Bioresource Technology 109 (2012) 131–139

Contents lists available at SciVerse ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Use of an osmotically sensitive mutant of *Propionibacterium freudenreichii* subspp. *shermanii* for the simultaneous productions of organic acids and trehalose from biodiesel waste based crude glycerol

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ARTICLE INFO

Article history:
Received 21 October 2011
Received in revised form 1 January 2012
Accepted 10 January 2012
Available online 20 January 2012

Keywords:
Osmotic sensitive mutant
Trehalose
Propionibacterium freudenreichii subspp. *shermanii*
Crude glycerol

ABSTRACT

Recently suitability of crude glycerol for trehalose and propionic acid productions was reported using *Propionibacterium freudenreichii* subspp. *shermanii* and it was concluded that presence of KCl in crude glycerol was the probable reason for higher trehalose accumulation with crude glycerol medium. To further improve trehalose production, an osmotic sensitive mutant of this strain (non-viable in medium with 3% NaCl) with higher trehalose yield was isolated. In mutant, trehalose yields achieved with respect to biomass and substrate consumed (391 mg/g of biomass, 90 mg/g of substrate consumed) were three and four times higher, respectively as compared to parent strain when crude glycerol was used as a carbon source. Other major fermentation products obtained were propionic acid (0.42 g/g of substrate consumed) and lactic acid (0.3 g/g of substrate consumed). It was also observed that in mutant higher activity of ADP-glucose pyrophosphorylase was probably responsible for higher trehalose accumulation.

1. Introduction

A large amount of biodiesel waste produced from biodiesel manufacturing industries pose a significant environmental risk (Yazdani and Gonzalez, 2007; Pyle et al., 2008). Thus many investigations reported utilization of this crude glycerol for various alternative uses like combustion, composting and biological conversions. Similarly, microbial conversion of crude glycerol into value added products is an alternative way for utilisation of this waste and it seems to be economically attractive. Several microbial products were produced from crude glycerol like 1,3-propionediol using *Clostridium* and *Klebsiella* (Himmi et al., 1999; Hiremath et al., 2011), hydrogen from *Enterobacter aerogens* (Ito et al., 2005), succinic acid using *Anaerobiospirillum succiniciproducens* (Lee et al., 2001) omega-3 polyunsaturated fatty acids from algal fermentation (Pyle et al., 2008), docosahexaenoic acid using *Schizochytrium* (Ethier et al., 2011), surfactin (Fonseca de Fariaa et al., 2011) and ethanol (Oh et al., 2011), respectively. In the present study, crude glycerol derived from biodiesel waste was used as a carbon source for the production of trehalose together with organic acids like propionic and lactic acids using an osmotic sensitive mutant of *Propionibacterium freudenreichii* subspp. *shermanii*.

Trehalose is a non-reducing sugar which is widely distributed in microbes (Argüelles, 2000). Trehalose is accumulated in microbes under environmental stress conditions including osmotic stress (Argüelles, 2000; Cardoso et al., 2004). The physiological roles of trehalose in bacterial cells include not only as a reserve carbohydrate, but also as a protectant against diverse adverse conditions including temperature, freezing and high osmotic pressure (Argüelles, 2000; Chi et al., 2009). On the other hand, trehalose as a sugar with nutraceutical value have many applications like as cryopreservative, effective component in cosmetics, stabilizer in clinical reagents and as preservative (Argüelles, 2000; Elbein et al., 2003; Cardoso et al., 2004; Chi et al., 2009). Trehalose synthesis is well known through OtsAB pathway in yeast and most of microbes (De Smet et al., 2000; Cardoso et al., 2007). OtsA (trehalose-6-phosphate synthetase) synthesizes trehalose-6-phosphate from UDP-glucose and glucose-6-phosphate and this trehalose-6-phosphate is further hydrolysed to trehalose by OtsB (trehalose 6-phosphate phosphatase) (De Smet et al., 2000). Another pathway is TreYZ pathway in which malto-oligosaccharides are converted into trehalose by consequent activities of TreY and TreZ enzymes (De Smet et al., 2000). The third enzyme is trehalose synthase (TreS) which interconvert maltose to trehalose (Cardoso et al., 2007).

Propionic acid is considered as an important chemical intermediate for the synthesis of cellulose fibres, herbicides, perfumes, pharmaceuticals and food preservatives (Zhang and Yang, 2009). Propionic acid is currently manufactured via petrochemical process

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doi:10.1016/j.biortech.2012.01.039

which requires petroleum products (non-renewable). However, rise in oil prices and demands for bio-based chemical products have generated significant commercial interests on producing propionic acid and other chemicals from bio renewable feedstock, including agricultural and industrial wastes (Zhang and Yang, 2009).

The production of trehalose is known through many enzymatic and fermentation methods (Schiraldi et al., 2002). Similarly, the uses of maltose, sucrose and glucose as carbon source for trehalose productions are well known (Chi et al., 2009). However, recently it was reported that crude glycerol was more suitable for trehalose and propionic acid productions in comparison to pure glycerol from *Propionibacterium freudenreichii subspp. shermanii* (Ruhal et al., 2011). But improvement in the process was needed to achieve higher trehalose yield. It was also concluded that the presence of KCl in the crude glycerol as impurity was probably responsible for enhanced accumulation of trehalose in the microbe (Ruhal et al., 2011). In particular, accumulation of higher trehalose under osmotic stress was reported previously (Cardoso et al., 2007). Thus it was envisaged to improve trehalose accumulation by isolating a mutant which should be osmotically more sensitive. This mutant was used for enhanced trehalose production from crude glycerol as a carbon source. Trehalose yield, other metabolites like propionic acid and lactic acid yields obtained with this mutant from crude glycerol are compared with parent strain.

2. Methods

2.1. Bacterial strain, media composition, biodiesel waste preparation and batch fermentation

The strain *Propionibacterium freudenreichii subspp. shermanii* NCIM 5137 was procured from NCL Pune India. For trehalose production study, the fermentation media reported by Cardoso et al. (2004) was used with slight modification. Composition of the complex media are as follows: tryptone (10 g L^{-1}), peptone (10 g L^{-1}), yeast extract (1 g L^{-1}), K_2HPO_4 (0.25 g L^{-1}), vitamin solution (20 ml L^{-1}) and carbon source (20 g L^{-1}). Filter sterilized vitamin solution was added in the autoclaved sterile media and had the following composition, biotin (1.1 mg L^{-1}), folic acid (1.1 mg L^{-1}), PABA (110 mg L^{-1}), riboflavin (110 mg L^{-1}), pyridoxine (220 mg L^{-1}), thiamine (220 mg L^{-1}), niacin amide (220 mg L^{-1}).

To evaluate the suitability of crude glycerol for trehalose, propionic and lactic acids productions, biodiesel waste was used which was prepared from base catalysed trans-esterification reaction of soya bean oil which was described elsewhere (Ruhal et al., 2011). The composition of crude glycerol and pre-treatment method were same as described elsewhere (Supplementary Table 1) (Ruhal et al., 2011).

For batch reactor and static flask studies, pre-treated crude and pure glycerol were used as carbon source. The autoclaved nitrogen sources tryptone (1%), peptone (1%), yeast extract (0.1%), and phosphate (0.025%) were added separately. Static flask studies were done in 500 ml Erlenmeyer flasks with 200 ml working media (initial pH 6.8) and incubated at 30 °C. Two litres, fermenter (Model BioFlo 110, with working volume of 2 L manufactured by New Brunswick Scientific Co. Inc.) was used for all batch fermenter studies; all the media constituents (except vitamins solution) were autoclaved in the fermenter except carbon source which was autoclaved separately. Carbon source, filter sterilized vitamin solution and 100 ml of inoculums were added aseptically. The pH was maintained at 6.8 by automatic addition of 2 M NaOH while dissolved oxygen was maintained at around 5–10% of air saturation by automatic passing of nitrogen gas at agitation rate of 200 rpm. The fermentation was carried out at 30 °C. Samples were collected at regular interval and rapidly centrifuged at low temperature (4 °C). The cell pellet and supernatant were stored at −80 °C till further use. Cell pellet was used for trehalose quantification while supernatant was used for lactic acid, propionic acid and glycerol quantifications.

2.2. Isolation of osmotic sensitive mutant

Twenty-five milliliters of culture broth was taken from mid exponential growth phase of *P. shermanii* NCIM 5137 under sterilised conditions. It was centrifuged and bacterial cell obtained as pellet were used for mutation study. For chemical mutagenesis 8% EMS was dissolved in 5 mM phosphate buffer (pH 7). The bacterial cell was suspended in the solution of 8% EMS and incubated for 45 min at 37 °C. After incubation cell pellet was washed thrice with $Na_2S_2O_3$ to neutralize the effect of mutagen. Further, different dilutions of bacterial cell from 10^{-1} up to 10^{-8} were incubated in agar plates with glucose as carbon source. From the dilution plates, single colony was picked and further replica plated on agar plates with glucose as carbon source containing 1%, 2%, 3%, 4% and 8% NaCl, 0.01%, 0.05% SDS and plates without SDS and NaCl. Colonies which did not grow on NaCl plates and SDS plates were selected for trehalose production determination.

2.3. Biomass quantification

Samples collected at regular interval were centrifuged at 12,000 rpm and 4 °C and the collected pellet was suspended in saline solution followed by taking optical density of the suspension at 600 nm. Dry weight (cell biomass) was calculated from a standard plot, between dry weight and optical density (OD) of different dilutions of cell suspensions: in brief 100 ml of fermentation broth was centrifuged at 12,000 rpm and 4 °C for 15 min and the resulting pellet was suspended in 10 ml of saline solution (0.85% NaCl); 3 ml of this suspension was kept at 105℃ for over-night and dry weight was determined while remaining 7 ml was used to make different dilutions of cell suspensions and consequently corresponding optical density of these suspensions were taken at 600 nm. Finally a standard plot was prepared by plotting optical density against cell concentration (after considering the NaCl present in the solution i.e., 8.5 g/l). (Supplementary Fig. 1).

2.4. Extraction of trehalose

For trehalose quantification, 10 ml of broth from the culture medium was collected at regular interval of time and rapidly centrifuged at 12,000 rpm and 4 °C for 10 min. It was further washed thrice with 0.85% NaCl solution. The washed cell pellet was suspended in 1 ml 80% ethanol (v/v) and boiled in water bath till the volume reduced to 0.2–0.3 ml. In this 0.2–0.3 ml of extract, citrate buffer of pH 5.7 was added to make the final volume to 1 ml and after centrifugation the clear supernatant was used for trehalose quantification.

2.5. Analytical methods for glycerol, trehalose, propionic acid and lactic acid

Glycerol was measured by periodate method as described previously (Bondioli and Bella, 2005). The concentration of trehalose in the cell extract was determined with enzyme trehalase method (Sigma–Aldrich) (Ruhal et al., 2011). The 100 μl of 0.012 U of enzyme was added to the 200 μl of cell extract while for control 100 μl of buffer was added instead of enzyme. This reaction mixture was kept over-night (around 12 h) at 37 °C in a shaking incubator. Standard trehalose of known concentration (0.5 g L^{-1}) was also incubated separately with the samples to confirm the complete hydrolysis of trehalose by trehalase enzyme. The glucose

formed after hydrolysis was quantified by DNS method (Miller, 1959).

Lactic acid and propionic acid were determined by HPLC method using a PL-Hi-Plex H column and an UV–Vis detector at 260 nm wavelength. The mobile phase used was 5 mM H_2SO_4 and flow rate was 1 ml/min. The concentrations of propionic and lactic acid were determined using standard plots obtained with pure propionic acid and lactic acid. All the results shown were average of three independent experiments with standard deviation as given.

2.6. Determination of yields of trehalose, propionic acid and lactic acid

Trehalose was estimated by enzyme trehalase (Sigma–Aldrich) method. The yields of trehalose were calculated with respect to substrate consumed (Y_{ts}) and biomass formed (Y_{tx}) as it was an intracellular product (Ruhal et al., 2011). The calculation for Y_{tx} is shown as Eq. (1). In case of Y_{ts} calculation, trehalose produced is divided by the corresponding substrate consumed

$$Y_{tx} = \frac{\text{trehalose}\left(\frac{\text{mg}}{\text{l}}\right)}{\text{dry weight of biomass}\left(\frac{\text{g}}{\text{l}}\right)} \quad (1)$$

Trehalose, dry weight of biomass and substrate concentration was determined as mentioned in previous sections. Similarly, yields of propionic acid (Y_{pa}) and lactic acid (Y_{la}) were calculated by dividing the metabolite (lactic acid, propionic acid) concentration with corresponding substrate consumed.

2.7. Analysis of enzyme activities

The cells were harvested at regular interval and washed thrice with 0.85% NaCl solution. Intracellular crude cell extract was obtained as reported previously in *Propionibacterium* (Tobiassen et al., 1996) and finally sonicated for 20 min (30 s pulse) using lab scale sonicator. The cell crude extract was used as the source of enzyme for various intracellular enzyme assays which were done at 37 °C with a final reaction mixture volume of one ml. The enzyme activities for trehalose-6-phosphate synthetase (OtsA), trehalase and TreS (Cardoso et al., 2007), phosphoglucomutase, phosphoglucoisomerase (Degeest and Vuyst, 2000), UDP-glucose/ADP-glucose/GDP-glucose pyrophosphorylase (Duan et al., 2008), pentose phosphate pathway enzyme glucose-6-phosphate dehydrogenase (Padilla et al., 2004), UDP-glucose dehydrogenase (Schiller et al., 1973) and TreYZ (Asthana et al., 2008) were determined as described elsewhere. Specific activities were expressed as nanokatal/g of biomass and one katal is defined as enzyme necessary for one mole of substrate conversion per second under specified conditions. For the enzyme reactions, involving $NAD^+/NADP^+$ absorbance of reaction mixture was measured at 340 nm at 37 °C (extinction coefficient used at absorbance 340 nm is $6.3 \times 10^3\ M^{-1}\ cm^{-1}$). All the specific activities were presented as average of three independent experiments with standard deviation. All the enzymes and chemicals used were procured from Sigma–Aldrich. For control, heat deactivated (10 min) crude extract was used in enzyme assay.

3. Results and discussions

3.1. Mutagenesis and trehalose yield in mutant in contrast to parent strain

The common defence strategy of microbes against environmental stresses includes accumulation of organic compatible solutes like trehalose, glutamate, proline, etc. (Argüelles, 2000). In particular, it is reported that there is enhanced trehalose accumulation under osmotic stress in *P. freudenreichii* (Cardoso et al., 2007; Ruhal et al., 2011). Based on these observations, it was predicted that osmotically sensitive mutant can improve the trehalose production in non-stress condition. In case of *P. shermanii* NCIM 5137 it was observed that trehalose production enhanced with only osmotic stress (results not shown). In fact it was observed that under oxidative stress and heat stress trehalose yield decreased with *P. shermanii* NCIM 5137(result not shown). Development of osmotically sensitive mutant was carried out with the objective of achieving higher trehalose yield without the necessity of using higher salt concentration in the production media. Instead of site directed mutagenesis (specific target to achieve osmotic sensitive mutant is unknown), it was decided to use chemical mutagenesis method for development of osmotically sensitive mutant. To this end, the advantage of chemical mutagenesis was utilized to isolate a bacterial mutant cell with poor viability in osmotic stress condition and exploring its potential for higher trehalose production in non-stress condition. Previously, this type of strategy involving mutant development by chemical mutagenesis for higher trehalose yield was successfully applied in yeast. In fact, trehalase mutant (obtained after chemical mutagenesis) had shown strong improvement in trehalose accumulation (Chi et al., 2003). Similarly a thermo sensitive mutant of yeast accumulating higher yield of trehalose was also reported (Wang et al., 2011).

The screening for isolation of mutant bacterial cell with osmotic sensitivity was done after treating cells of *P. shermanii* NCIM 5137 with chemical mutagen EMS (ethyl methane sulphonate or methane sulphonic acid ethyl ester) as described in material and methods section. About thousand single cell colonies were screened for osmotic sensitivity using the above mentioned methodology. Finally four mutants which were sensitive to 3% NaCl (unable to grow in media with 3% NaCl) were selected and designated as osmotically sensitive mutant (as compared to parent strain). Parent strain was able to grow in agar plate containing 4% NaCl. Additionally, it was also observed that mutants were non-viable in media containing 0.05% SDS but parent strain was able to grow at 0.05% SDS. Thus it indicates that there was modification in cell surface of mutant as compared to parent cell. Therefore these mutants were considered osmotically sensitive and for surviving in osmotic stress conditions it should have the potential to accumulate osmolytes like trehalose. However effort to screen mutant with still higher osmotic sensitivity (not able to grow at 1% or 2% NaCl) was not carried out as it was observed that these osmotic sensitive mutants have slow growth as compared to parent strain (commercially unattractive). The four mutants were screened for trehalose production ability in media with carbon source glycerol (0% NaCl) and yield values are listed in Table 1. Glycerol was chosen as carbon source because it was decided to produce trehalose from crude glycerol. In Table 1, trehalose yields obtained with respect to biomass and substrate consumed at stationary phase are shown and comparison with parent strain is presented. Thus it can be concluded that mutant 1 is better suited for trehalose production as yield obtained with respect to substrate consumed was highest.

Interestingly, during the evaluation of physiological changes in mutant cell it was found that mutant cells became nisin resistance

Table 1
Comparison of trehalose yields obtained based on biomass (mg/g of biomass, Y_{tx}) and substrate consumed (mg/g of substrate consumed, Y_{ts}) of isolated osmotically sensitive mutant and parent strain in media with glycerol as carbon source and static flask conditions.

Yield	Y_{tx}	Y_{ts}
Mutant 1	233 ± 2	11.8 ± 1
Mutant 2	191 ± 4	8.8 ± 0.5
Mutant 3	66 ± 2	6.2 ± 1
Mutant 4	184 ± 6	8.62 ± 0.4
Parent	88 ± 4	0.87 ± 0.03

Table 2
Nisin resistance in osmotically sensitive mutants at different concentration of nisin (μg ml^{-1}) in contrast to wild strain.

Mutant No.	0.001	0.01	0.5	1	10	40	80	120
Mutant 1	++++++	++++++	++++++	++++++	++++++	++++++	++++++	++++++
Mutant 2	++++++	++++++	++++++	++++++	++++++	++++++	++++++	++++++
Mutant 3	+++	+++	+++	+++	+++	+++	+++	+++
Mutant 4	+++	+++	+++	+++	+++	+++	+++	+++
Parent	+	+	+	+	–	–	–	–

++++++, Very good growth (within 24 h).
+++, Good growth (with in 48 h).
+, Poor growth (>48 h).
– No growth.

as compared to parent strain. It was predicted that nisin binding capacity was lost in mutants as it was resistant up to the concentration of 120 μg ml^{-1} of nisin as compared to parent strain which was unable to grow above 10 μg ml^{-1} of nisin (Table 2). However, mutants and parents were equally sensitive to other antibiotics like penicillin and ampicillin at similar concentrations. There was no report on nisin resistance and trehalose content of cell in any microbes. Nisin resistance mechanism was studied in detail with *Listeria monocytogenes*. (Gravesen et al., 2001) *L. lactis* (Kramer et al., 2006), *Staphylococcus aureus* (Peschel and Otto, 1999), *Streptococcus bovis* (Mantovani and Russell, 2001), etc. From all these studies it was concluded that nisin resistance in various organisms can be acquired through alterations in the expression of genes that are involved in cell wall and cytoplasmic membrane biosynthesis. However other mechanisms were also suggested for nisin resistance in bacteria like nisin degrading enzyme. So in the present study it was difficult to predict mechanism of the nisin resistance in mutant. As it was reported that nisin resistance mostly associated with cell wall or cell-membrane alteration so it could be the reason for nisin resistance in the mutant. As osmotic sensitivity was also associated with nisin resistance so chances of cell wall or cell membrane alteration is higher with this mutant. However, this needs to be experimentally verified with different sets of experiments. In the present study our objective was to produce more trehalose with osmotic sensitive mutant. This improvement of trehalose yield in a nisin resistant and osmotic sensitive mutant is first time reported here. However, trehalose yields obtained with various mutants does not correlate with nisin sensitivity study.

On the basis of trehalose yield in Table 1, mutant 1 was chosen for further study of trehalose production. During a study with parent strain, it was found that parent strain accumulates higher trehalose in carbon source glycerol thus in this study mutant was studied for trehalose production from carbon source glycerol only. In mutant, the highest trehalose yield with respect to biomass was 331 mg (g of biomass)$^{-1}$ and final trehalose yield in stationary phase was 228 mg (g of biomass)$^{-1}$ (Fig. 1) while in parent strain final trehalose yield was 88 mg (g of biomass)$^{-1}$ which was 2.5 times lower than mutant strain (Fig. 1). Under static flask condition, mutant was slow growing in comparison to parent strain (Fig. 1). In view of these results it was concluded that this approach of developing mutant sensitive to particular stress has proven effective for enhanced production of trehalose in non-stress condition. Therefore this strategy showed remarkable improvement of final trehalose yield in mutant as compared to parent strain and this can be ascribed to lower rate of trehalose degradation with growth in late exponential phase.

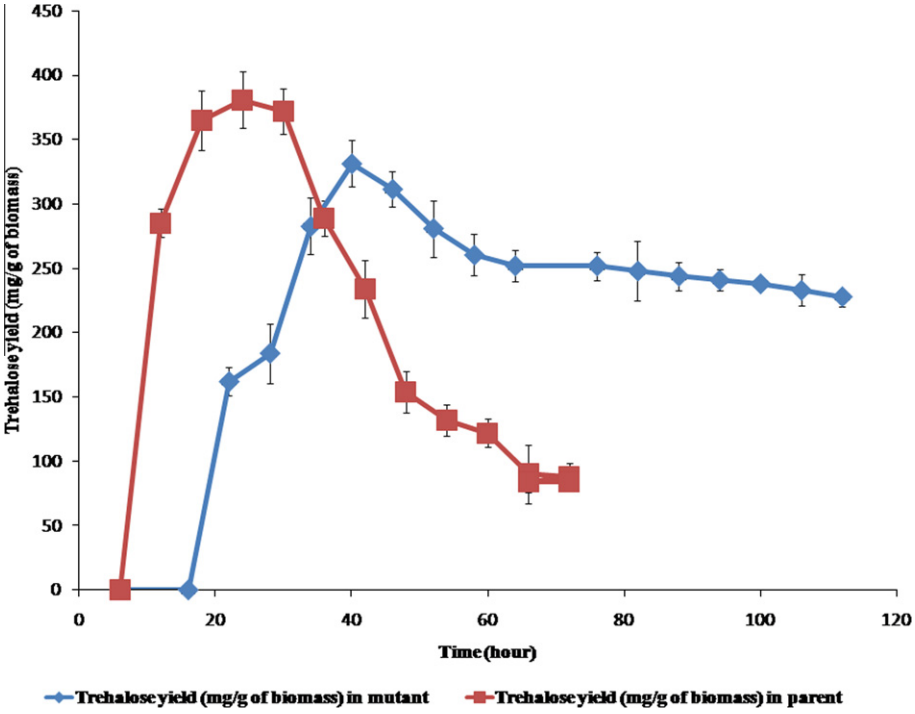

Fig. 1. Trehalose yields obtained in parent and mutant strain with pure glycerol media.

3.2. Production of trehalose with crude glycerol as carbon source

Production of chemicals from microbes is always advantageous as microbes can use waste materials to synthesize many industrially important chemicals while conventional chemical synthesis methods depend a lot on petroleum based substrates. Besides easy scalability of microbial process makes them most suitable for synthesis of molecules like trehalose as compared to other conventional methods (like extraction) which requires large amount of natural sources. Since highest trehalose yield achieved was with substrate glycerol, hence for trehalose production from an alternative cheap source of glycerol known as crude glycerol was explored in the present study. In a similar study, crude glycerol was found more suitable for higher trehalose production with parent strain (Ruhal et al., 2011).

During static flask studies with crude glycerol as carbon source, highest trehalose yield with respect to biomass was 685 ± 25 mg (g of biomass)$^{-1}$ and final yield was 412 ± 22 mg (g of biomass)$^{-1}$, this is probably the highest yield ever reported (Fig. 2). In parent strain, corresponding final trehalose yield with respect to biomass was 128 ± 6 mg (g biomass)$^{-1}$ under similar experimental condition. Similarly, final trehalose yield with respect to substrate consumed was improved around six fold as compared to that in parent strain (Fig. 2). In a recent study with mutant strain of *Saccharomycopsis*, trehalose yield of 28% with respect to biomass (in the present study 41%) was reported using cassava starch as carbon source (Wang et al., 2011). Similarly *Corynebacterium* recombinant strain was able to produce trehalose yield of 31% of biomass with glucose as carbon source (Carpinelli et al., 2006).

The yields of microbial metabolites are functions of cultivation conditions and hence studies on trehalose production in bioreactor were carried out to avoid the lowering of pH as observed in static flask study. In case of static flask study it was observed that complete substrate conversion was not achieved which may either due to lowering of pH of fermentation broth or toxicity effects of impurities present in crude glycerol. Thus, study of trehalose production with mutant was done under controlled conditions of pH and dissolved oxygen in batch reactor. In batch reactor with pure glycerol as substrate, highest trehalose yield achieved with respect to biomass was 376 ± 16 mg (g of biomass)$^{-1}$ while final trehalose yield was 138 ± 2 mg (g of biomass)$^{-1}$ on other hand maximum yield of trehalose with respect to substrate consumed was 80 ± 5 mg (g of substrate consumed)$^{-1}$ and final yield was 27 ± 2 mg (g of substrate consumed)$^{-1}$ (Fig. 3). In crude glycerol media, highest trehalose yield was 530 ± 16 mg (g of biomass)$^{-1}$ while final yield was 391 ± 14 mg (g of biomass)$^{-1}$ and maximum trehalose yield with respect to substrate consumed was 142 ± 10 mg (g of substrate consumed)$^{-1}$ while final yield was 90 ± 5 mg (g of substrate consumed)$^{-1}$ (Fig. 3). Thus in comparison to parent strain, trehalose yields with respect to biomass and substrate consumed were also improved significantly in mutant during trehalose production in bioreactor. In crude glycerol media, final trehalose yield achieved with respect to biomass was three fold higher (same as static flask study) while with respect to substrate it was four fold higher in mutant as compared to parent strain. While complete substrate consumption was not achieved and propionic and lactic acid yields were 0.46 and 0.3 g (g of substrate consumed)$^{-1}$, respectively (Fig. 4). In comparison to parent strain, propionic acid yield was lower in mutant strain with crude glycerol during trehalose production in bioreactor. Thus it indicates that mutant strain was superior to parent strain for production of trehalose in batch reactor with crude glycerol substrate. This study has shown that conversion of one gram of crude glycerol results to 0.46 g of propionic acid, 0.3 g of lactic acid and 0.09 g of trehalose.

The advantages of the present fermentation study with respect to trehalose production were developed mutant was able to produce higher trehalose yield with crude glycerol in comparison to pure glycerol, very high trehalose accumulation (41% of biomass) with crude glycerol (highest yield reported till now), trehalose

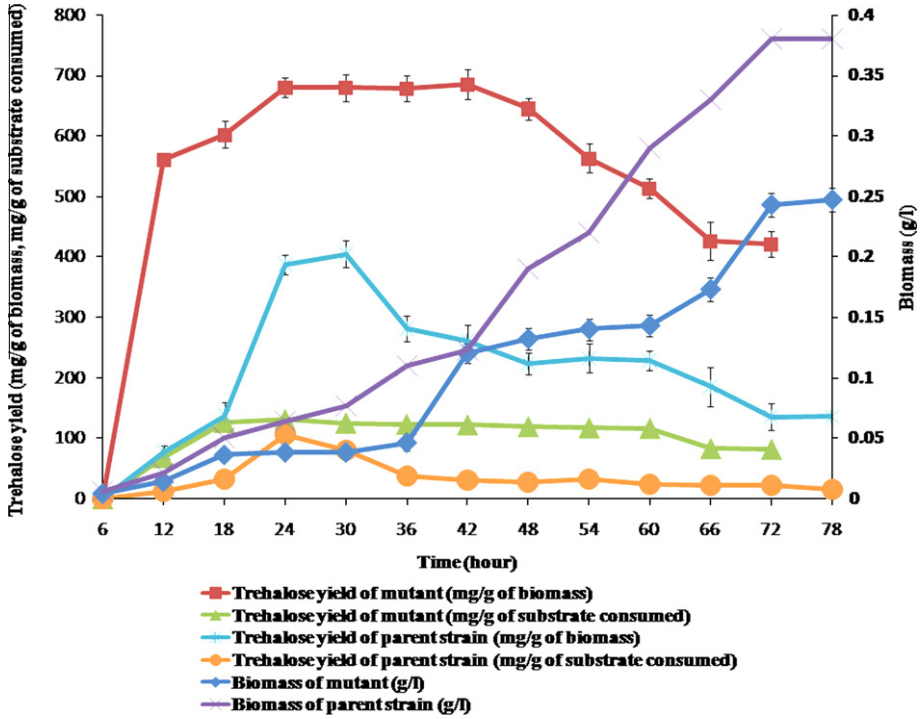

Fig. 2. Comparison of trehalose yields obtained in static flask conditions with mutant and parent strain using crude glycerol as a carbon source.

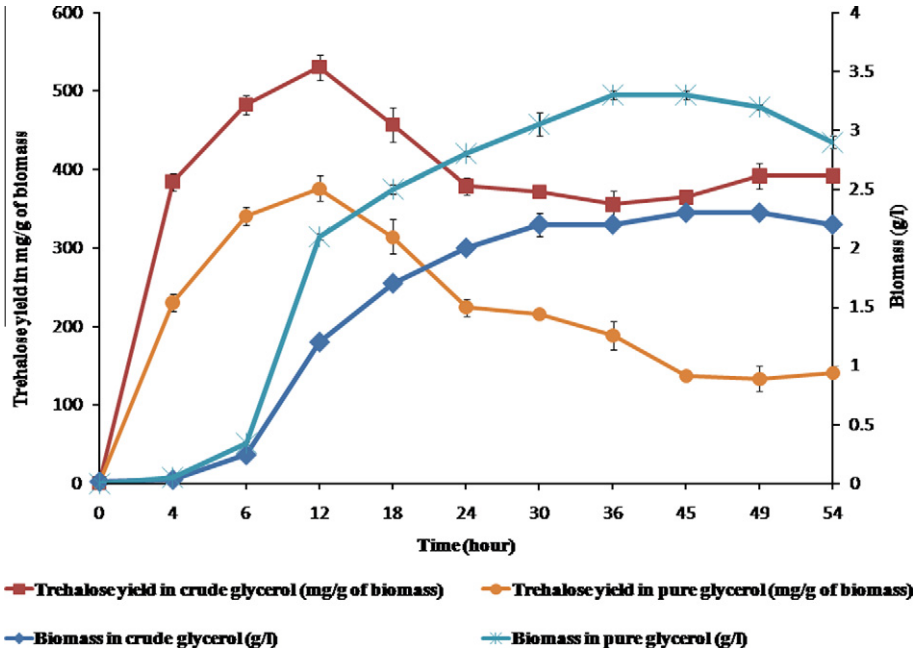

Fig. 3. Batch reactor fermentation study of mutant with pure and crude glycerol as carbon source.

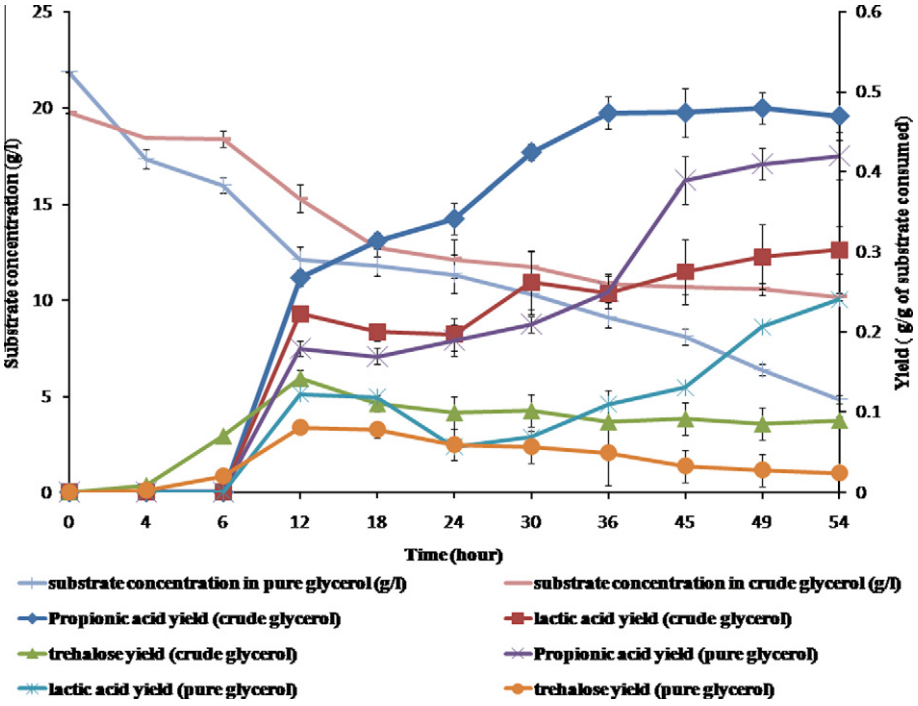

Fig. 4. Trehalose, propionic acid and lactic acid yields achieved in medium with pure and crude glycerol as carbon source in batch reactor.

accumulation was also higher in comparison to recombinant strain of *Corynebacterium* which reported 50 mg (g of substrate consumed)$^{-1}$ (Carpinelli et al., 2006). Thus mutant strain was efficient for production of trehalose using cheap source of glycerol.

3.3. Changes in the physiology of mutant with respect to variation in enzyme activities

Trehalose accumulation was enhanced in mutant and it was maximum with crude glycerol. Thus an effort was made to understand the molecular mechanisms responsible for enhanced trehalose production. Comparison of enzymes activities associated with trehalose biosynthesis in mutant and parent strain can be a good approach for identifying most influential metabolic parameters responsible for enhanced trehalose yield with mutant. Three pathways of trehalose biosynthesis are known OtsAB, TreYZ and TreS pathway. In *P. freudenreichii*, OtsAB pathway is followed (Cardoso et al., 2007). In the present study no activities of TreS and TreYZ were observed in mutant. Thus OtsAB pathway was considered for the present study. NDP-glucose(UDP-glucose ADP-glucose and

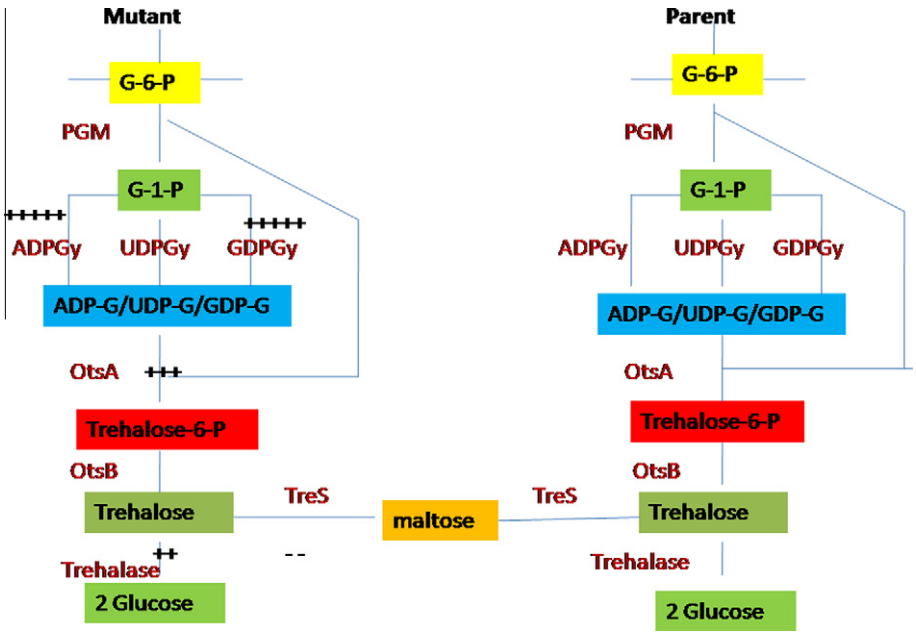

Fig. 5. Comparison of changes in enzyme activities in parent and mutant strain in relation to trehalose biosynthesis. Degree of enzyme activities increase are shown by + sign, where PGM (phosphoglucomutase), ADP-G/UDP-G/GDP-G pyr (ADP-G/UDP-G/GDP-G pyrophosphorylase), G-6-P is glucose-6-phosphate, glucose-1-phosphate (G-1-P).

Table 3
Specific enzyme activities in nanokatal/g of biomass, OtsA (trehalose-6-phosphate synthetase), Pgm (phosphoglucomutase), Iso (isomerase), G6PD (glucose-6-phosphate dehydrogenase), UDPgD (UDP-glucose dehydrogenase), F16diP (fructose 1, 6 diphosphtase), TreS (trehalose synthase), Tase (trehalase), ADP-G/GDP-G/UDP-G pyrophosphorylase (ADP-G/GDP-G/UDP-G pyr), trehalose yield Y_{tx} (mg/g of biomass and Y_{ts} (mg/g of substrate consumed). Main enzymes which have different enzyme activities in mutant and parent strains are shown by bold figures.

Hour	OtsA	Pgm	Iso	G6PD	UDPgD	F16diP	TreS	Tase	ADP-G pyr	GDP-G pyr	UDP-G pyr	Y_{tx}	Y_{ts}
Mutant													
24	3116 ± 12	102 ± 8	1113.9 ± 24	1531 ± 12	139.7 ± 8	3994 ± 24	0 ± 0	555 ± 6	751.8 ± 1	598.7 ± 6	125.3 ± 11	132 ± 8	48 ± 3
30	8816 ± 22	200 ± 11	2497 ± 21	3124 ± 18	337 ± 4	4087 ± 54	0 ± 0	**939** ± 21	**1337.8** ± 0.8	**1122** ± 4	328.8 ± 21	331 ± 14	50 ± 3
36	6803 ± 31	174 ± 13	1353 ± 11	1982 ± 15	314 ± 14	2193 ± 76	0 ± 0	582 ± 18	877.6 ± 0.5	828 ± 8	264.5 ± 22	311 ± 11	42 ± 2
42	4981 ± 14	109 ± 7	769 ± 8	1324 ± 8	192 ± 15	2003 ± 23	0 ± 0	124 ± 8	352.7 ± 0.3	312 ± 4	216.4 ± 18	257 ± 9	14.4 ± 3
Parent													
12	7293 ± 9	302 ± 8	1193 ± 4	2478 ± 9	**1948** ± 11	1845 ± 12	0 ± 0	**41** ± 3	**71** ± 1	**172** ± 1	238 ± 4	381 ± 11	2.1 ± 0.3
18	3419 ± 11	264 ± 4	1131 ± 5	926 ± 11	1066 ± 12	1957 ± 11	0 ± 0	18 ± 3	27 ± 1	55 ± 3	195 ± 3	270 ± 8	2.8 ± 0.2
24	2351 ± 8	234 ± 2	957 ± 3	403 ± 4	272 ± 4	1923 ± 9	0 ± 0	18 ± 3	4 ± 1	7.9 ± 0.1	159 ± 2	219 ± 9	1.18 ± 0.1
30	2313 ± 4	160 ± 1	471 ± 4	225 ± 9	423 ± 2	793 ± 5	0 ± 0	11.7 ± 4	1.5 ± 0.01	7.8 ± 0.2	107 ± 5	130 ± 4	0.62 ± 0.04

GDP-glucose) and glucose-6-phosphate are substrates of OtsA. Thus in the present study, enzymes associated with NDP-glucose and glucose-6-phosphate synthesis and metabolism were considered. OtsA is trehalose-6-phosphate synthetase which synthesises trehalose-6-phosphate from substrates NDP-glucose (UDP-glucose/GDP-glucose/ADP-glucose) and glucose-6-phosphate (Fig. 5). NDP-glucose synthesis is involved by numerous enzymes like ADP-glucose/UDP-glucose/GDP-glucose pyrophosphorylase while glucose-6-phosphate is involved in glycolysis and pentose phosphate pathway hence activities of phosphoglucoisomerase and glucose-6-phosphate dehydrogenase were determined in parent and mutant strain when grown under identical conditions (Fig. 5). Similarly, phosphoglucomutase activity was also monitored as it utilizes glucose-6-phosphate. UDP-glucose dehydrogenase is a part of UDP-glucose metabolism thus its activity was also determined in parent and mutant strain.

All these enzymes activities were determined for four stages of growth including the maximum trehalose accumulation stage. Comparison of individual enzyme activity in parent and mutant strain was restricted to maximum activity value. The activities which are significantly different in mutant and parent are shown as bold in Table 3 and with + sign in Fig. 5. The activity of OtsA was 1.2-fold higher in mutant in comparison to parent strain (Table 3). Interestingly, levels of expressions of enzymes synthesising NDP-glucose were different from each other (Table 3). Amongst them activity of ADP-glucose pyrophosphorylase was around 18-fold higher in mutant, similarly activities of GDP-glucose pyrophosphorylase and UDP-glucose pyrophosphorylase were 6.5- and 1.3-folds higher in mutant in comparison to parent strain (Table 3). It is first time reported here that NDP-glucose synthesising enzymes may vary in their expression during trehalose biosynthesis. On the other hand, activity of trehalose degrading enzyme trehalase was 20-fold higher in mutant strain which may be a response against higher trehalose accumulation as was noted in yeast. No TreYZ and TreS activities were found in mutant and parent strain (Table 3).

Other intracellular activities of isomerase, glucose-6-phosphate dehydrogenase (PPP), phosphoglucomutase, fructose 16 diP did not show any mark difference (maximum two times difference) in parent and mutant strains while activity of UDP-glucose

dehydrogenase was almost six times lower in mutant as compared to parent strain.

From the enzymes activities profile it was clear that there was over-expressions of ADP-glucose/GDP-G pyrophosphorylase together with OtsA was effective for higher trehalose yield. It seems that by diverting flux from UDP-glucose towards ADP-glucose and GDP-glucose pathway favoured higher trehalose yield. Fig. 5 shows the difference in enzyme activities in mutant and parent strain. In previous reports in yeast higher activity of OtsA, UDP-G pyrophosphorylase and phosphoglucomutase under osmotic stress were reported (Voit, 2003), similarly in *Corynebacterium* over-expression of OtsA together with UDP-G pyrophosphorylase has resulted in higher yield of trehalose (Carpinelli et al., 2006). However, in the present study important role of ADP-glucose pyrophosphorylase was highlighted which was not studied in other cases.

4. Conclusions

In the present work the improvement of trehalose accumulation was achieved with an osmotically sensitive mutant of *P. freudenreichii subspp. Shermanii.* In mutant, trehalose yield achieved with respect to biomass was three times improved as compared to parent strain with crude glycerol. Likewise, trehalose yield achieved with respect to substrate consumed was increased from 21 mg (g of substrate consumed)$^{-1}$ (parent strain) to 90 mg (g of substrate consumed)$^{-1}$ (mutant strain). Along with this 0.42 g/g of propionic acid and 0.31 g/g of lactic acid were also obtained from crude glycerol. A new insight was also gained on the prominent effect of ADP-G pyrophosphorylase in enhanced trehalose synthesis. It was already shown that lower rate of decrease of trehalose content in late stationary phase with mutant was probably responsible for achieving higher final trehalose yields with mutant as compared to parent strain (Fig. 1). Comparison of enzyme activity profile of parent and mutant strain indicates that decrease (in comparison to maximum activity value) in enzyme activity with growth at late exponential phase was low in mutant as compared to parent strain. Specifically, ADP-glucose pyrophosphorylase activity was decreased by three times with growth in mutant whereas same enzyme activity in parent strain decreased by 50 times with growth. Similarly, GDP-glucose pyrophosphorylase activity was decreased by two times in mutant while it was decreased by 20 times in parent with growth. A similar phenomenon was also observed with OtsA and fructose 1,6-biphosphotase. Thus this indicates that in mutant not only higher trehalose is produced at mid exponential phase due to higher activities of ADP-glucose pyrophosphorylase, GDP-glucose pyrophosphorylase and OtsA but rate of decrease of these activities were lower with growth as compared to parent strain. At the same time it is worth to note that even with high trehalase activity in mutant significant lowering of trehalose content was not observed with growth. This indicates that rate biosynthesis of trehalose with growth was not decreased significantly in mutant as compared to parent strain and thus net rate of decrease of trehalose content of cell was minimal even in presence of high trehalase in mutant as compared to parent strain.

Acknowledgement

This work was supported by Department of Biotechnology (DBT), Govt. of India, with Grant No. BT/PR-7493/PID/20/289/2006.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biortech.2012.01.039.

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