

Sweet sorghum juice as an alternative carbon source and adaptive evolution of *Lactobacillus brevis* NIE9.3.3 in sweet sorghum juice and biodiesel derived crude glycerol to improve 1, 3 propanediol production

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Abstract

Sweet sorghum juice (SSJ) is considered as an ideal complement for carbon supplement in ethanol fermentation for its ease of cultivation. Extraction of fermentable sugars from the sweet sorghum is very simple in comparison to lignocellulosic biomass. Hence sweet sorghum is a suitable candidate as a feedstock. In the present study, batch fermentations were carried out using *Lactobacillus brevis* NIE9.3.3, a facultative anaerobe, isolated through onsite enrichment technique to produce 1,3-propanediol and other co-metabolites, in glucose-glycerol co-fermentation. To make the process more sustainable, the glucose supplemented in the production media was replaced with SSJ. The supplementation of 40 g/L sorghum juice and 40 g/L crude glycerol in the production media resulted in the titre of 25.9 g/L 1, 3-PDO with a volumetric yield of 0.64 g_{1,3-PDO}/g_{glycerol}. Adaptation of the microorganisms and cultivation under controlled conditions of temperature and substrate concentrations followed by selection was carried out, that is, adaptive evolution. Among the adaptively evolved strains, PD 20.100 has displayed better performance and increased the titres up to 38.4 g/L with a volumetric yield of 0.64 g_{1,3-PDO}/g_{glycerol}. The industrial applicability of the fermentation process was checked in pilot scale and the production yield was comparable with that of flask scale. The utilization of agricultural and biodiesel industrial waste for the production of 1,3-PDO by a GRAS organism and the strain improvement through ALE for better utilization and conversion of substrates indicates the novelty of this work.

Keywords: Sweet sorghum juice; 1,3-PDO; Adaptive evolution; *Lactobacillus brevis* NIE9.3.3; Co-fermentation

1. Introduction

The conversion and utilization of renewable feedstock for the production of valuable materials is an important research area in recent years because of the decreasing supply of non-renewable resources, increasing global energy demand and negative environmental impact (Tabah et al., 2016). Glycerol is a by-product of biodiesel industries where 1 ton of raw glycerol is produced for every 10 tons of biodiesel. The crude glycerol produced from the biodiesel industry contains impurities such as alcohols, salts, heavy metals and water (Johnson and Taconi, 2007). Sweet sorghum juice is a well-established carbon source for the production of ethanol. SSJ contains an adequate amount of sugar content which can be readily utilized by the microbes for fermentation. Sweet sorghum, from which SSJ was derived, is considered as an eligible option as a feedstock for fermentation processes due to the high content of sugars and cultivation conditions. It can also withstand the driest seasons which make it highly drought-resistant (Sree et al., 1999; Woods, 2000). The management of by-products from the biodiesel industry and unexpended agro-residues are better options as the carbon source in microbial growth media for the production of metabolites such as citric acid, succinic acid, propionic acid and 1,3-PDO (Kośmider et al., 2010). Among these chemicals, 1, 3-propanediol has attracted more attention worldwide due to its vast application.

1,3-PDO has numerous applications in polymers, cosmetics, foods and adhesives (Cheng et al., 2007). The major application of 1,3-PDO is the synthesis of polytrimethyleneterephthalate (PTT), a new group of polyester having application particularly in textile and fibre industries due to its superior stretching and stretch recovery characteristics (Kurian, 2005). The chemical synthesis of 1,3-PDO has many disadvantages in that it requires high pressure, high temperature and catalysts. So as an alternative to chemical synthesis, biological methods can be adopted.

DuPont is producing 135 g/L of 1, 3- PDO using a genetically modified *E. coli* strain using corn sugar as the sole carbon source (Nakamura and Whited, 2003). Bioconversion of glycerol to 1,3-PDO involves a coupled oxido-reductive process, yielding more reducing equivalents and energy molecules like ATP for growth and secondary metabolite production (Vivek et al., 2016). The production of 1,3-PDO from glycerol is a reduction process that consumes 1 mol NADH per mol of 1,3-PDO. In the reductive pathway, the first enzyme, adenosylcobalamin dependent glycerol dehydratase, GDHt (E.C.4.2.1.3.0), removes a water molecule from glycerol to form 3-HPA (an intermediate in 1, 3-propanediol synthesis) by a radical mechanism (Sun et al., 2003). 3-HPA is further reduced to 1,3-PDO by NADH+ H⁺-dependent enzyme 1, 3-propanediol dehydrogenase (1, 3-propanediol-oxidoreductase) (Ahrens et al., 1998). The conversion of glycerol to dihydroxyacetone is catalyzed by NAD⁺-dependent glycerol dehydrogenase in the oxidative pathway. The phosphorylation of the latter product is catalysed by the enzyme dihydroxyacetone kinase and finally glycolysis (Daniel *et al.*, 1995; Zhu et al., 2002). The by-product formation is different based on the organisms and culture conditions. The change in NADH/NAD⁺ ratio can influence the metabolite distribution as mediated by the oxidation state of the carbon used (San et al., 2002). *Lactobacillus* sp. which are non-pathogenic, having probiotic applications in food and pharmaceutical industries are found to be native producers of 1,3-PDO. Among them, *L. reuteri*, *L. panis* and *L. diolivorans* were reported for 1,3-PDO production, whereas *L. diolivorans* was considered being industrially relevant strain producing high titres of 85 g/L 1,3-PDO (Pflügl et al., 2014).

Various physical and chemical factors affect product formation during fermentation (Drozdzyńska et al., 2011). The microbial processes have certain disadvantages such as high cost, low yields, productivity and tedious downstream processing. The cost of the process

depends on the cost of the organic and inorganic supplements in the production media, cost of operation and efficiency of bioconversion. Selection of the cheap raw material and its efficient utilization are the critical factors in the economization of the product (Milessi et al., 2013). To increase the efficiency of the process, adaptive laboratory evolution can opt which reduces the inherent intolerance of the biocatalytic cell towards the contaminants in the feedstock (Dragosits and Mattanovich, 2013). It is performed by allowing the microbial cells to grow at a specific condition for the long term and selecting the strain which fulfils the criteria the best. It has been practised widely for the improvement of secondary metabolites with major industrial applications (Reyes et al., 2014).

In this study, the microbial production of 1,3-PDO by a non-pathogenic strain, *Lactobacillus brevis* N1E9.3.3 using SSJ as a carbon source along with crude glycerol was evaluated. Based on the analysis, pH optimization studies have been done to increase the bioconversion and yield. Adaptive laboratory evolution (ALE) was adopted to improve the substrate uptake by the microbial cell for better production of metabolites. For the best results, pH studies were done on the selected ALE strain. Batch fermentation was carried out in Schott bottles under anaerobic condition with varying concentrations of glucose and crude glycerol to check the production of 1,3-PDO and other co-metabolites. The comparative analysis between wild type and evolved strains were done. To check the reproducibility of results from 100 mL flasks to pilot scale, batch fermentation has been carried out in a bioreactor. This study validates the efficiency of *L. brevis* N1E9.3.3 to utilize SSJ as the carbon source to form end products such as 1, 3-propanediol, lactic acid and acetic acid. The improved efficiency of ALE strain for the better 1,3-PDO yield was also evaluated.

2. Materials and methods

2.1. Microbial Strains

Lactobacillus brevis N1E9.3.3, a potent LAB strain to produce 1,3-PDO along with lactic acid and acetic acid was isolated from onsite enrichment technique (Vivek et al., 2016) and adaptively evolved strains from this LAB species have been used in this study.

2.2. Adaptive laboratory evolution of *L. brevis* N1E9.3.3

For ALE, the wild type strain was cultured in plates of modified MRS (mMRS) media with higher concentrations of glycerol and glucose. Isolation criteria were the fast growth in agar plates with the same glucose and glycerol concentrations in each plate with 60 g/L and 80 g/L. Strains underwent 20-day adaptive evolution where the incubation period of each generation was 24 hrs. The pure cultures were named as PD isolated strains followed by generation number and the colony number in the respective individual mMRS plate. The isolated colonies were then inoculated to the production media to analyze the 1,3-PDO production.

2.3. Substrates and growth conditions

Wild type strain and ALE strains were isolated and maintained in commercial MRS (de Man, Rogosa and Sharpe) medium. Isolated cells were then plated for maintenance. For long term storage, the cells were centrifuged and kept at -20°C with 40% (w/v) glycerol. The composition of mMRS media for the production of 1,3-PDO and other by-products is as discussed by Vivek et al. in 2016. The pH was adjusted to 8.5 using 5 N NaOH. Incubation was done at 37°C, 200 rpm under anaerobic condition by purging nitrogen at a flow rate of 0.2 vvm. In this study, sweet sorghum juice, obtained upon request from IIMR, Hyderabad has been used as the carbon

substrate to replace glucose in the production media. It was then stored at 4° C for future use. pH optimization studies were carried out by manually adjusting the pH every 24 hrs.

2.4. Experimental procedures

The effect of SSJ concentration with respect to glycerol concentrations were optimized in shake flask experiments in batch mode of operation. The varying concentrations of SSJ (20 g/L - 80 g/L) were used to analyse the 1,3-PDO and other by-product formation by wild type. The pH was maintained manually using 10 N NaOH in pH optimization study accordingly. Batch fermentations were carried out under anaerobic conditions. The samples were taken for HPLC analysis after 96 hrs of incubation.

A bioreactor experiment was carried out in a 3 L Infors reactor using suspended cells with pH stability. 1L of the production media containing 60 g/L of glucose with 60 g/L of glycerol was inoculated with 5% of 16 hrs old inoculum. The experiment conditions were maintained at 37°C, 200 rpm. pH was maintained automatically with 5 N NaOH. Sampling was done after 96 hrs for HPLC analysis.

2.5. Analytical methods

The concentration of acetic acid, lactic acid, ethanol, 1,3-PDO and residual glycerol in the sample was analyzed using High-Performance Liquid Chromatography (HPLC (Shimadzu prominence UFLC) Rezex-ROA Organic acid column, 300 × 7.8mm (Phenomenex), RI detector under the following conditions: mobile phase: 0.01N H₂SO₄ with a flow rate of 0.6 mL/min, sample volume - 20 µL and column temperature 65°C. The standards were prepared in 50 g/L (w/v) concentration. It was then diluted to different concentrations (10, 20, 30, 40, 50 g/L) and the standard graph was plotted. From the regression equation obtained, the concentrations of the

products were determined. The glucose and fructose concentration in the sweet sorghum syrup was determined by HPLC: degassed Milli-Q water as mobile phase with the conditions: Flow rate: 0.6 mL/min, Phenomenex Rezex RPM-Monosaccharide Pbp2 (300 × 7.8 mm) column, 80°C column temperature, RI detector.

2.6. Data calculation

The concentrations of end products in terms of yield i.e., bioconversion of gram substrate converted to gram product was calculated using equation 1;

$$Y_{\text{g of 1,3-PDO/g of SSJ}} = \frac{C_{\text{1,3-PDO (g/L)}}}{C_{\text{glycerol (g/L)}}} \dots\dots\dots (1)$$

3. Results and discussion

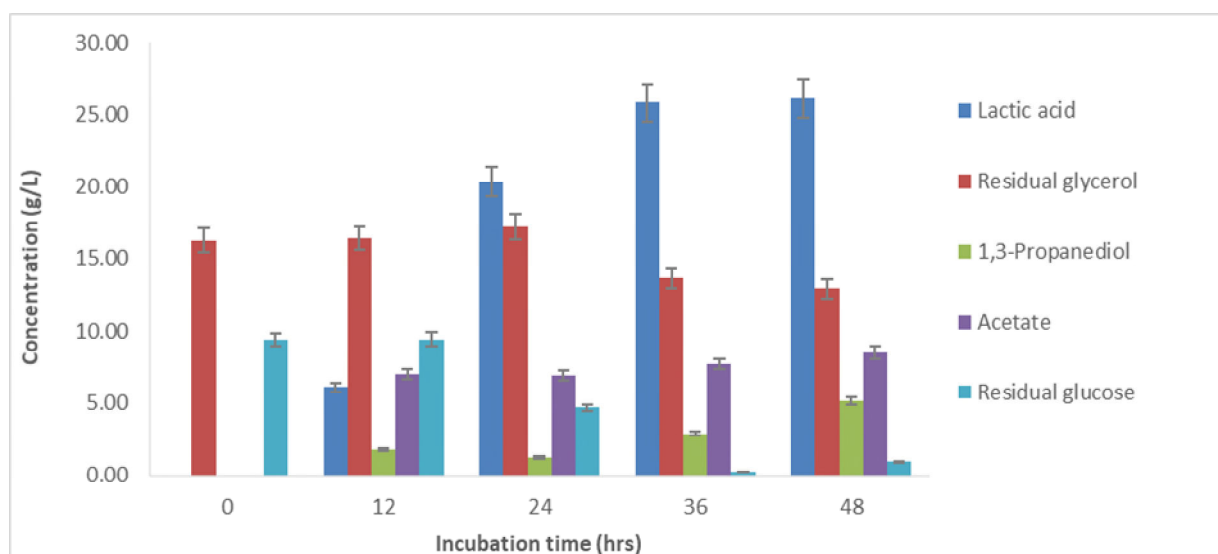
3.1. Effect of SSJ as carbon source

SSJ has been considered as an alternative source of carbon for biotransformation processes in various studies. Batch mode of operation has been carried out to analyze the efficiency of SSJ to replace glucose in the production media to make the fermentation more sustainable. Several researchers used SSJ as the carbon source for ethanol fermentation by adopting various approaches (Laopaiboon et al., 2021; Wu et al., 2010). Lipid synthesis by *Schizochytrium limacinum* SR21 and L-lactic acid synthesis by *Bacillus coagulans* are some of the examples which proves the ability of SSJ as a carbon source for the production of important metabolites (Liang et al., 2010; Olszewska-Widrat et al., 2019). In microbial fermentation processes, glucose is the simplest sugar that can be easily metabolized by a bacterial cell for proliferation and energy generation. The sugar content in the SSJ was analyzed by HPLC and it contains 47 g/L fructose and 78.4 g/L glucose which makes it one of the suitable candidates for glucose

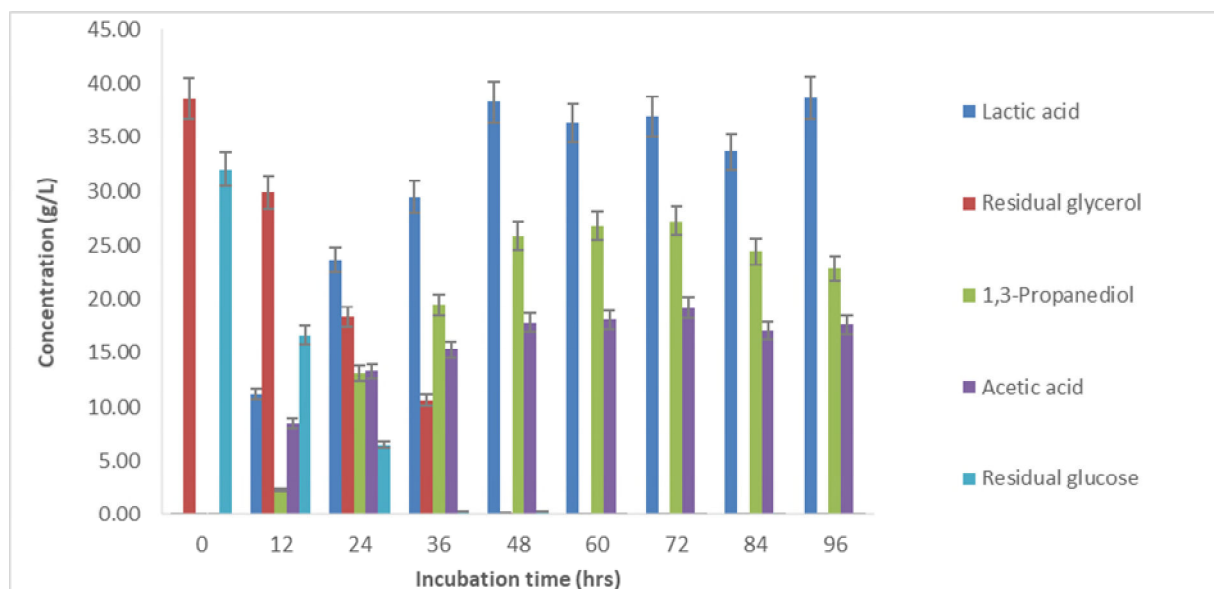
substitute in production media. Along with SSJ, crude glycerol has been used as the carbon source in this bioconversion process. Glycerol content is $\geq 99.0\%$ in purified glycerol whereas, in crude glycerol, it changes according to the presence of other components which includes approximately 15–20% water, 5–7% salt, 0.5–2% organic compounds, and a very small amount of methanol and sodium hydroxide (Domingos et al., 2019). If purified glycerol is used, there will be a slight increase in the production of 1,3-PDO in lower concentrations of glycerol. But in higher concentrations, a drop of production of 1,3-PDO will occur based on the tolerance level of the microbial cell. There are reports regarding the production of 1,3-PDO along with other byproducts when glucose crude glycerol co-fermentation strategy was adopted for this particular strain (Vivek et al., 2016).

To evaluate the production and yield of 1,3 PDO and hence for the optimization, batch fermentation with sorghum juice as a carbon source was done where varying concentrations (20 g/L-80 g/L) of sorghum juice and crude glycerol in a 1:1 ratio have been used. The period of incubation was a minimum of 48 hrs with pH stability. The results indicate that 1,3-PDO productivity is significantly influenced by pH (Yen et al., 2014). To obtain the maximum production, the experiment was carried out by maintaining the pH at 8.5 using 5 N NaOH manually with a time interval of 24 hrs. From the data, it was observed that the microbial cell utilized glucose from SSJ for cell growth but the uptake of glycerol was limited and thus resulted in the low concentration of 1,3-PDO. When the microbial cells were supplied with a lower concentration of SSJ, it resulted in the carbon flux towards the oxidative branch of the metabolic pathway. After 48 hrs of incubation, lower concentrations of SSJ and crude glycerol were well consumed by the bacterial cells compared to higher concentrations of carbon sources. The batch fermentation with 40 g/L crude glycerol and 40 g/L SSJ as carbon sources in the production

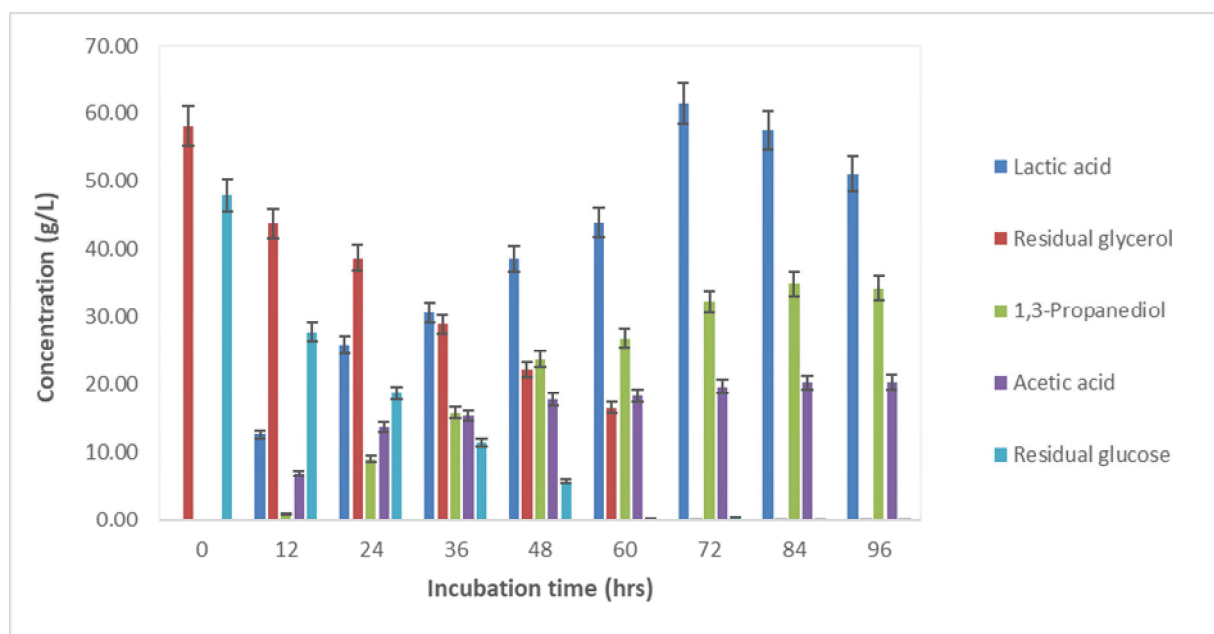
media showed the highest glycerol utilization and maximum 1,3-PDO production with a titre of 25.9 g/L 1,3-PDO and 0.64 g_{1,3-PDO}/g_{glycerol} of product yield. When the media supplemented with higher concentrations of carbon sources, the production of all the metabolites was abated. This indicates that the microbial cell is unable to utilize higher concentrations of SSJ and crude glycerol. The results of the experiments were illustrated in Fig. 1. As noted by Yang et al., 2012, the accumulation of impurities existing in the natural raw feedstock can be the reason for the above plot. The conversion of sugar content in SSJ was better utilized than glycerol since the unbalanced carbon flux towards the oxidative pathway resulted in the increased lactic acid production. There was a positive correlation with the production of lactic acid and acetic acid which once again validates the above observation.



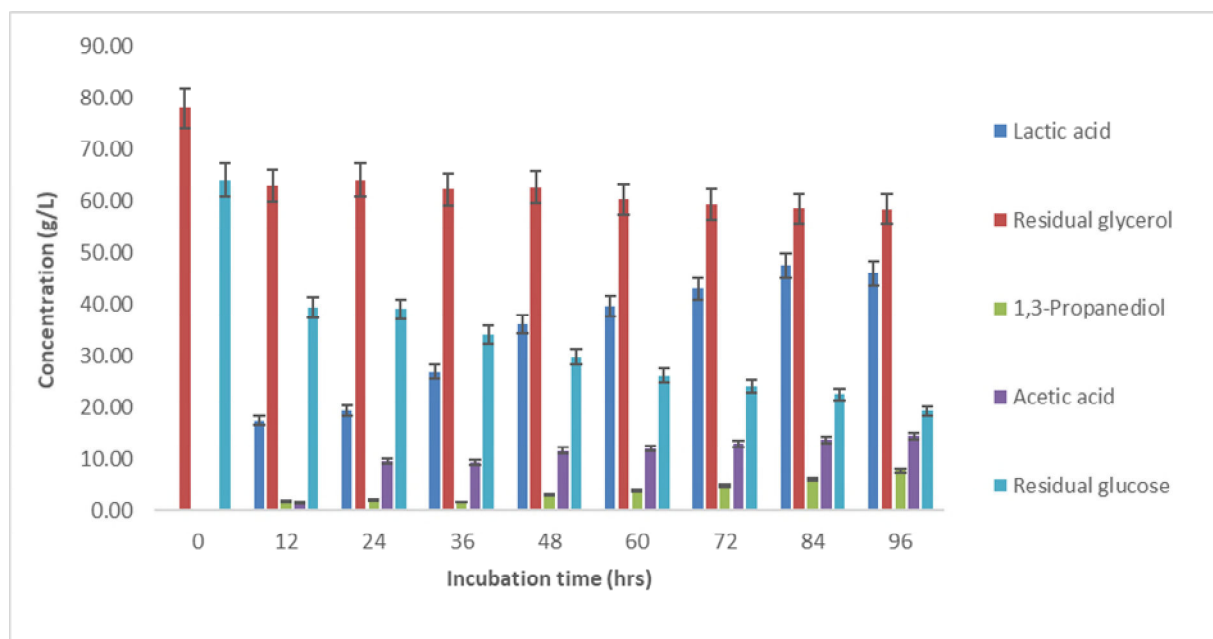
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Fig.1. Effect of varying concentrations of SSJ for the production of 1,3-PDO and other metabolites with crude glycerol. A) 20 g/L SSJ and 20 g/L crude glycerol, B) 40 g/L SSJ and 40 g/L crude glycerol, C) 60 g/L SSJ and 60 g/L crude glycerol and D) 80 g/L SSJ and 80 g/L crude glycerol

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

The concentration of H^+ ions plays a major role in the overall efficiency of a biological process. pH is one of the environmental factors having an important role in the production of diols. It influences the regulation of enzymes in both the oxidative and reductive pathways and alters NAD^+ regeneration pathways (Sittijunda and Reungsang, 2017). To analyse the effect of pH, experiments were carried out in the pH range of 5.0 to 8.5 in batch mode. The pH was adjusted manually to the required value by the addition of 10 N NaOH. The samples were taken for HPLC analysis after 96 hrs. Incomplete utilization of carbon sources was the major problem that has to be tackled down for increasing the efficiency of the substrate conversion. The results conveyed

the complete utilization of both SSJ and crude glycerol occurred after 96 hrs except at pH 5.0. The highest conversion of substrate occurred in pH 7.5 with a production of 16 g/L 1,3-PDO (Fig. 2). At lower pH, the glycerol utilization was compromised which once again proved the effect of pH in maintaining the reducing energy as well as the enzyme activity. Along with 1,3-PDO, lactic acid and acetic acid were produced proportionally as they are the major products of the oxidative pathway.

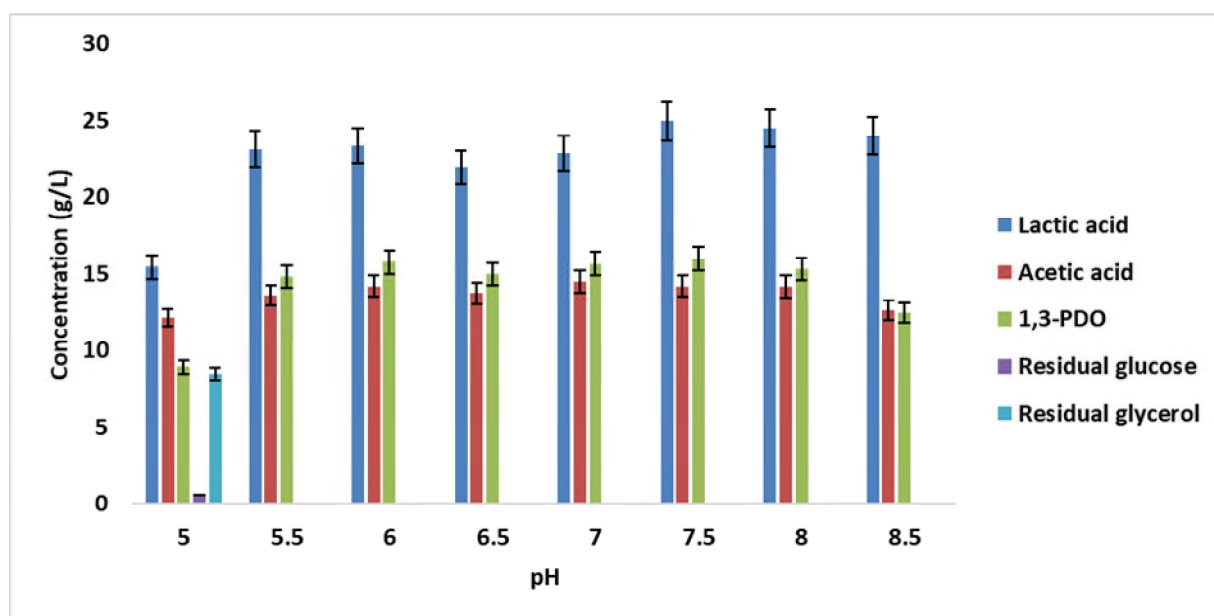


Fig.2. Effect of pH in the production of 1,3-PDO and other metabolites

3.3. ALE and Batch fermentations

Intolerance to substrates lessens the efficacy of product conversion. Hence, efforts have been made by many researchers to improve the tolerance towards carbon sources by adaptation (Jensen et al., 2012; Portnoy et al., 2011). The contaminants in industrial and agro-industrial waste feedstock lead to low yield and productivity due to the intolerance of the microbial cell. To overcome this limitation, adaptive laboratory evolution can be employed which in turn improves the tolerance level of the microbial cells for the better uptake and utilization of substrates. ALE

is having certain advantages such as the need for simple culture media and nutrients for cultivation within lab facilities and comparatively fast results due to fast generation time (Dragosits and Mattanovich, 2013). To obtain a strain with tolerance to higher substrate tolerance, the wild type strain was streaked on MRS media plates supplemented with higher amounts of SSJ and crude glycerol. As mentioned in the materials and methods section, the colonies with the faster growth were selected and the production of 1,3-PDO was analysed. Selected colonies were named PD10.80, PD10.100, PD15.80, PD15.100, PD20.80, PD20.100 based on the generation number and colony number in the plates. From the above-mentioned strains, PD20.80 and PD20.100 selected for further studies due to their fast growth in higher concentrations of SSJ and crude glycerol compared to the other selected strains. To check the efficiency of the evolved strain (selected strain was PD20.100) to convert glucose and crude glycerol to 1,3-PDO and other co-metabolites, pH optimization studies have been carried out in 60 g/L concentrations of each of the carbon sources (Fig. 3). The metabolite profiling was done through HPLC. As observed before, maximum utilization and conversion of substrates to products occurred when the pH of batch fermentation was maintained at pH 7.5. An optimum pH level should be maintained in the fermentation process for a better production rate of metabolites.

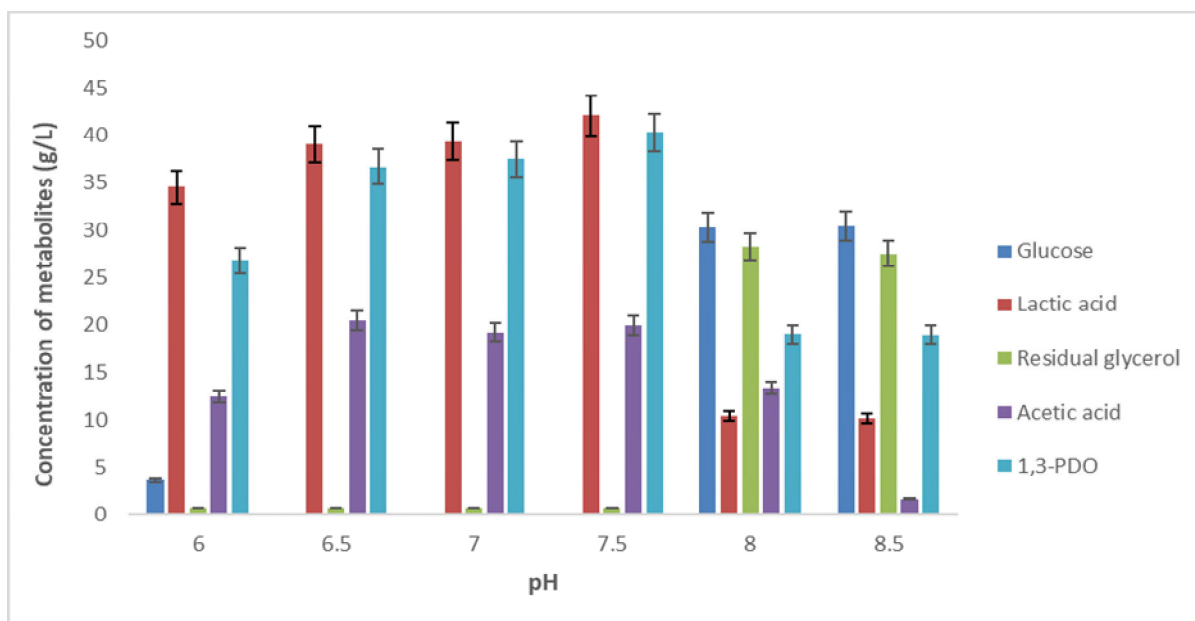
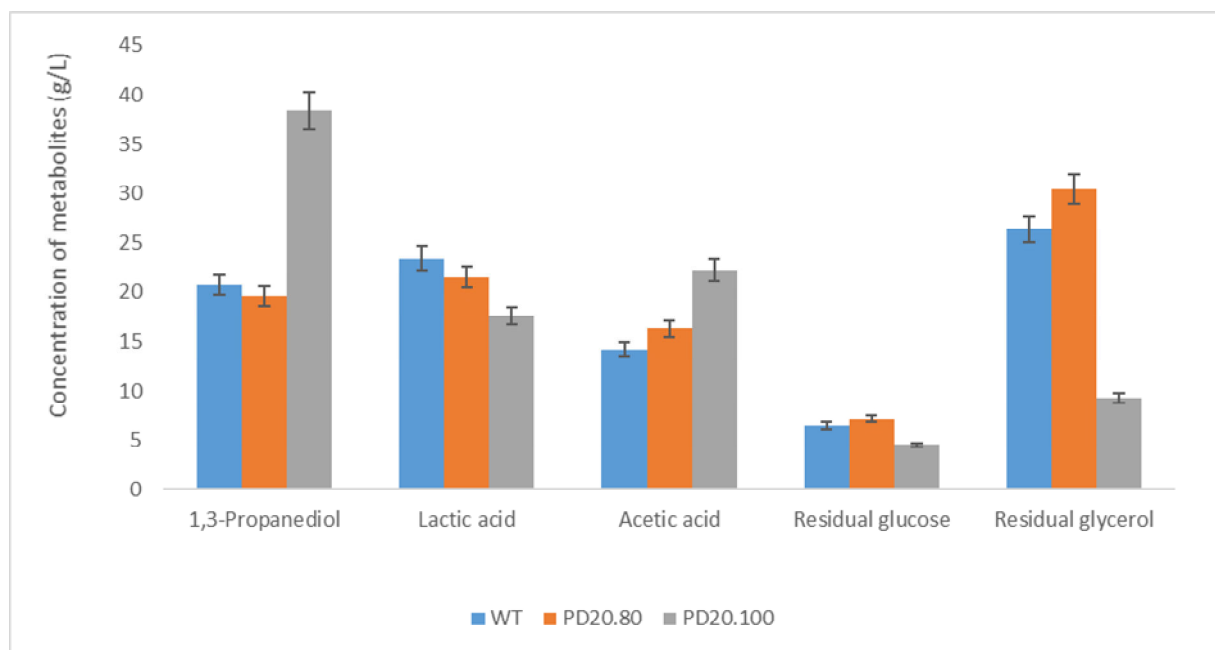


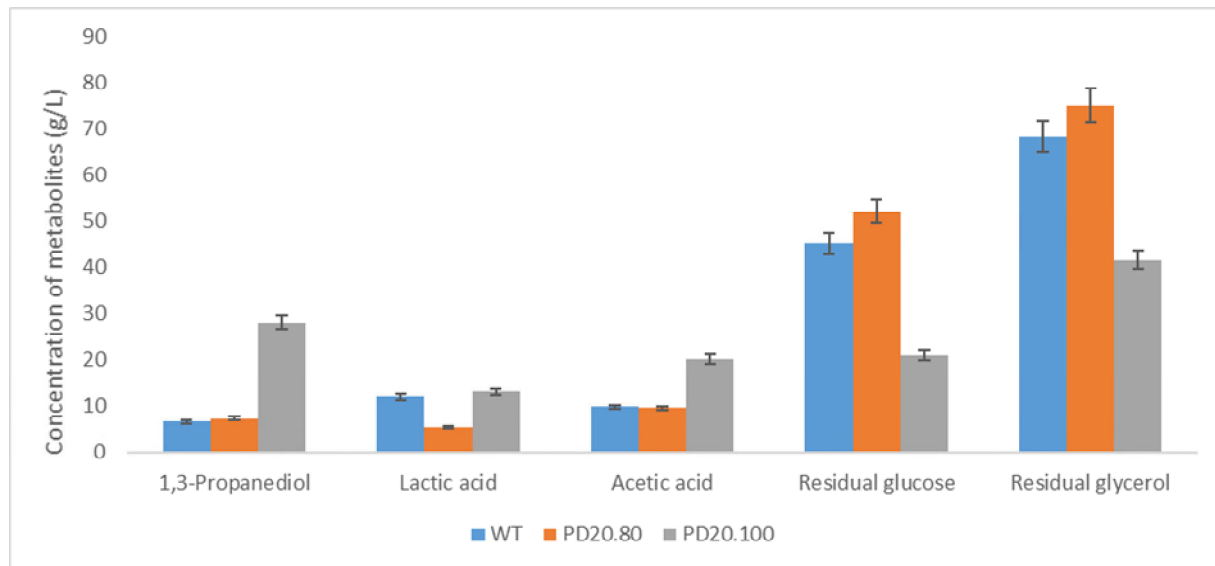
Fig.3. Effect of pH in the production of 1,3-PDO and other metabolites by PD20.100

To check the efficiency of the selected adaptively evolved cultures for 1,3-PDO production and substrate utilization, batch fermentations were done with varying concentrations of glucose and crude glycerol by comparing with the wild type. The operation conditions of experiments were done accordingly as optimized earlier. It has been observed that PD20.100 gained the attributes through ALE to increase the production rate of metabolites. In another study for ethanol fermentation, five cycles of acclimatization resulted in the improved growth rate of yeast cells along with an increase in ethanol yield by 5-fold (Zhang et al., 2015). Here, compared to the wild type, in specific concentrations of carbon source, a 2-fold increase in the production of 1,3-PDO was observed. ALE strain PD20.80 was able to produce 1,3-PDO but the production rate was comparable with that of wild type. Co-fermentation of glycerol and glucose by *Lactobacillus* sp. was reported to increase the biomass and alter the end product profile by suppressing the production of other co-metabolites (Vivek et al., 2016). Different combinations of glucose and

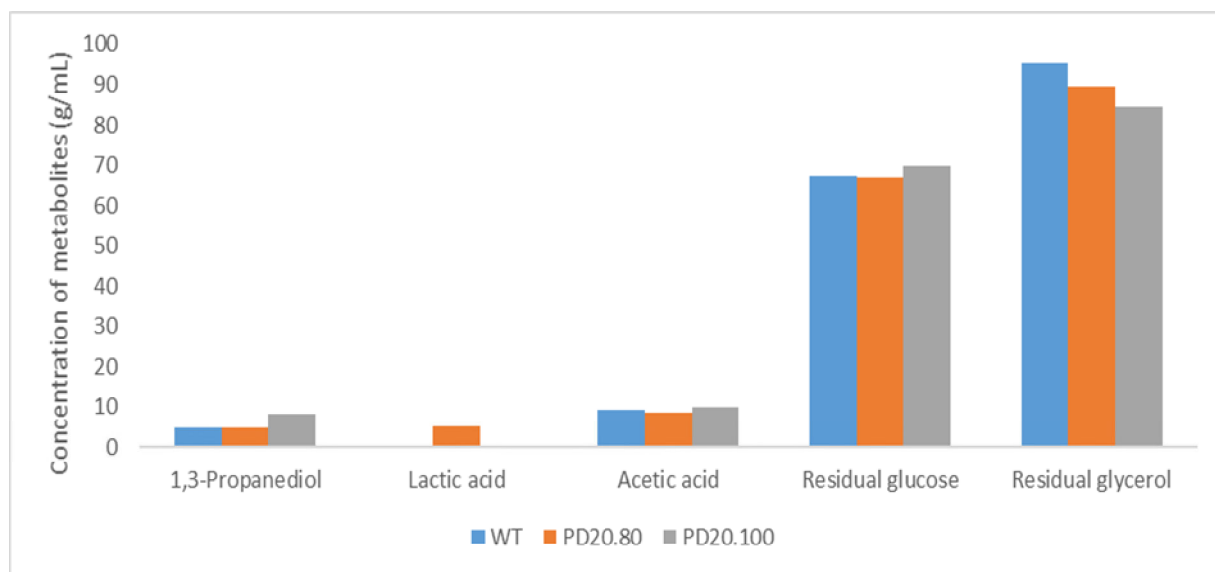
crude glycerol concentrations were selected to find out the optimal conditions for substrate concentration in the fermentation media. Considerable concentrations of substrates or products lead to high osmotic pressure in the fermentation medium and the presence of toxic substances together will lead to the inhibition of product formation (Nicolau et al., 2010). When the production media was supplemented with 60 g/L of glucose and 60 g/L of crude glycerol, PD20.100 showed 0.64 g_{1,3-PDO/g glycerol} whereas, for wild type and PD20.80, the yield was 0.34 and g_{1,3-PDO/g glycerol} and 0.32 g_{1,3-PDO/g glycerol} respectively. The utilization of glycerol was high in the PD20.100 that of the other strains proved that the above strain obtained tolerance towards the carbon sources. From the results, it was observed that while adopting the co-fermentation strategy, the amount of glucose and glycerol should be the same for PD20.100 strain to obtain the maximum yield. When crude glycerol is provided in the production media with twice the concentration of glucose, the yield was reduced from 0.64 g_{1,3-PDO/g glycerol} to 0.068 g_{1,3-PDO/g glycerol}. Another major observation was the reorientation of the metabolic flux of the carbon sources. The results were illustrated in Fig. 3. In the wild type, along with 1,3-PDO, lactic acid was the major product. But in PD20.100, the concentration of acetic acid was higher compared to the lactic acid. From the above observation, it can be concluded that along with the improved carbon source utilization and 1,3-PDO production, PD20.100 have altered carbon flux in the oxidative pathway of glycerol utilization (Fig. 4).



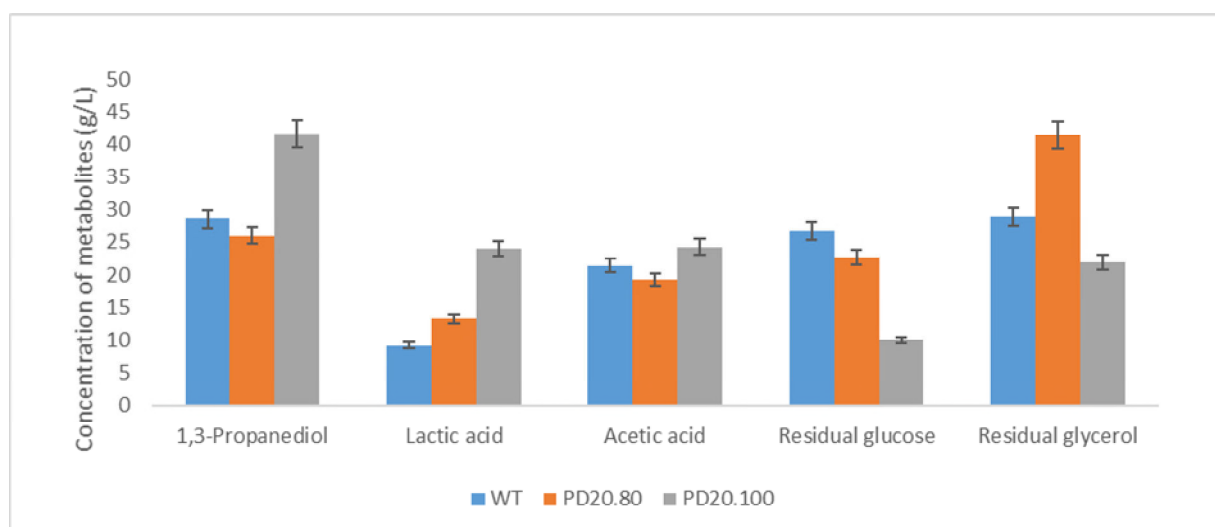
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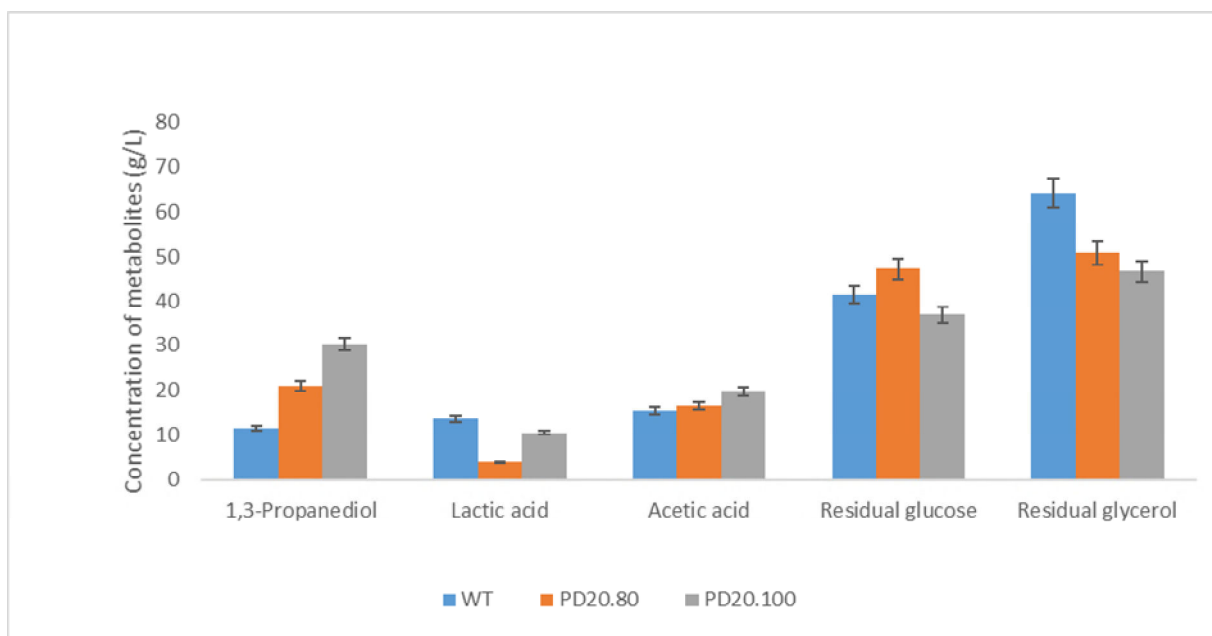
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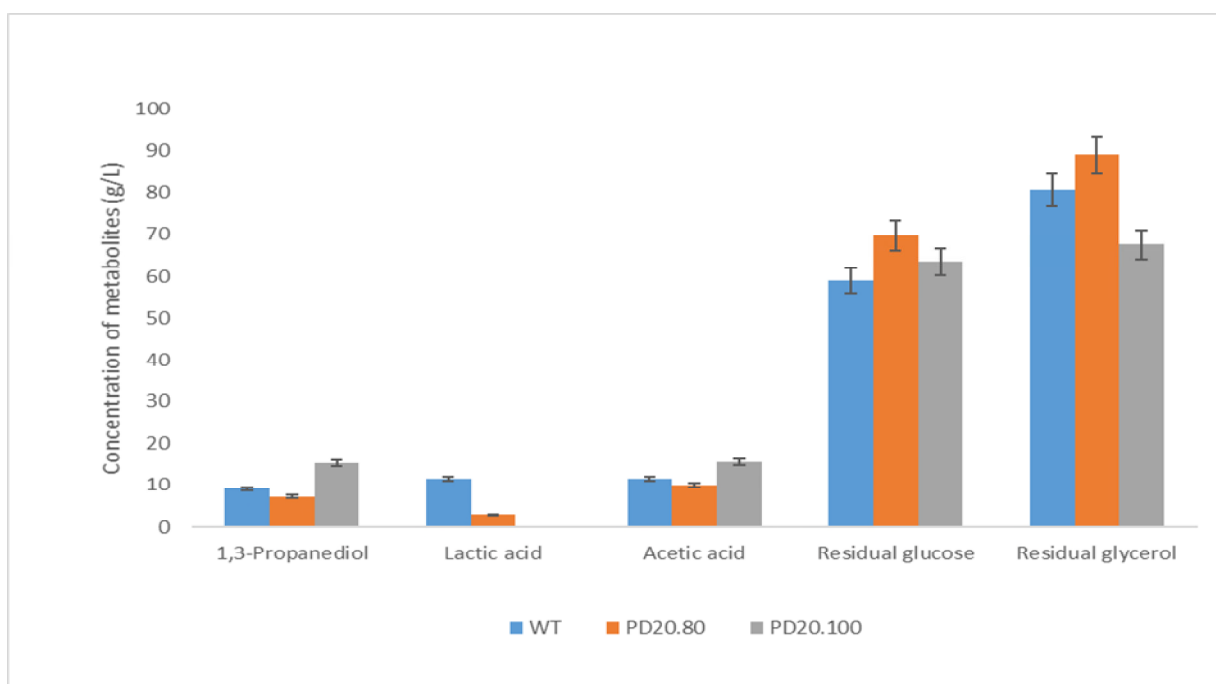
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Fig.4. Metabolite profiling by HPLC after 96 h of batch fermentation by wild type, PD20.80 and PD20.100 in mMRS with various concentrations of glucose and crude glycerol. A) 60 g/L

glucose and 60 g/L crude glycerol, B) 60 g/L glucose and 80 g/L crude glycerol, C) 60 g/L glucose and 120 g/L crude glycerol, D) 80 g/L glucose and 80 g/L crude glycerol, E) 100 g/L glucose and 100 g/L crude glycerol, F) 120 g/L glucose and 120 g/L crude glycerol

For the acceptance of a bioprocess at the industrial level, the production of metabolites should be consistent on a small scale and large scale. To observe the stable level of production on a pilot scale, batch fermentation was carried out. 1 L production media containing 60 g/L crude glycerol: 60 g/L glucose was used for the experiment. After 96 h of incubation, 39.8 g/L of 1,3-PDO was produced with a production yield of 0.66 g_{1,3-PDO}/g_{glycerol}. The production of 1,3-PDO and lactic acid were in proportion with a yield of 0.60 g_{1,3-PDO}/g_{glycerol}.

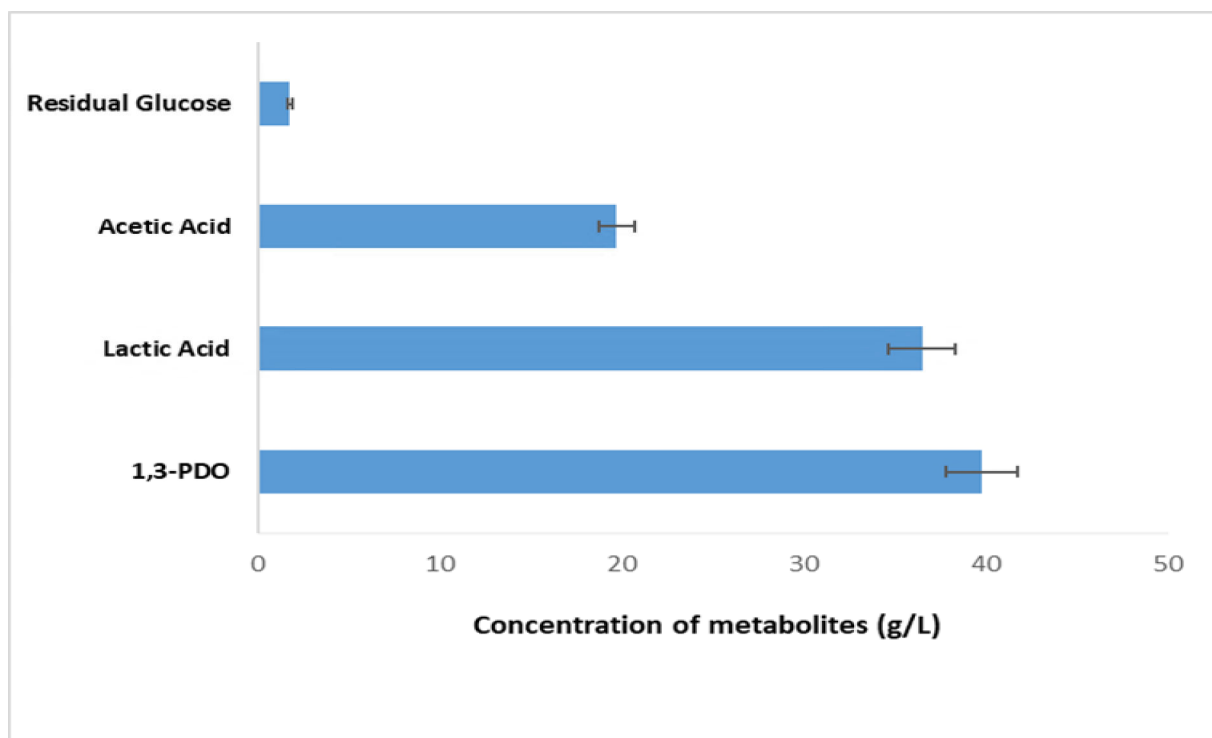


Fig.5. Metabolite profiling by HPLC after 96 h of batch fermentation by PD20.100 in pilot scale in mMRS production media with 60 g/L glucose and 60 g/L crude glycerol.

4. Conclusions

SSJ serves as a potential carbon source in the production media for 1,3-PDO fermentation. In batch experiments, 0.64 g_{1,3-PDO}/g_{glycerol} yield was obtained in production media with SSJ and crude glycerol as carbon sources. However, complete utilization of substrates was ceased due to the presence of inhibitory substances in the feedstock. To reduce the effect, pH optimization studies that were done succeeded in the complete utilization of carbon sources. ALE, as discussed before, improved the tolerance of strains towards the substrates resulted in a better product yield compared to the wild type. At specific carbon sources concentration combination, the 1,3-PDO yield increased by 2-fold and 3-fold. Bioreactor study demonstrated the redundancy of the production rate on a large scale. The study revealed the application of waste residues from agricultural and biodiesel industries to be metabolized biologically to industrially important chemicals. The substrate tolerance and the implementation of ALE to overcome this has been successful in this study and also gives an idea about the operating conditions to produce 1,3-PDO and co-metabolites from glucose and crude glycerol with pilot-scale production.

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