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Original Research

Enhancing Profitability of Ethanol Fermentation through Gamma Ray Mutagenesis of *Saccharomyces cerevisiae*

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Abstract

Invertase has a vital role in ethanol fermentation, especially on sucrose-containing media. In this study, catabolite-resistant mutants of *Saccharomyces cerevisiae* were isolated through gamma ray mutagenesis with improved productivity of ethanol and invertases. Enzyme productivity of mutant yeast was compared with that of the parent. Various fermentation parameters were optimized, including substrate concentration, pH, and temperature on the production of invertase and ethanol. Applying optimized conditions (pH 4.6, 37ºC) at lab scale, industrial scale experiments in 300 m^3 fermenters were performed. Mutants had completed fermentation with high efficiency in 28 hrs irrespective of parents that took 36 hrs. The mutant obtained is of great commercial value as it has the potential to enhance the overall profitability of the fermentation process through advancement in the quality of the final product.

Keywords: invertase, ethanol, mutagenesis, *Saccharomyces cerevisiae,* fermentation, enzyme

Introduction

World energy consumption is growing at rising rates, facing the progressive depletion of its energetic resources based on non-renewable fuels that also lead to the increase in the generation of polluting gases released into the atmosphere, which have caused changes in the global climate. The solution is to find alternative sources of energy [1]. The use of biomass is an environment friendly way of power generation [2-11]. Ethanol is the main liquid biofuel currently replacing 3% of gasoline. Worldwide, >50% bioethanol is produced from sugarcane juice and molasses. The principal sugar, sucrose, present in the molasses is generally the mostly

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available economical sugar for the industrial utilization of the yeast *Saccharomyces cerevisiae* [12].

It is commonly believed that yeast cells express an extracellular invertase capable of splitting sucrose into glucose and fructose. *Saccharomyces cerevisiae* can use different kinds of carbon sources, but the glucose and fructose are ideal for its growth. When these sugars are present in the medium, the production of enzymes required for utilizing other carbon sources is enormously withdrawn or totally blocked. The trend is called carbon catabolite repression [13], altering a number of phenomena in yeast in the central and peripheral metabolism.

Therefore, sugars present in the media are used in an orderly way, creating a lag phase and resulting in prolonged production time [14]. The process will be more proficient by simultaneous use of all the sugars by the deregulated strain through a reduction in production time.

Keeping in view the above-stated problem and industrial need, the *Saccharomyces cerevisiae* strain presently being used at a distillery was irradiated with gamma rays. 2-Deoxy-D-glucose (DOG) has been widely used to select glucose-derepressed mutant [15-16]. The glucose-deregulated mutants with high invertase activity were selected. The strain's efficiency is dependent on invertase activity to hydrolyze the sucrose even under the inhibitory conditions existing in molasses [17]. Invertase production was optimized on the laboratory scale on industrial complex media (molasses) irrespective of synthetic media that is highly favorable over the conventional complex media. Ethanol fermentation was done in 300 m^3 industrial fermenters.

The objective of this work was to characterize and optimize invertase production at laboratory scale and further implement on a large scale. This study is new of its kind to quantify the effect of invertase activity on ethanol production on an industrial scale.

Materials and Methods

The culture was maintained at yeast culture medium. Molasses with the addition of N and P source [18] was used in all the experiments otherwise mentioned. Commercial sulfuric acid was used to adjust pH [19]. The presently used strain in ethanol production process of Shakarganj Mills Distillery Jhang Pakistan, of SAF-Instant France was subjected to gamma ray-irradiated mutagenesis as described by Sridhar and Elliott [20] in three repeats. For preparation of inoculums, well-grown single colonies of each parent and mutant culture were picked up by a loop and inoculated in 100 ml of yeast inoculum medium in an Erlenmeyer flask (sterilized at 121ºC and 1.5 PSI) and incubated at 37ºC in an orbital shaker (Toshiba, Japan) at 120 rpm for 24 hrs. Forty-five ml of molasses medium was transferred to each 250 ml Erlenmeyer flask. The flasks were cotton plugged and autoclaved for 15 min and cooled at room temperature. Five ml of inoculum were transferred to each flask under sterile conditions. Flasks were then incubated in an orbital incubator shaker. Various conditions and process variables, temperature, nitrogen, phosphorus source ratios, and pH were optimized for the maximum production of invertase and ethanol. Concentrations of total fermentable sugars (TFS) in diluted molasses were measured calorimetrically as described earlier [21]. The concentration of TRS in diluted molasses, after sucrose inversion using HCl and amounts of residual sugars in fermented wash, was measured by the Fehling-Soxhlet method. As 10% TFS were inactivated, this loss was made up by adding firter-sterized glucose to the fermentor. But for large-scale study, this practice was not adopted as fermentation was performed on unsterilized molasses. Ethanol in fermented samples was determined with an ebulliometer and confirmed on GC as described previously [22].

Fermenter studies were carried out in a microprocessor-controlled 23-l stainless steel fermenter (Biostat C5, Braun Biotechnology, Melsungen, Germany) (15-l working-volume vessel) equipped with instruments and controllers for parameters such as agitation, temperature, pH, and dissolved oxygen, and fitted with a reflux cooler in the gas exhaust to minimize evaporation. The vessel was filled with medium-containing sugars (15% TRS, found optimum) in molasses supplemented with an optimum concentration of $(NH_4)_2SO_4$ (7.5 g/ l) and other nutrients. The pH was adjusted to 4.6 (optimum) and the medium was steam-sterilized *in situ* for 30 min. The fermenter was inoculated with 10% (v/v) active inoculum. The aeration was carried out through a sparger at 15 l/min for 8 h to enhance biomass production before switching over to 3 l/min. This process lasted up to 72 h, during which foaming was controlled by adding silicone oil as an antifoaming agent. Substrate, nitrogen source, pH, and temperature-dependent formation of ethanol occurred along with minute quantities of acetic acid, succinic acid, and glycerol. pH dropped due to the formation of acetic acid, so pH was controlled automatically at 4.6 using KOH. In all studies, 100 ml samples in triplicate were collected from both parental and mutant cultures after every 8 h for different analysis periodically.

In 1 ml of the 0.2 M sucrose and 1 ml sodium acetate buffer mixtures, 100 µl of appropriately diluted invertase solution was added. The reaction mixture was agitated at 50ºC for 30 min in a shaking water bath. Then 50 µl reaction mixtures were added to 950 µl distilled water and boiled for 10 min to deactivate the enzyme. The amount of glucose formed was determined using a glucose oxidase kit [21]. Treatment effects were compared by the protected least significant methods with ANOVA-II using MstatC software.

For genetic variability assessment, total genomic DNA from parent and mutant strains (24 h grown cultures) of *Saccharomyces cerevisiae* were isolated separately using the CTAB (cetyltrimethylammonium bromide) method with little modification. The method is employed for purification of DNA from plant tissues. After RNA

treatment, the quantity of DNA was measured using a fluorimeter (Hoefer USA), and quality was checked by running 0.8 ng of both parent and mutant strain DNA on agarose gel (0.8%). Data attained through the analysis of the primers (RAPD assay amplification by PCR technique) were utilized to assess the genetic similarity based on the number of shared amplification products. The similarity index was approximated through bivariate (1-0) data. pH determination was done as reported earlier [23]. The quantity of ethanol produced in alcoholic fermentation of molasses was estimated on an ebulliometer. However, it was confirmed on gas chromatography afterwards [22].

Results and Discussion

Composite mutant was developed through off and on exposure to γ-rays. Only 8 mutants developed larger pink colour in sucrose-DG medium and the best single mutant in plate tests of the mutant strain *S. cerevisiae* was selected, designated as SML-1. The potential mutant SML-1 was grown several times and streaked until it became stable. Gamma ray-induced mutations are reliable methods for developing mutant derivatives [20]. Finally, a stable mutant was used for further studies in liquid culture. The parent and mutant strains of *Saccharomyces cerevisiae* were tested in time course studies (Fig. 1) for ethanol production and conditions were optimized at laboratory scale. Initially the effect of TFS on cell mass and ethanol production was tested in a 23 l fermentor (working volume 15 l; Fig.1), as mentioned previously. Representative time course of ethanol production by both parental and mutant strains for molasses batch fermentation containing 120, 150, and 180 g sugars L^{-1} is presented in Fig. 1.

Fig. 1 also shows time course of invertase production as well (bottom far right). Time consumed by the mutant derivative was 8 h (28 h) less than that taken by the wild strain (36 h). Maximum ethanol (80 g L^{-1}) was obtained with mutant derivative (Fig. 1 180 g/L). The growth curves (Fig. 1) indicated that cell mass concentration in both strains reached maximum values after 28 and 36 h in the case of mutant and parental strains, respectively. These curves also indicated that production of ethanol was apparently growth-associated up to 24 h of fermentation, after which ethanol production was non-growth associated.

The results of kinetic parameters for substrate consumption and cell mass formation are presented in Table 1. The effect of carbon source concentrations on all growth parameters, namely cell mass formation rate (Q_x), growth yield coefficient ($Y_{x/s}$), specific substrate consumption rate (q_s), and maximum specific growth rate (μ_m). The interactive effect of carbon sources and organisms was found to be highly significant with respect to all growth kinetic parameters. Maximum specific growth rate (μ_m), specific substrate uptake rates (q_S), and volumetric rate of substrate consumption (Q_S; Table 1)

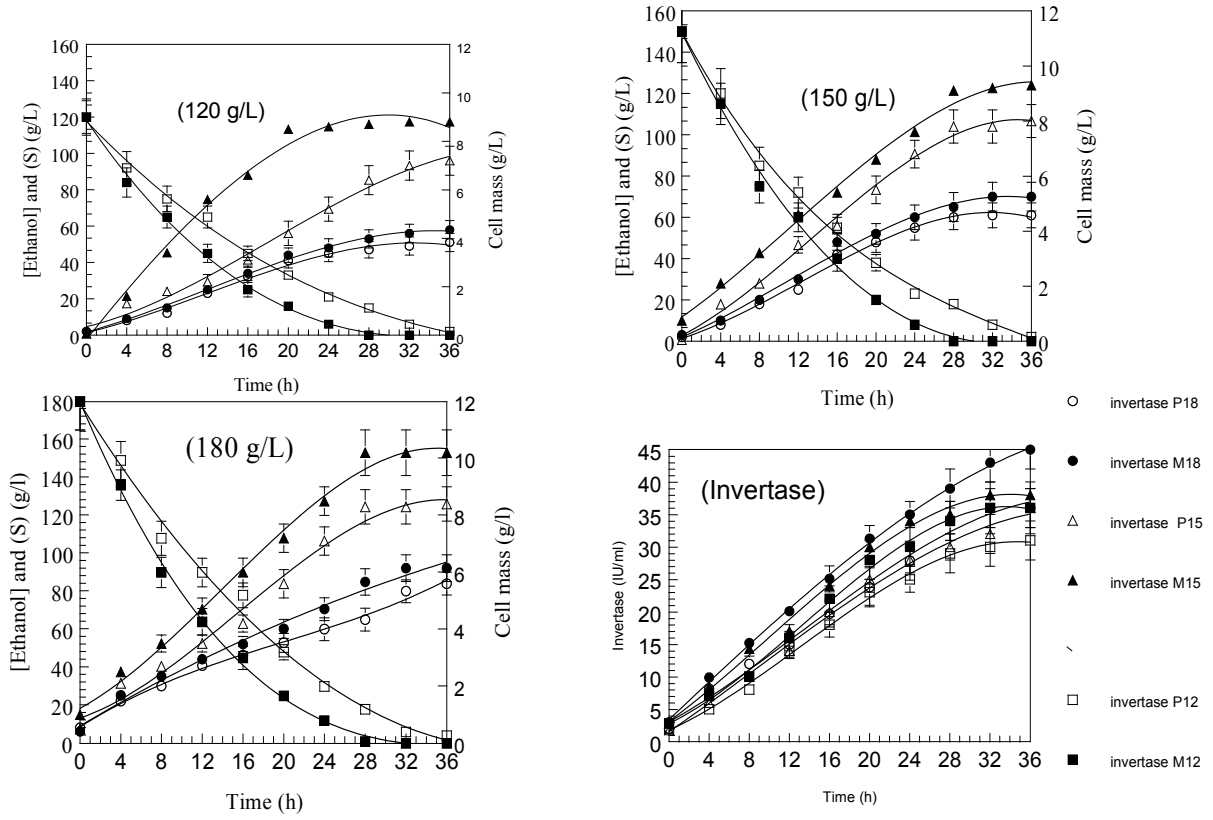

Fig. 1. Comparative depiction of fermentation kinetic parameters of *S. cerevisiae* (P) and its mutant for ethanol formation.

Table 1. Comparative fermentation kinetic parameters of *S. cerevisiae* (P) and its multiple mutant (SML-1, M) for ethanol formation (kinetic parameters following growth on molasses under optimized cultivation conditions).

Carbon source (L^{-1})		Q_X (g cells L^{-1} h^{-1})	Q_S ($g.L^{-1}$ h)	$Y_{X/S}$ ($g.g^{-1}$sub.)	q_S ($g.g^{-1}$ h^{-1})	μ_m (h^{-1})
120 g	P	0.12 ± 0.02^b	3.5 ± 0.2^b	0.06 ± 0.02^b	3.83 ± 0.2^b	0.23 ± 0.02^b
	M	0.22 ± 0.2^a	4.4 ± 0.2^a	0.065 ± 0.02^a	$4.15.\pm0.2^b$	0.27 ± 0.02^b
150 g	P	0.28 ± 0.02^b	3.9 ± 0.2^b	0.061 ± 0.02^b	5.08 ± 0.2^a	0.31 ± 0.02^a
	M	0.35 ± 0.02^a	4.6 ± 0.2^a	0.066 ± 0.02^a	5.15 ± 0.2^a	0.34 ± 0.02^a
180 g	P	0.12 ± 0.2^b	3.85 ± 0.2^b	0.056 ± 0.02^b	5.36 ± 0.2^a	0.30 ± 0.02^a
	M	0.25 ± 0.2^a	4.45 ± 0.2^b	0.060 ± 0.02^a	5.33 ± 0.2^a	0.32 ± 0.02^a
P		≥0.0001	≥0.0001	≥0.001	≥0.0001	≥0.0003

Each value is a mean ±SD of three replicates; values followed by different letters in superscript in each column represent differences among carbon sources for the production of ethanol

Table 2. Comparative fermentation kinetic parameters of *S. cerevisiae* (P) and its multiple mutant (SML-1, M) for ethanol formation (kinetic parameters following growth on 15% TFS in molasses under optimized cultivation conditions).

Carbon source (L^{-1})		Q_P ($g\ L^{-1}\ h^{-1}$)	$Y_{P/X}$ ($g\ g^{-1}$ cells)	$Y_{P/S}$ (g. g^{-1} sub.)	q_p ($g\ g^{-1}\ h^{-1}$)	Theoretical yield (%)
120 g	P	2.1 ± 0.2^b	7.1 ± 0.2^b	0.43 ± 0.02^b	1.6 ± 0.2^b	84 ± 2^b
	M	2.8 ± 0.2^a	8.7 ± 0.2^a	0.48 ± 0.02^a	2.8 ± 0.2^a	93 ± 3^a
150 g	P	2.2 ± 0.2^b	7.8 ± 0.2^b	0.47 ± 0.02^b	1.8 ± 0.2^b	92 ± 4^a
	M	2.9 ± 0.2^a	9.2 ± 0.2^a	0.48 ± 0.02^a	2.6 ± 0.2^a	93 ± 3^a
180 g	P	1.9 ± 0.2^b	7.7 ± 0.2^b	0.40 ± 0.02^b	2.2 ± 0.2^b	78 ± 3^c
	M	3.1 ± 0.2^a	8.9 ± 0.2^a	0.48 ± 0.02^a	2.9 ± 0.2^a	93 ± 3^a
P		≥0.001	≥0.001	≥0.005	≥0.001	≥0.001

Each value is a mean ±SD of three replicates; values followed by different letters in superscript in each column represent difference among carbon sources for the production of ethanol

were supported at a significantly higher value (P<0.001) by the mutant SML-1 than the values supported by the wild organism, and were considered the factors that might control product synthesis by different concentrations of substrate. There were variations in specific growth rates and substrate uptake rates during growth on all carbon source concentrations in the case of mutant derivatives, permitting larger variation in synthesis of invertase and ethanol by this organism (Table 2, Fig. 1). Lower concentrations of carbon source in the medium supported lower growth of yeast, resulting in less yield of invertase (Fig. 1) and ethanol (Table 2).

Multiple mutations enhanced the specific ethanol yield ($Y_{P/X}$), product yield ($Y_{P/S}$), volumetric rate of ethanol formation (Q_p), and specific rate of ethanol formation (q_p) up to 119, 120.9, 142.6, and 149% over respective values of parental strain, respectively (Table 2), while invertase productivity (1,560±50 IU/L h) was 220% higher than that of the wild organism. Volumetric yields of ethanol (Table 1) were higher than those reported by other authors (1, 13 ,17). Our mutant strain showed better results than those by the wild organism on all concentrations of sugars in molasses and temperatures. These values were also higher than those exhibited by a multiple mutant strain of *Kluyveromyces marxianus*, which took 60 h to consume 150 g glucose/l and commercial strain of S. cerevisiae consumed 150 g glucose/l in 48 h at 30-32ºC (23), but our mutant completed industrial scale fermentation in 28 h.

Thus multiple mutagenesis improved results of both substrate consumption (Table 1) and product formation parameters (Fig. 1, Table 2). These results suggested that the multiple mutagenesis resulted in an excellent improvement of the multi-genes-controlled characters of yeast strain for improved ethanol and invertase production. The mutant strain demonstrated a more industrious mode showing improved behavior for thermotolerance, osmotolerance, substrate consumption, and ethanol yield.

Genetic Variability between the Mutant and Parent Strains

Samples of genomic DNA of parental and mutant strains were analyzed by RAPD-PCR method. They was used to verify that the mutant isolate was genetically different from the parental organism. A total of 35 primers were employed. A total of 204 loci were amplified, with an average of ~6 loci per primer. Out of the 35 primers, 9 primers (OPA-03, OPB-01, OPB-03, OPB-05, OPE-02, OPE-05, OPK-01, OPK-03, and OPK-05) amplified polymorphic DNA fragments

Table 3. Comparative fermentation kinetic parameters of *S. cerevisiae* (P) and its multiple mutant (SML-1, M) for ethanol formation (kinetic parameters following growth on molasses under optimized cultivation conditions).

Temperature		$Q_{P\ invertase}$ (IU L^{-1} h^{-1})	$Y_{P\ ethanol/S}$ (gg^{-1}.)
27	P	615±30[c]	0.42±0.03[a]
	M	950±45[b]	0.45±0.03 [a]
32	P	715±30[c]	0.44±0.03[a]
	M	1560±50[a]	0.47±0.03 [a]
37	P	679±30[c]	0,32±0.03[b]
	M	1600±60[a]	0.480.03 [a]
42	*P*	460±20[d]	0.25±0.03[b]
	M	1530±45 [a]	0.45±0.03[a]
P		>0.001	>0.001

Each value is a mean ± SD of three replicates; values followed by different letters in superscript in each column differ significantly at $P \leq 0.05$

in the mutant strain. A maximum 19 fragments were amplified with primer OPD-05 while the minimum number of fragments were amplified by OPA-02, OPA-03, OPB-05, OPL-01, OPL-03, OPK-05, and OPD-03. The size of DNA fragments amplified varied from 0.5kb to 2kbOf these polymorphic primers, three produced polymorphic loci in the parent strain and six primers produced polymorphic loci in the mutant strain of *Saccharomyces cerevisiae*. Thus the mutant strain showed a nearly uniform RAPD pattern, whereas the parental strain gave a heterogeneous amplification pattern. RAPD analysis was capable of readily differentiating between parent and mutant strain and production pattern of the tested strains. Both strains with some primers showed a correlation of genetic variability with enzyme production. Some other researchers used RAPD markers to verify genetic diversity and phenotypic correlation [24]. In this study 56% genetic similarity (2 x numbers of common bands/ total bands) was calculated between original and mutant strains. It is therefore suggested that RAPD assay is sufficient to differentiate the wild and the mutant strains.

Invertase Production Studies at Laboratory Scale

For industrial-scale ethanol fermentation, *Saccharomyces cerevisiae* is the distinctive yeast strain universally employed by ethanol producers. Although many strains have been isolated/screened and characterized for improved growth rate and enhanced ethanol formation, a glucose repression problem during utilization of mixed carbon sources has yet to be resolved.

Invertase activity, ethanol percentage, and residual sugars were measured for both parent and mutant strains at varying concentrations of carbon source (12, 15, 18%, w/v), pH, temperature, and dilution (Tables 1-5).

Table 4. Effect of controlled pH of the fermentation medium in 23 L fermenter on kinetic parameters of *S. cerevisiae* (W) and its mutant strain (M) on ethanol and invertase formation using molasses medium (15% sugars).

pH	Strain	Q_p (g $L^{-1}h^{-1}$)	$Y_{p\ ethanol/s}$ (g/g subs)	$Qp_{\ invertase}$ (IU $L^{-1}h^{-1}$)
pH 4.0	W	2.1[d]	0.36[d]	859[e]
	M	3.6[a]	0.42[b]	960 [c]
pH 4.5	W	2.4[d]	0.41 [b]	950 [c]
	M	3.2[b]	0.48[a]	1292[a]
pH 5.0	W	2.3[d]	0.39[d]	930 [c]
	M	3.2[b]	0.46[a]	1100[b]
pH 5.5	W	2.13[e]	0.32[e]	887 [c]
	M	3.28[b]	0.44[b]	1010[b]
pH 6.0	W	2.05[e]	0.29[f]	884 [c]
	M	3.15[b]	0.41[b]	985 [c]
pH 6.5	W	1.90[e]	0.26[g]	750[d]
	M	2.90[c]	0.39[d]	840 [c]
P		0.0001	0.0001	0.0001

Each value is a mean ± SD of three replicates; values followed by different letters in superscript in each column differ significantly at $P \leq 0.05$

Temperature is one of the leading factors that has a serious effect on the production of invertase [29]. The invertase activity at different temperatures 27, 32, 37, and 42°C was assessed (Table 3).

At 37ºC enzyme activity was much higher in the case of the mutant as compared to that of the parent strain. Maximum production of invertase (33.8 U/ml, Q_p = 1,439±60 U/lh) (Table 3) was obtained when incubation temperature was maintained at 37ºC for the mutant and 32°C for the parent. At high temperatures, low enzyme secretion may be due to thermal inactivation of yeast culture [30]. Therefore, fermentation at elevated temperatures needs a thermotolerant strain. Further increase in incubation temperature resulted in marked decline in invertase production by both strains, but more severely in the parent. At 27°C, enzyme production was low, which might be due to the fact that the temperature was not suitable for yeast growth. The invertase activity was found at maximum with in the temperature range of 34-39ºC in parent and mutant, respectively. At higher temperatures after the optimal value, a decrease was observed and there was more decrease in the parent.

Kaur and Sharma et al.[31] obtained maximum invertase activity at initial pH 5.0 in synthetic fermentation medium. In both strains, invertase production was enhanced up to pH 5.0 then decreased as the variables increased. Shafiq et al. [29] reported that yeast always desires to grow at high pH, but for the invertase secretion pH (4.6-5.0) was the best. The inhibitory influence of high pH (above 5.0), could be attributed

Table 5. Comparative fermentation kinetic parameters of *S. cerevisiae* (P) and its multiple mutant (SML-1, M) for ethanol and invertase formation kinetic parameters following growth on molasses used as 1:2 dilution and 1:1.5 dilution in an industrial-scale fermentation process.

Dilution Used	Strain	$Q_{Pinvertase}$ (IU L^{-1} h^{-1})	$Y_{Pethanol/S}$ (L. $Tonne^{-1}$)	Q_{pEth} (g /L.h)	Q_S (g/L.h)
1:2 (15% TFS)	P	609±30[c]	218±5[b]	1.19	7.2* (0.40)**
	M	890±45[b]	232±6[a]	1.56	8.2* (0.47)**
1:1.5 (18% TFS)	P	600±30[c]	222±8[b]	1.29	8.19* (0.47)**
	M	990±50[a]	240±9[a]	1.67	9.2* (0.51)**
P		>0.001	>0.0214		

Each value is a mean ± SD of three crops; values followed by different letters in superscript in each column differ significantly at $P \leq 0.05$; *rate of substrate accumulation; **rate of substrate uptake

to the lower ATP formation during the metabolic changes in *Saccharomyces cerevisiae*. In both strains invertase production was significantly affected by pH. Today it is necessary to move to alternate energy sources that will reduce over-dependence on non-renewable energy sources like fossil fuels. This will definitely be a cost-effective and eco-friendly approach [32-45].

Conclusions

The mutant strain completed fermentation with high efficiency in 28 hours as compared with the parent that took 36 hours for completing fermentation. The mutants obtained are of great commercial value as it has the potential to decrease the overall cost of the fermentation process and improve the quality of ethanol. The strain is characterized and optimized for invertase production at laboratory scale and has been trialed successfully at full industrial scale.

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Conflict of Interest

The authors declare no conflict of interest.

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