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BIOENERGY/BIOFUELS/BIOCHEMICALS

Improved trehalose production from biodiesel waste using parent and osmotically sensitive mutant of *Propionibacterium freudenreichii* subsp. *shermanii* under aerobic conditions

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Abstract Trehalose is an important nutraceutical of wide commercial interest in the food processing industry. Recently, crude glycerol was reported to be suitable for the production of trehalose using a food microbe, *Propionibacterium freudenreichii* subsp. *shermanii*, under static flask conditions. Similarly, enhanced trehalose yield was reported in an osmotically sensitive mutant of the same strain under anaerobic conditions. In the present study, an effort was made to achieve higher production of trehalose, propionic acid, and lactic acid using the parent and an osmotically sensitive mutant of *P. freudenreichii* subsp. *shermanii* under aeration conditions. Under aeration conditions (200 rpm in shake flasks and 30 % air saturation in a batch reactor), biomass was increased and approximately 98 % of crude glycerol was consumed. In the parent strain, a trehalose titre of 361 mg/l was achieved, whereas in the mutant strain a trehalose titre of 1.3 g/l was produced in shake flask conditions (200 rpm). In the mutant strain, propionic and lactic acid yields of 0.53 and 0.21 g/g of substrate were also achieved with crude glycerol. Similarly, in controlled batch reactor culturing conditions a final trehalose titre of approximately 1.56 g/l was achieved with the mutant strain using crude glycerol as the substrate. Enhanced production of trehalose using *P. freudenreichii* subsp. *shermanii* from waste under aeration conditions is reported here. Higher production of trehalose was not due to a higher yield of trehalose but to a higher final biomass concentration.

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Keywords Trehalose · Osmotically sensitive mutant · *Propionibacterium freudenreichii* · Crude glycerol · Aeration · Biodiesel

Abbreviations

Y_{tx} Trehalose yield with respect to biomass
Y_{ts} Trehalose yield with respect to substrate consumed

Introduction

Trehalose is a non-reducing, extremely stable disaccharide in which two α-glucose units are linked by an α,α-1, 1-glucosidic bond. Trehalose has mild sweetness, high solubility and low hygroscopicity. It is also stable during processing and storage of several food preparations, thereby preventing Maillard reactions; hence, it can be used in dried food (milk powder, fruit juices), frozen food), confectionary (cake, baked confectionary, jam, cream, sweet been jam), beverages, fermented food (bread and yoghurt), ice-cream, sauce, sweetener and seasoning [21, 34]. It is also used as a food additive in dried or processed food for human consumption and it is also naturally found in honey and mushrooms [16]. Trehalose is also reported to have nutraceutical value [18]. It is half as sweet as sucrose, provides sustained energy and elicits a very low insulin response [9, 15, 23]. Trehalose was also reported to work as a stress protectant in *Lactococcus lactis* [6].

Emerging applications of trehalose in foodstuffs and other areas can only be realized if it can be produced economically. The conventional process for the production of trehalose by *Saccharomyces cerevisiae* has a relatively low production yield and has been replaced by an enzymatic conversion process [27]. In comparison to such

enzymatic methods, various microbial-based processes have the advantage of utilizing agricultural or industrial wastes as the substrates for the production of valuable metabolites. Trehalose was also produced from beer waste brewing yeast by using a high intensity pulsed electric field [20]. Similarly effort was also made to produce trehalose from corn starch [7, 36]. Therefore, microbial production of higher trehalose titres using waste like crude glycerol could offer an economically advantageous and environmentally friendly alternative to the currently employed enzymatic processes. The large amount of biodiesel waste produced by the biodiesel manufacturing industries poses a major environmental risk [30, 37]. Thus many investigations reported the 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 use of this waste and it seems to be economically attractive. Several microbial products were produced from crude glycerol e.g. 1,3-propanediol using *Clostridium* and *Klebsiella* [16, 17], hydrogen from *Enterobacter aerogenes* [19], succinic acid using *Anaerobiospirillum succiniciproducens* [24], vitamin B12 [22], omega-3 polyunsaturated fatty acids from algal fermentation [30], docosahexaenoic acid using *Schizochytrium* [10], surfactin [13] and ethanol [29]. In the present study, crude glycerol from biodiesel waste was used as a carbon source for the parent and osmotically sensitive mutant of *Propionibacterium freudenreichii* subsp. *shermanii* during the production of trehalose together with organic acids like propionic acid and lactic acid. *Propionibacterium* are gram-positive, non-sporing, non-motile pleomorphic rods. They are anaerobic to aerotolerant. They grow optimally at 30 °C and neutral pH [11, 35]. *Propionibacterium freudenreichii* has been used for a long time as a ripening culture in Swiss-type cheese manufacture. It is well known for its various probiotic properties and is regarded as a safe food microbe [29]. Many *P. freudenreichii* strains are reported to play an important role in adaptation, cheese ripening, biopreservation and probiotic effects [35]. Their ability to produce antifungal compounds (mainly propionic acid) makes *P. freudenreichii* ideal as a biopreservative in food products [26]. Propionibacteria were also used for the production of propionic acid, 1,4-dihydroxy-2-naphthoic acid and menaquinone [12, 14]. Propionic acid bacteria are known to be anaerobic but it was reported that they are not very sensitive to aerobic conditions [8, 31]. It was also reported that *P. freudenreichii* can grow under aerobic conditions which led to an increase in biomass and changes in the organic acid formation profiles [31].

This prompted us to design and investigate an experiment in which an osmotically sensitive mutant and the parent strain of *P. freudenreichii* subsp. *shermanii* NCIM 5137 were cultured under aeration conditions (in shake flasks at 200 rpm and 30 % air saturation in a bioreactor) with pure and crude glycerol as the carbon source. Trehalose content and biomass growth profiles were measured at regular intervals of time. Final yields of organic acids like propionic acid and lactic acid were estimated at the stationary phase. The production of trehalose under anaerobic conditions was previously reported in the parent and mutant strains of *P. freudenreichii* subsp. *shermanii* [32, 33].

Materials and methods

Microorganism and culture media composition

Strain and culture media were the same as those described previously [32, 33]. *Propionibacterium shermanii* NCIM 5137 was procured from NCL Pune, India. For trehalose production, the media composition used was the same as that reported elsewhere [4, 32, 33] and comprised tryptone (20 g/l), peptone (20 g/l), yeast extract (1 g/l), K_2HPO_4 (0.25 g/l), vitamin solution (20 ml/l) and carbon source (20 g/l). The vitamin solution comprised biotin (1.1 mg/l), folic acid (1.1 mg/l), PABA (110 mg/l), riboflavin (110 mg/l), pyridoxine (220 mg/l), thiamine (220 mg/l) and niacinamide (220 mg/l).

Chemical mutagenesis and screening, isolation of mutant

Isolation and screening of osmotically sensitive mutant were reported previously [33]. In brief, 25 ml of culture broth was taken from the mid exponential growth phase of *P. shermanii* NCIM 5137. It was centrifuged and the bacterial cells obtained in the pellet were suspended in 5 mM phosphate buffer of pH 7.1. For chemical mutagenesis, 8 % EMS was dissolved in 5 mM phosphate buffer (pH 7). The bacterial cells were suspended in the solution of 8 % EMS and incubated for 45 min at 37 °C. After incubation, the cell pellet was washed three times with $Na_2S_2O_3$ to neutralize the effect of the mutagen. Further different dilutions of bacterial cells (10^{-1}–10^{-8}) were incubated in agar plates with glucose as the carbon source. From the agar plates, a single colony was further selected and replica plated on agar plates containing glucose as the carbon source and 1, 2, 3, 4 and 8 % NaCl, 0.01 and 0.05 % SDS and plates with no SDS and NaCl. Colonies which did not grow or grew weakly on NaCl plates and SDS plates were selected for trehalose production. The stability of mutants was checked by repeated simultaneous plating on agar plates containing 0 and 3 % NaCl. After around 50 repeated platings, any colony unable to grow in

3 % NaCl but able to grow in 0 % NaCl was selected for this study. Thus the stability of the mutants was checked with respect to their osmotic sensitivity.

Shake flask studies

The cultures were grown in 500-ml culture flasks containing 100 ml of liquid media. The entire flasks were inoculated with freshly grown culture and incubated at 30 °C at 200 rpm. Growth was monitored by measuring the optical density spectrophotometrically. For optical density measurement, the culture broth was centrifuged at 20,000×*g* for 10 min at 4 °C and the resulting pellet was washed and resuspended in isotonic solution (0.85 % NaCl) followed by optical density measurement at 600 nm. Further, cell culture samples were harvested at regular intervals and rapidly centrifuged at 20,000×*g* for 15 min at 4 °C; supernatants (cell-free broth) were stored at −20 °C until being used for substrate concentration analysis, whereas the washed cell pellet was kept in a refrigerator (−80 °C) until further analysis of trehalose content.

Batch reactor studies

A 2-l New Brunswick autoclavable fermenter was used for all batch reactor studies and 100 ml of 24 h grown static flask culture was used as the inoculum. After autoclaving the fermenter with media, sterilised carbon source solution was added separately and the inoculum was finally added. The final working volume was 2 l, pH was maintained at 6.8 by automatic addition of NaOH and dissolved oxygen was maintained at 30 % of air saturation by automatic increase in agitation. Samples were taken at regular intervals (4–6 h) and were used for substrate and product analysis.

Crude glycerol or biodiesel waste preparation and pretreatment

To evaluate the suitability of crude glycerol for the production of trehalose and propionic acid, biodiesel waste was used which was prepared by base-catalysed transesterification of soya bean oil [32, 33]. The composition of the crude glycerol was reported previously [32]. Crude glycerol was mixed with distilled water at a ratio of 1:4 (v/v) to reduce the viscosity of the fluid, and then the pH of the fluid was adjusted to 3 with hydrochloric acid to convert soap into free fatty acids. The formed precipitate was separated from the crude glycerol solution by centrifugation at 5,000×*g* and subsequently the pH of the supernatant was adjusted to 12 with base (KOH), followed by separation of the formed precipitate by centrifugation. Finally the pH of the supernatant obtained after the second centrifugation was adjusted to 6.8. Methanol was removed during autoclaving.

Extraction and analysis of trehalose

Extraction of trehalose was carried out as reported previously [4, 32, 33]. For intracellular trehalose, the washed cell pellet was suspended in 2 ml of 80 % ethanol and boiled in a water bath till the volume reduced to 0.2–0.3 ml. In the extract, citrate buffer (pH 5.5, 0.1 M) was added to make the final volume up to 1 ml and after centrifugation the clear supernatant was used for trehalose quantification. The concentration of trehalose in the cell extract was determined by the enzyme trehalase method (Sigma-Aldrich) as described previously [32, 33]. Then 100 µl of 0.012 U of enzyme was added to the 200 µl of cell extract, whereas for control 100 µl of buffer was added instead of enzyme. This reaction mixture was kept overnight (around 12 h) at 37 °C in a shaking incubator. Standard trehalose of known concentration (0.5 g/l) was also incubated separately with the samples to confirm the complete hydrolysis of trehalose by the trehalase enzyme. The glucose formed after hydrolysis was quantified by the DNS method [28].

HPLC analysis of organic acid

Lactic acid and propionic acid were determined by HPLC using a PL-Hi-Plex H column and a 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.

Biomass quantification

Biomass quantification was done as described previously [32]. Samples collected at regular interval were centrifuged at 12,000×*g* and 4 °C and the washed collected pellet was suspended in saline solution followed by measurement of the 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.

Substrate analysis

Glycerol was measured by the periodate method as described previously [2]. The concentration of glycerol was estimated from the calibration curve of known glycerol concentrations.

Results and discussion

Trehalose production with pure and crude glycerol from *P. shermanii* NCIM 5137 in shake flask studies

Production of trehalose is reported using various carbon sources like sucrose, glucose, maltose and starch [9, 34]. Recently, trehalose production using pure and crude glycerol was also reported [32, 33]. Interestingly, a higher trehalose yield was obtained with crude glycerol in comparison to pure glycerol with *P. shermanii* NCIM 5137 [26]. With crude glycerol media (crude glycerol 20 g/l) a trehalose yield of 128 mg/g of biomass was obtained in static flasks, and a trehalose titre of 261 mg/l was obtained under anaerobic conditions in a batch reactor [32]. Thus there was a need to increase the final trehalose titre. In the present study, the feasibility of increasing the final trehalose titre by increasing the biomass concentration without compromising the trehalose yield was evaluated. An effort was made to delineate the effects of aerobic conditions on growth, trehalose yield (mg/g of biomass and mg/g of substrate) and final trehalose titre (absolute concentration in mg/l). It was reported previously that the cell biomass concentration of *Propionibacterium* increases with aeration [8, 31]. A similar observation was reported with *P. freudenreichii* where the final biomass and biomass yield were increased under aeration (50 % air saturation) [4]; hence, we planned an experiment in which *P. shermanii* NCIM was cultured in shake flask conditions instead of static flask conditions. During shake flask culture of *P. shermanii* NCIM 5137 with pure glycerol as the carbon source, a final biomass of 5.1 g/l was obtained (Fig. 1). The final trehalose titre (absolute trehalose) was 156 mg/l together with propionic acid and lactic acid yields of 0.31 and 0.34 g/g of substrate consumed respectively (Table 1). As it was reported previously that trehalose yield was higher with crude glycerol media as compared to pure glycerol media in *P. shermanii* NCIM 5137 [32], trehalose production was studied with crude glycerol (cheap source of glycerol from biodiesel waste) under aeration conditions in static flasks. In crude glycerol media, a final trehalose titre of 361 mg/l was achieved. The final biomass achieved was 5.7 g/l (Fig. 1) while substrate consumption was 87.8 % as compared to 100 % conversion achieved with pure glycerol (Table 1). Similarly, a higher yield of propionic acid (0.56 g/g of substrate) was achieved with crude glycerol as compared to pure glycerol media under similar experimental conditions (Table 1). The lactic acid yield was 0.22 g/g of substrate consumed. Interestingly, the trehalose titre was approximately 2.3-fold higher with crude glycerol together with achievement of a higher yield of propionic acid under aeration conditions. If we compare the trehalose yield with respect to the biomass in aerobic and anaerobic conditions with crude glycerol media it can be observed that the yield decreased by twofold in aerobic conditions as compared to static flask culturing. However, the yield with respect to substrate consumed increased by twofold in aerobic cultures as compared to static flask cultures (Tables 1, 3). Thus this study indicates that under aerobic conditions, cell growth was favoured and this resulted in a

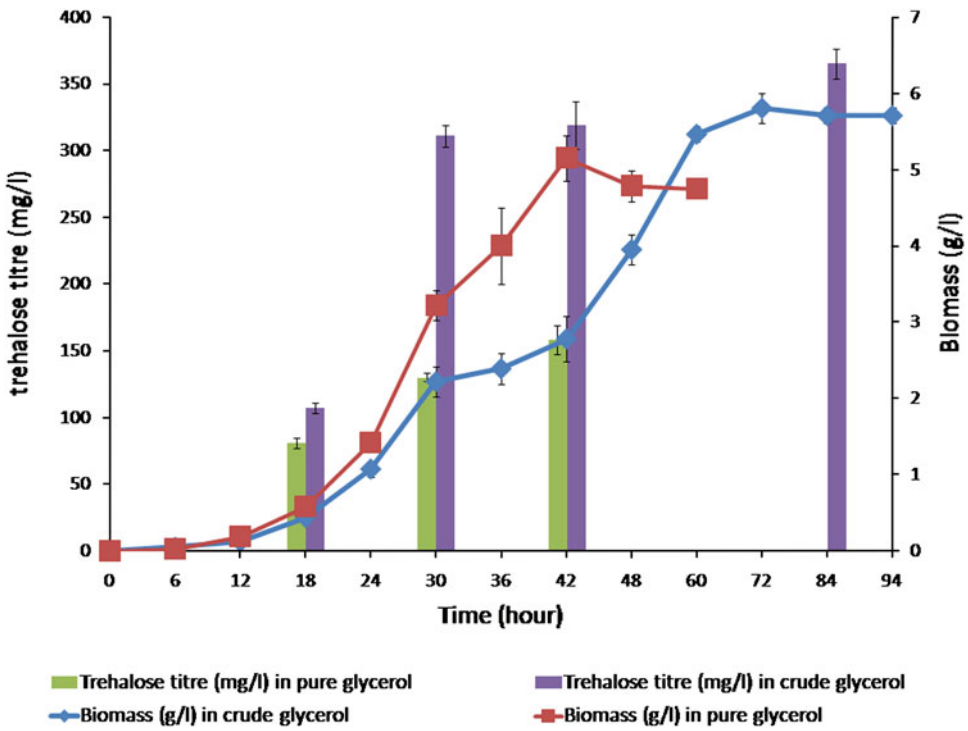

Fig. 1 Trehalose titre and biomass production in media with pure and crude glycerol in *P. shermanii* NCIM 5137

Table 1 Comparison of trehalose titre and propionic acid and lactic acid yields in parent strain of *P. shermanii* with pure and crude glycerol

Parameters	Pure glycerol	Crude glycerol
Final biomass (g/l)	5.1 ± 0.5	5.7 ± 0.8
Trehalose titre (mg/l)	158 ± 6	361 ± 11
Propionic acid yield (g/g of substrate consumed)	0.31 ± 0.01	0.56 ± 0.03
Lactic acid yield (g/g of substrate consumed)	0.34 ± 0.03	0.22 ± 0.01
Substrate consumption (%)	100	87.8

lower trehalose yield with respect to biomass. Thus it can be concluded that even though the trehalose yield with respect to biomass decreased by twofold, the 20 times higher final biomass concentration resulted overall in a 2.3-fold increase in trehalose titre under aeration conditions (Table 1). Unfortunately, the trehalose titre achieved was not encouraging, hence effort was made to improve the trehalose titre by using an already developed osmotically sensitive mutant under aeration conditions [33].

Trehalose production with pure and crude glycerol using osmotically sensitive mutant of *P. shermanii* NCIM 5137 in shake flask studies

Trehalose accumulation increased under various environmental stress conditions including osmotic stress [1]. An enhancement of trehalose production was reported to result from osmotic stress in *P. freudenreichii* [3, 4]. Similarly, with *P. shermanii* NCIM 5137 a higher trehalose yield was reported with crude glycerol media and this was due to the presence of KCl in crude glycerol [32]. This led us to isolate an osmotically sensitive mutant (unable to grow in the presence of 3 % NaCl in contrast to the parent strain) and the application of this osmotically sensitive mutant for enhanced yield of trehalose under anaerobic conditions was already reported [33]. The development of an osmotically sensitive mutant was carried out with the objective of achieving a higher trehalose yield without the necessity of using a higher salt concentration in the production media. Instead of site-directed mutagenesis (the specific target required to achieve an osmotically sensitive mutant is unknown), it was decided to use a chemical mutagenesis method for the development of an osmotically sensitive mutant. Previously, this type of strategy involving mutant development by chemical mutagenesis for higher trehalose yield was successfully applied in yeast. A thermosensitive mutant of yeast accumulating a higher yield of trehalose was also reported [36]. Isolation and screening of the osmotically sensitive mutant is described in the "Materials and methods" section and mutants were screened on the basis of higher trehalose yield achieved in static flask conditions. One mutant with higher trehalose yield (233 mg/g of biomass obtained with pure glycerol media in static flask conditions [33]) was chosen for further study and detailed screening for enhanced trehalose yield with this osmotically sensitive mutant under static flask conditions was reported in our previous work [33]. In the present study, the osmotically sensitive mutant was cultured with

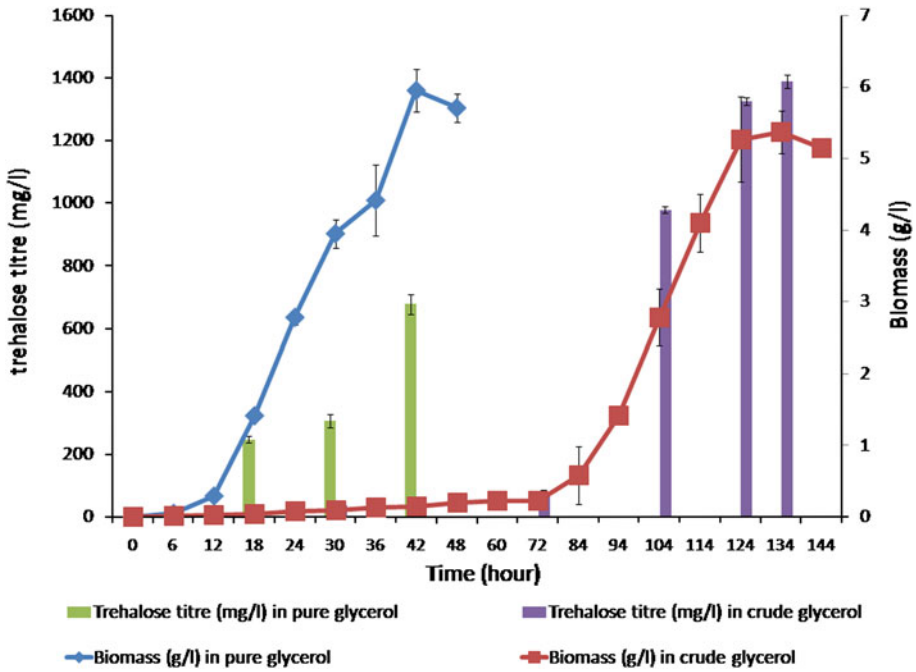

Fig. 2 Trehalose titre and biomass growth in media with pure and crude glycerol in osmotically sensitive mutant of *P. shermanii* NCIM 5137

Table 2 Comparison of trehalose titre and propionic acid and lactic acid yields in mutant strain of *P. shermanii* with pure and crude glycerol

Parameters	Pure glycerol	Crude glycerol
Final biomass (g/l)	5.9 ± 0.6	5.4 ± 0.7
Trehalose titre (mg/l)	678 ± 14	1,303 ± 22
Propionic acid yield (g/g of substrate consumed)	0.38 ± 0.03	0.53 ± 0.02
Lactic acid yield (g/g of substrate consumed)	0.28 ± 0.02	0.21 ± 0.01
Substrate consumption (%)	100	94

Table 3 Comparison of trehalose yield with respect to biomass (Y_{tx}) and substrate consumed (Y_{ts}), trehalose titre (absolute trehalose) in mutant and parent strain of *P. shermanii* NCIM 5137 with crude glycerol (20 g/l) in aerobic and anaerobic conditions

Parameter	Y_{tx} (mg/g of biomass)	Y_{ts} (mg/g of substrate)	Trehalose titre (mg/l)	Biomass (g/l)
Static flask (parent)	136	15.8	28.5	0.21
Static flask (mutant)	421	81	101	0.24
Shake flask (parent)	63.5	36.4	361	5.7
Shake flask (mutant)	241	69.4	1,303	5.4

pure glycerol as the carbon source under shake flask conditions and the final biomass achieved was 5.9 g/l (Fig. 2) while a trehalose titre of 678 mg/l was achieved (Fig. 2). This trehalose titre was higher as compared to the parent strain with pure and crude glycerol media under aeration conditions. Propionic acid and lactic acid yields were 0.38 and 0.28 g/g of substrate consumed respectively (Table 2). Complete substrate consumption was achieved with the mutant (Table 2). In a study under similar experimental conditions with crude glycerol as the carbon source, a final trehalose titre of 1.3 g/l was achieved with the mutant. Interestingly, a propionic acid yield of 0.53 g/g of substrate was achieved together with a lactic acid yield of 0.21 g/g of substrate (Fig. 2; Table 2). A substrate consumption of 94 % was achieved with crude glycerol (Table 2). Thus the trehalose titre achieved with crude glycerol was 3.6-fold higher with mutant as compared to the parent strain under similar experimental conditions. Thus a cumulative benefit of a 13-fold increase in the trehalose titre was obtained by using the mutant and aerobic culturing conditions as compared to the parent strain in static flask culturing conditions (Tables 2, 3). If a comparison of trehalose yield with respect to biomass is made between aerobic and static culturing conditions in the mutant with crude glycerol media then it can be concluded that the yield decreased by 1.74-fold in aerobic conditions (Table 3). Similarly, the trehalose yield with respect to substrate consumed was marginally less in aerobic conditions of growth as compared to static flask culturing conditions (Table 3). A 25-fold increase in biomass concentration under aerobic culturing conditions with the mutant resulted in a 3.6-fold increase in the trehalose titre even when there was a 1.74-fold decrease in the trehalose yield with respect to biomass. Thus our adopted strategy ensured a 3.6-fold higher trehalose titre without compromising the trehalose yield with respect to substrate consumed. These results encouraged us to study the trehalose production with crude glycerol under controlled conditions of the batch reactor.

Batch reactor study for higher trehalose titre with osmotically sensitive mutant of *P. shermanii* NCIM 5137

Because the highest trehalose titre was obtained with crude glycerol using the mutant, a similar experiment was conducted in a batch reactor under controlled conditions of pH and dissolved oxygen (30 % of saturation). A final trehalose titre of 1.56 g/l was obtained along with 6.5 g/l of biomass (Fig. 3). Recently, a trehalose titre of 1.7 g/l was reported with pure glycerol in recombinant *Escherichia coli* (OtsAB overexpression) and in the presence of a trehalase inhibitor (validamycin) (extracellular) [19]. In the present work, a trehalose titre of 1.56 g/l was obtained with crude glycerol (biodiesel waste, an alternate cheap source of glycerol) with an osmotically sensitive mutant of *P. shermanii* NCIM 5137 without the addition of a trehalase inhibitor (not economical as it is costly). The lactic acid and propionic acid yields were 0.53 and 0.21 g/g of substrate, respectively, in aerated conditions with the mutant. The productivity achieved with the mutant in crude glycerol media under controlled conditions (batch fermenter) was 31 mg/l/h and this value is comparable with another reported value (47 mg/l/h) obtained with recombinant *E. coli* along with a trehalase inhibitor [19]. In comparison to the productivity obtained with a recombinant *Corneybacterium* sp. at 21 g/l of biomass concentration, this productivity is significantly low but improvement can be achieved by using high cell density fermentation [5]. In the case of *Saccharomycopsis fibuligera* A11, a productivity of 122 mg/l/h was achieved with a biomass concentration 22.8 g/l [36]. In the present study, a higher trehalose titre was obtained in aerobic conditions as compared to static flask culturing conditions but with a marginal compromise in the trehalose yield (Y_{ts}). Therefore, future efforts should be directed toward improving the productivity by increasing the biomass concentration in aerobic fermentation without compromising the trehalose

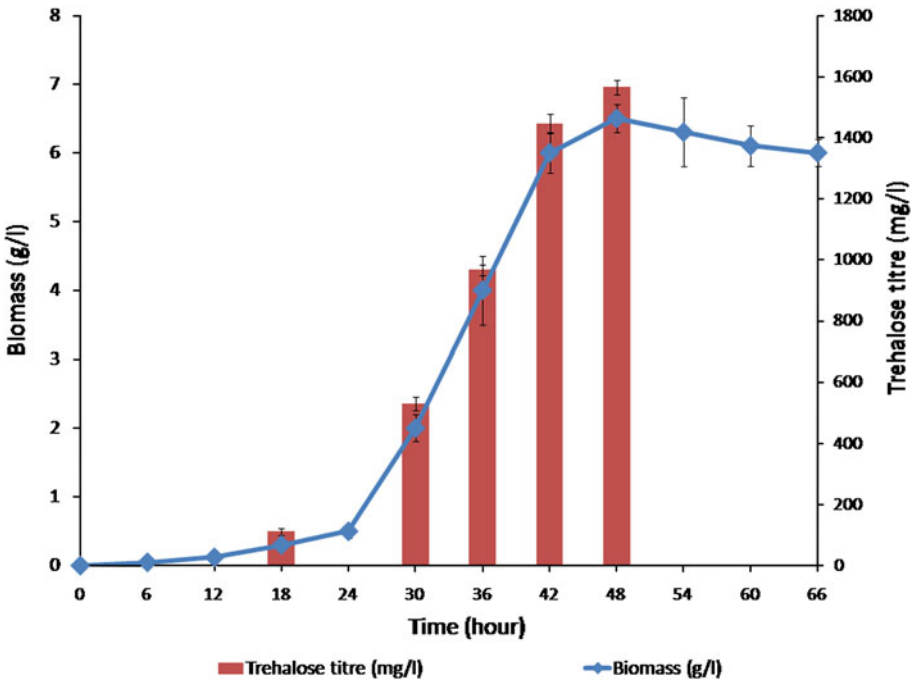

Fig. 3 Trehalose titre and biomass growth in media with crude glycerol in osmotically sensitive mutant of *P. shermanii* NCIM 5137 under batch reactor conditions

yield with respect to substrate consumed. But the advantage of present study was the production of trehalose with crude glycerol, whereas pure glycerol, glucose and starch were used with *E. coli*, *Corynebacterium* and *S. fibuligera*, respectively [5, 25, 36]. In the present study, as there was no improvement in the trehalose yield under aeration conditions, no effort was made to evaluate the effect of aeration with respect to enzyme activities associated with the trehalose biosynthesis pathway. However this study shows that a fed batch fermentation strategy may improve the trehalose titre as well as the trehalose yield. An effect of the crude glycerol concentration on the final trehalose titre cannot be ruled out.

Conclusions

The present study described a novel process for the conversion of crude glycerol from biodiesel waste into the nutraceutical trehalose using an osmotically sensitive mutant of food microbe *P. freudenreichii* subsp. *shermanii*. The trehalose titre was enhanced from 156 mg/l in the parent strain with pure glycerol to 1.56 g/l in the mutant strain with crude glycerol. Thus an efficient environmental friendly process for the production of trehalose was developed which utilises waste with the help of a safe food microbe. The strategy of improving trehalose titre by using aerobic culturing conditions seems to be effective and it can be further explored for higher productivity. The trehalose yield with respect to biomass was four times higher with the mutant as compared to that with the parent strain under similar experimental conditions. This remarkable improvement in yield resulted in a fourfold higher trehalose titre with the mutant as compared to that with the parent strain.

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