Improvement of Invertase Synthesis by the Mutant *Saccharomyces cerevisiae* through UV Mutagenesis

S. Saravanan¹, Abdul Nasar Kalanthoden², R. Karthikayan¹, M. Kavitha^{3,*}, S. Kutti Rani²

¹School of Life Sciences, B.S. Abdur Rahman Crescent University, India

²Department of Chemistry, B.S. Abdur Rahman Crescent University, India

³Department of Pharmaceutical Technology, Vinayaka Mission University, India

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Abstract In fermentation technology, strain improvement of baker's yeast has traditionally relied on random mutagenesis followed by screening for mutant exhibiting enhanced properties of interest. Such mutant organisms are useful in several industries. *Saccharomyces cerevisiae* can use sucrose as the sole source of both carbon and energy; hydrolysis of this sugar is catalyzed by the enzyme invertase. The main objective of this work is to overcome the glucose repression of invertase by invertase

constitutive mutants through UV mutation. This may occur in any glucose repressible genes as a single or double mutation in repressor gene (s) which might cause constitutive synthesis of invertase. These mutated screened strains were optimized with various glucose concentration and different incubation hours for higher invertase production. The maximum synthesis of invertase was 1.066 units/ml in hour from mutant *Saccharomyces cerevisiae* type-2 strain.

Graphical Abstract

Keywords Constitutive Synthesis, Invertase, Mutation, Optimization, *Saccharomyces cerevisiae*, UV Mutagenesis

1. Introduction

Saccharomyces cerevisiae is the most thoroughly investigated eukaryotic microorganism, which aids our understanding the biology of the eukaryotic cell. For several decades, it has been used in the production of food and alcoholic beverages, and at present it is used in number of different food and pharmaceutical industries. The advantage of using this microorganism is to speed up and reduce the cost of process and to allow continuous production of high-value biomolecules in a subject of great current interest [1].

Invertase is encoded by a family of six unlinked structural genes namely SUC 1 to SUC 5 and SUC 7 are known to exist in this genome [2-6]. Each SUC gene encodes for both a secreted and an intra cellular form of invertase. A specific yeast strain may possess none, one or several functional SUC genes [7, 3]. The level of enzyme secretion depends on the concentration of glucose in the culture medium [9]. Repression of the external invertase synthesis has been described by the level of transcription [10] and [11], translation, and glycosylation [12] of the protein. *S. cerevisiae* growing under repressible condition (1% glucose or more) could produce a burst of external invertase when shifted to higher temperature. The secretion of this invertase requires protein synthesis, but it was found to be independent of RNA formation. The level of accumulated and translated mRNA was inversely proportional to the glucose present in the growth medium [13]. These results are consistent with the hypothesis that invertase is continuously synthesized both in the presence and absence of glucose, but it is degraded under repressible condition [14]. Two types of mutants affected in the regulation of invertase synthesis. Mutation in any of the six *snf* genes such as *snf1*, *snf2*, *snf3*, *snf4*, *snf5*, and *snf6* prevents the de-repression of invertase synthesis and thus identifies elements of positive control [15, 16]. Mutation causing constitutive synthesis of invertase, thus defective in negative control (glucose-insensitive) isolated suppressors of *snf*, which prevents the de-repression of glucose repressible genes. Mutation occurs in one of the suppressor genes, *snf6*, causes constitutive synthesis of invertase and other repressible enzymes [17]. The *snf1* gene [18- 20] product is required to depress the expression of many glucose repressible genes including the SUC2 structural gene of invertase [16]. In wild-type strains, synthesis of this mRNA is regulated by glucose repression [21, 22]. A 1.8 kb mRNA transcribed constitutively from the SUC2 gene encloses the cytoplasmic invertase, which plays no major role in sucrose fermentation [11]. Mutation causes constitutive synthesis of high level invertase.

An increase in productivity to reduces the overall cost of

the product, as well as the production cost; hence, it is one of the important topics for the research. Usually, enhanced productivity can be achieved by strain improvement and optimizing the process parameters [23]. Therefore, random ultraviolet (UV) light mutagenesis of Backer's yeast could be promising strategy for improving invertase productivity [24]. Screening the large number of mutant strain selection by using a direct invertase filter assay method was highly relaying to isolate a higher invertase synthesizing strains [17]. Media optimization, which is still one of the most critically investigated phenomena, is carried out before any large-scale metabolite production. It became more vibrant, effective, efficient, economical and robust in fermentation industry [24]. For designing the most suitable fermentation conditions and appropriate medium components and its percentages must be identified and optimized then only maximum product concentration could be achieved [24].

In this study, we attempted to improve the invertase productivity of the wild type yeast by UV mutagenesis. First, wild type baker's yeast was irradiated by UV and then mutant strains were selected based on invertase filter assay method. Then, screened strains were conformed the glucose repression of invertase constitutive mutants. Finally, optimize the fermentation conditions for the excess production of invertase by isolated mutant *S. cerevisiae* strain was deliberate.

2. Materials and Methods

2.1. Chemicals

Baker's yeast was obtained from a ("saf-instant red" commercially available) local market. All chemicals were analytical grade purchased from Sigma-Aldrich and Merck.

2.2. Screening of High Sucrose Fermenting Yeast Strain

Wild type baker's yeast 0.5gm was dissolved in 10ml of 0.1M phosphate buffer then 0.1 ml of suspended yeast was spread on YEPD (Yeast extract peptone dextrose) agar medium [25, 26] containing 10gm of yeast extract, 20gm of peptone, 20gm of dextrose, and agar adjusted to pH 7.2 in 1000 ml of distilled water along with streptomycin (100 µg/ml) and ampicillin (50 µg/ml) containing plates incubated at 30°C for 30 h and slants its used for further studies. For isolated pure strains were inoculated on YEPS (Yeast extract peptone sucrose) agar medium containing 10gm of yeast extract, 20gm of peptone, 20gm of sucrose, and agar was adjusted to pH 7.2 in 1000 ml of distilled water containing bromocresol purple as an indicator which was used to screen yeast capable of high sucrose fermenting.

2.3. UV Mutagenesis, Mutation Analysis and Strain Selection

Isolated culture inoculated in YEPD (50 ml) medium, as

described previously. After the incubation cells were harvested at 5000 rpm for 10 min. The pellet was washed twice with 0.1M phosphate buffer (pH 7.0) and the harvested cells were suspended in 10 ml of 0.1M phosphate buffer (pH 7.0), then 1 ml of the suspended cells were aliquoted in to sterilized glass petri plates, along with 5 ml of phosphate buffer (pH 7.0). The aliquots were exposed to UV radiation for varying time intervals (0, 5, 10, 20 and 30 min) to provide varying UV dosage under the laminar flow hood and UV source for 48cm. 100 μ l of each sample were used to spread on YEP glucose plates and incubated at 30°C for 30 h then analysis of mutation frequency [27] and survival rate percentages to be calculated by widely used Luria and Delbruck method [28].

2.4. Filter Assay of Invertase Activity

This technique was used to screen large numbers of mutants, for detecting invertase activity in yeast grown in glucose medium [17]. Mutant yeast colonies grown in YEP glucose on agar plates were transferred to dry whatman no. 3 filters (9cm diameter) by pressing the filters on to the plates. A second dry filter was stapled to the first filter to make a sandwich with yeast colonies in between. The filter sandwich was placed in 100 ml of distilled water to remove any glucose or other reducing sugars. The filters were then wetted with a solution containing 5% sucrose in 10mm sodium acetate buffer (pH 4.6) and incubated at room temperature for 3 to 5 min and transferred to a separate petri dish floating in a 50°C water bath. A portion (3 ml) of 0.1% triphenyl tetrazolium chloride in 0.5N sodium hydroxide was added. The red color spots indicate the invertase positive colonies through mutation (1 to 2 min). The filters were removed, dried, and stored in refrigerator.

2.5. Invertase Assay

Invertase assay was performed by Nelson-Somagy's method [29], 1 mg/ml of standard glucose stock solution was prepared. From this, different concentrations (10, 20, 30, 40, and 100 μ g/ml) of standard glucose solution were prepared and final volume was adjusted to 2 ml using buffer, (Table 1) vortexed and measured at 520 nm. A standard graph was

drawn based on the data obtained (absorbance vs concentration). Invertase assay was carried out to compare the enzymatic activity of wild type and mutant type-1 to type-4 yeasts. All strains were grown on YEP+glucose and YEP+sucrose medium for 30 h at room temperature, respectively. After incubation, the culture was centrifuged at 5000 rpm for 10 min. Then the supernatant was carefully collected and stored. 50 μ l of the culture supernatant was used for invertase assay.

2ml of 0.1M sodium acetate buffer (pH 4.5), 0.5ml of 0.1M substrate and 50 μ l of culture supernatant mixtures were incubated at 37°C in a water bath for exactly 15 min, and color intensity was measured at 520 nm. 2ml of 0.1M sodium acetate buffer used as blank, without invertase as substrate blank and without substrate as enzyme blank used for invertase assay.

Protein concentration in enzyme sample was estimated by Bradford's [30] method using bovine serum albumin (BSA) as a standard. To prepare standard BSA, 1 mg/ml of BSA stock solution was prepared using distilled water and the concentration starting from 0 to 100 μ g. The final volume was made up to 500 μ l with distilled water. The experiment was done in triplicates. 4.5 ml of Bradford reagent was added, mixed, and measured at 590 nm. The data were plotted in a graph as concentration of BSA versus absorbance at 590 nm. One unit enzyme invertase activity is defined as the amount of enzyme, which liberates one micromole of reducing sugar per minute under the condition of the assay. Specific activity is expressed in Units/mg of protein [31].

2.6. Invertase Assay for Wild and Mutant Yeast

All subsequent experiments carried out with the initial inoculum yeast cell count approximately 10^7 CFU/ml. Isolated mutant strains type-1, type-2, type-3, type-4 and wild type strain 1ml culture inoculated individually in 50ml of the YEP glucose and YEP sucrose medium for incubate at 30°C for 30 h. After incubation, all the strains invertase enzyme activity estimated according to the previously described methods.

Table 1. Reaction mixtures for invertase assay

Components	Blank	Substrate Blank	Enzyme Blank	Test Sample Growth	
				Sucrose	Glucose
0.1M sodium acetate buffer (pH 4.5)	2 ml	1.5 ml	1.5 ml	1.5 ml	1.5 ml
0.1M substrate	_____	0.5 ml	_____	0.5 ml	0.5 ml
1 mg/ml invertase	_____	_____	50 μ l	50 μ l	50 μ l
Total	2 ml	2 ml	2 ml	2 ml	2 ml

2.7. Optimization of Invertase Production

2.7.1. Effect of Various Concentration of Glucose

Mutant strain type-2 were inoculated separately to different concentrations (0.1, 0.2, 0.5, 1.0, 2.0, 5.0, and 10%) of glucose in YEP broth. Then it was incubated 30°C for 30 h and then invertase activity was assayed as described above method.

2.7.2. Effect of Various Incubation Periods

The type-2 mutant yeast culture was inoculated in 50 ml YEP glucose broth and kept for 30°C for incubation at various time intervals such as 10, 20, 30, 40, 50, and 60 h. After incubation, the culture was assayed for the invertase activity.

2.7.3. Statistical Analysis

All data point represents the mean of three independent assays. Statistical implication was determined by standard deviation. The data are represented as the mean $\pm$ standard error (Figures 1-5).

3. Results and Discussion

After incubation, large white colonies of yeast cells were observed and change of color from purple to yellow around the colonies as indicator for sucrose fermentation. This isolated single colony was named as wild type strain and sub cultured in YEPD medium along with streptomycin 100

$\mu\text{g/ml}$ and ampicillin 50 $\mu\text{g/ml}$ containing plates for further screening studies.

High invertase producing mutants were selected from UV mutation and invertase filter paper assay. In filter paper assay, red color spots were developed in the filter paper indicating the positive colonies of invertase. [17], previously reported the same results. This indicates the ability of the mutated culture to produce invertase in YEP glucose medium. This red color spotted colonies were positive selection for further subculture. Based on UV exposure time, type-1, type-2, type-3, and type-4 mutant strains derived from wildtype strain. As shown to the Table 2, approximately 100% survival rate, 1×10^7 mutation frequency and no more invertase positive colonies on wild type strain but type-1 strain have 15 invertase positive colonies with 15×10^{-8} mutation frequency and their survival rate also high 85.5% compare to other mutant. In type-4 strain 42.8% survival rate with 3×10^{-8} mutation frequency and lower invertase positive colonies compare to other mutant; type-2 have 5 and type-3 have 4 positive colonies. As per the Fig. 1 wild type strain survival rate of percentage were gradually decreases from 100% to 42.8% this comparable report previously reported [32] but the time of exposure was in seconds. More than 30 mins exposure are usually lethal for yeast in their reproduction. But, this isolated strains actively grown in subsequent cultures However, all the mutant strains having significant mutation frequency and invertase positive colonies on filter paper assay, these results Table 2 and Fig 1 shown initial evaluation of mutant strains.

Table 2. Generation of mutant strains by UV mutagenesis and mutation analysis of wild type baker's yeast.

UV Exposure time(min)	Strains	Survival rate %	Mutation frequency	Number of invertase positive colonies (Invertase filter assay)
0	wild type	100	1×10^7	-
5	type-1	85.5	15×10^{-8}	15
10	type-2	78.5	5×10^{-8}	5
20	type-3	68.2	4×10^{-8}	4
30	type-4	42.8	3×10^{-8}	3

Figure 1. UV radiation mutagenesis survival rate % of wild type strain. For the analysis of survival rates by UV mutagenesis, cells grown for 24h at 30°C on a YPD medium plate were collected and suspended in phosphate buffer. After the cell concentration was determined by counting, cells were spread on YPD medium plates. The plates were placed under a UV lamp at a distance of 48 cm and were irradiated for various periods of time. Following irradiation, the plates were incubated at 30°C for 30h, and the numbers of colonies were counted to determine survival rates.

3.1. Invertase Assay for Wild and Mutant Yeast

Isolated mutant strains and wild type yeast cells were grown in YEP glucose and YEP sucrose medium for invertase assay (Fig 2) to compare their invertase synthesis ability. In wildtype yeast invertase activities in glucose, sucrose was 3.1, and 25.0 Units/ml, respectively. Although in mutant strain type-1 very less 20.2 Units/ml in sucrose medium. In type-3 highest 28.4 Units/ml in sucrose, but lowermost in glucose medium 1.2 Units/ml. comparably type-4 not much improvement in mutagenesis almost similar observations as wild type strain 3.4, and 22.8 Units/ml. Ultimately type-2 strain in glucose medium was progressively increased from 3.1 to 32.0 Units/ml and almost near 24.1 Units/ml in sucrose medium as compared to the wild type. Therefore, mutant type-2 strain was assumed as could be a constitutive mutant for the production of invertase (Table 3-4, Fig 2). This mutation may have occurred in any glucose repressible genes such as SSn, snf1, snf3, snf4, snf6, and including the SUC2 structural gene [11], [33], [34]. This single or double mutant leads to de-repress the repressible gene [20]. Unfluctuating outcomes in hourly invertase synthesis increased from 0.103 to 1.066 UNITS/ml in glucose medium and almost similar 0.833 and 0.803 Units/ml in sucrose medium as compared to the wild type (Fig 3). Its accord this mutation causes constitutive synthesis of invertase and overcome the glucose repression of the secreted invertase through mutation.

Table 3. Invertase assay of wild type yeast

	YEP + Glucose 2% medium	YEP + Sucrose 2% medium
Substrate blank (OD)	0.29	0.29
Enzyme blank (OD)	0.07	0.07
Test solution (OD)	0.56	1.98
Net reading (OD)	0.20	1.62
Enzyme activity (Units/ml)	3.1	25.0
Total protein (mg)	3.9	11.0
Specific activity (Units/mg)	0.79	2.27

*OD- optical density

Table 4. Invertase assay of mutant type-2 yeast

	YEP + Glucose 2% medium	YEP + Sucrose 2% medium
Substrate blank (OD)	0.32	0.32
Enzyme blank (OD)	0.09	0.09
Test solution (OD)	2.44	1.98
Net reading (OD)	2.04	1.56
Enzyme activity (Units/ml)	32.0	24.3
Total protein (mg)	15.0	11.6
Specific activity (Units/mg)	2.10	2.09

Net glucose = Test solution – Enzyme blank

O.D O.D

Net reading = Net glucose – substrate blank

O.D O.D

Figure 2. Invertase activity of *Saccharomyces cerevisiae* wild type and mutant strains

Figure 3. Invertase activity in hourly for wild and mutant type-2 yeast strain

Figure 4. Different glucose concentrations in mutant type-2 growth medium for invertase assay

Figure 5. Different incubation periods for mutant type-2 strain for the invertase assay.

The effect of glucose concentration (0.1, 0.2, 0.5, 1.0, 2.0, 5.0, and 10%) and incubation duration time (10, 20, 30, 40, 50, and 60 h) was tested on mutant type-2 strain. The highest invertase production was observed at 2% (28.3 Units/ml) and at 5% (28.8 Units/ml) of glucose. Beyond 5% glucose, the

enzyme activity was almost reduced in 10% (Fig: 4). In different invertase production time duration experiment, the maximum enzyme activity was observed at 30 h (21.0 Units/ml) of incubation. Further increase in the incubation time significantly reduces the invertase activity its shown in

Fig:5 Similar observation has been reported previously with pH 3.5-5.0 [35] and optimum temperature 70°C [36] observation also reported.

In conclusion, this work is to overcome the glucose repression of the secreted invertase through traditional UV mutation. This mutational effort has given an industrially potent strain and the above conditions are valuable for the excess production of invertase to speed up the industrial processes. Further studies of genetic engineering, pathway analysis, transcriptome, and proteome analysis are needed to improve more economical and biologically safer products in the fermentation industry.

Conflict of Interest

The authors declare that they have no conflict of interest in the publication.

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