid produced (gL^{-1})
		Initial	Residual	T (gL^{-1})	Y (gg^{-1})	P ($gL^{-1}h^{-1}$)	
7	6.68 ± 0.64	21.04 ± 0.15	BQL	9.65 ± 0.13	0.48	1.40	2.99 ± 0.01
10.5	13.21± 0.15	42.55 ± 1.13	19.73 ± 0.36	11.11 ± 0.15	0.48	1.05	3.80 ± 0.24
13.5	24.15 ± 0.07	66.20 ± 1.51	41.99 ± 1.40	11.65 ± 0.82	0.48	0.86	3.54 ± 0.28

Note: T-titer; Y- Yield; P-productivity; CG-Crude Glycerol

Compared to PG, the titer and productivity of 1,3-PDO at $20gL^{-1}$ CG concentration was higher, suggesting that the bacterium was highly robust. Earlier Yang et al., also reported improved 1, 3-PDO titers when PG was replaced by waste glycerol in the case of *Klebsiella pneumoniae* [24]. They argued that the salts present in CG acted as micronutrients and resulted in higher growth and thus higher 1, 3-PDO titers. Table 12, compares the performance of the *C. freundii* IIPDR3 with other wild *Citrobacter* strains reported in the state of the art, wherein CG was used as the substrate and fermentation was carried out under batch mode.

Table 12: Comparative evaluation of *C. freundii* IIPDR3 with other wild *Citrobacter* strains reported with CG as substrate in a batch fermentation

Wild *Citrobacter* strains	CG procured from (% glycerol)	Initial CG (gL^{-1})	1,3-PDO		By-products formed	References
			T (gL^{-1})	P ($gL^{-1}h^{-1}$)		
Citrobacter freundii ATCC 8090	Petrobras, Brazil (80%)	16	4.35	0.18	acetate and succinate	[48]
Citrobacter werkmanii	CG from rapeseed-derived biodiesel (80%)	20	9.6	-	-	[13]
Citrobacter freundii VK-19	ADM, Germany (81%)	20.1	10.6	1.06	acetate and lactate	[16]
Citrobacter freundii VK-19	Petrobras, Brazil (77.7%)	31.6	15.7	0.88	acetate, ethanol formate, lactate & succinate	[17]
Citrobacter freundii FMCC-8		20.9	10.7	0.63		
		41.6	18.6	0.60		
Citrobacter freundii NRRL-B 2645	Verd SA, Greece (74.9%)	51.5	28.8	0.62	Acetate and Lactate	[49]
Citrobacter freundii IIPDR3	CBDA, India **(42.14%)**	**21.04**	**9.65**	**1.40**	Acetate, lactate* and succinate*	This study
		42.55	**11.11**	**1.05**		

Note: T-titer; P-productivity; CG-crude glycerol; *represents qualitative detection of organic acids

Despite lesser purity of glycerol in the CG, the strain showed appreciable 1, 3-PDO titers and productivity compared to other *Citrobacter* strains. Recently, *Citrobacter freundii* NRRL-B 2645 accumulated exceptionally high 1,3-PDO titres (28.8 g/L) from 51.5 g/L of initial CG under anaerobic conditions, when grown in a 3.6L bioreactor [49].As evident from Table 12, most of the prior art studies have used CG having a glycerol content of 75% and above. However, in the present case, the glycerol content in CG was merely 42.14% and contained varied impurities (salts, methanol, unreacted fatty acids) as discussed elaboratively [50]. With increasing glycerol conc. (40 and $60gL^{-1}$), these impurities also increased. It is possible that some of these impurities reached their threshold inhibitory concentration, impeding

glycerol uptake and halting 1,3-PDO production at higher CG concentrations (Table 11).The pretreatment of CG might become imperative at a later stage and authors are optimistic that this strategy would significantly improve 1,3-PDO titers.

Future studies will be targeted towards enhancing TYP matrices of the strain at ≥50 g/L initial glycerol. Fed-batch studies with PG and pretreated CG (PCG) will be attempted to improve the TYP matrices of the strain. If necessary, in the second stage TYP matrices of 1,3-PDO will be improved using the Design of Experiments (DoE). Alternately, evolutionary engineering approaches may also be considered to increase the strain's tolerance towards organic acids and glycerol. Deciphering the maximum potential of *C. freundii* may be rewarding as it has an advantage over popular natural 1,3-PDO producers namely *Klebsiella* and *Clostridium*. If *Klebsiella* is a BSL-2 microbe representing high pathogenicity, *Clostridium* demands obligate anaerobiosis. Unlike these microbes, the current strain is BSL-1 bacterium which produces appreciable titers of 1,3-PDO under microaerobic conditions. Above all, pH studies prove the alkalophilic nature of the strain, making it a lucrative candidate for biodiesel-derived waste glycerol.

4. Conclusion

The present study concluded with the fact that under batch mode, *C. freundii* IIPDR3 transformed 39.57 ± 0.7 gL^{-1} of PG to 18.04 ± 0.76 gL^{-1} 1,3-PDO after optimizing various media components and physiochemical parameters using the OVAT approach. To the best of the author's knowledge *C. freundii* IIPDR3 is the first reported alkalophilic *Citrobacter* strain. Post-optimization and by replacing NaOH with liquor NH_3 as a neutralizing agent, the 1,3-PDO productivity improved significantly from 0.82 to 1.72$gL^{-1}h^{-1}$. When the robustness of the strain was assessed on CG at 21.04 ± 0.15 gL^{-1}, 9.65 ± 0.13 gL^{-1}1,3-PDO was produced after nearly complete utilization of glycerol and productivity being 1.4 $gL^{-1}h^{-1}$.

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Author's contribution

Mridul Budakoti: Conceptualization, Investigation, Methodology, Formal analysis, Writing-Original Draft; Shivani Bhandari: Investigation, Formal analysis; Vinod Kumar: Review & Editing; Deepti Agrawal- Conceptualization, Supervision, Writing-Original Draft and Detailed Analysis

Competing Interest

The authors declare no competing interests

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Unveiling the potential of a novel alkalophilic Citrobacter freundii IIPDR3 strain in valorizing glycerol to 1,3-propanediol

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