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# ISOLATION AND OPTIMIZATION OF CONSTITUTIVELY SYNTHESIZED INVERTASE, FROM *SACCHAROMYCES CEREVISIAE* MUTANT TYPE- 2 STRAIN

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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 is code by six SUCI to SUC5 and SUC7 un linked genes. The main objective of this work is to overcome the glucose repression of invertase by invertase constitutive mutants, and to optimize various parameters such as temperature, incubation time, and pH. This mutation may occur in any glucose repressible genes as a single or double mutation in repressor gene (s) which might cause constitutive synthesis of invertase. The maximum constitutive synthesis of invertase was obtained with aerobic conditions for growth, 2-5% of glucose concentration, pH 4-5 of medium, and incubation period at 30°C for 30 h using UV mutated type-2 strain. Using this mutant strain, natural substrates containing sucrose + glucose mixture can be used as raw materials, for the excess production of fermentation products.*

***KEYWORDS:*** *Constitutive Synthesis, Invertase, Mutation, Optimization, Saccharomyces cerevisiae*

## *Article History*

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## 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 (Eduardo JS Bras et al, 2016).

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. 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 (Carlson and Botstein, 1983; Carlson et al, 1985). The level of enzyme secretion depends on the concentration of glucose in the culture medium (Gascon and Ottolenghi, 1972). Repression of the external invertase synthesis has been described, by the level of transcription (Elorza et al, 1977 and Perlman et al, 1981), translation, and glycosylation (Chu and Maley, 1980) of the protein. In this case, the non-glycosylated enzyme could be degraded by specific degradation steps and are responsible for the glucose dependent, through activation of the malate dehydrogenase (Abrams et al, 1978). *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. 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 (Carlson M et al, 1982).

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 (Celenza and Carlson, 1984; Neigeborn and Carlson, 1984). 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 (Robert J. Trumbly, 1986). The snf1 gene product is required to depress the expression of many glucose repressible genes including the SUC2 structural gene of invertase (M. Carlson et al, 1984). In wild-type strains, synthesis of this mRNA is regulated by glucose repression. A 1.8 kb mRNA transcribed constitutively from the SUC2 gene encloses the cytoplasmic invertase, which plays no major role in sucrose fermentation (Pearlmann et al, 1981). The cytoplasmic invertase and 1.8 kb of mRNA are produced normally by SNF1 mutants. Hence, the SNF1 mutation only affects the glucose-regulated expression of the SUC2 gene (Neigerborn, 1987-25). The phenotype of the mutants suggests that the SNF1 gene product act positively to de-repress gene expression in response to lower glucose concentration in the environment (M. Carlson et al, 1984). The SNF6 mutant produce low levels of secreted invertase under de-repressing conditions; however, no pleiotropy was detected. The SNF3 mutant produce 10-35% of secreted invertase under de-repressed conditions in the wild type, but grow less in sucrose than expected from invertase activity; in addition, these mutants synthesized some invertase under glucose repressing condition. Mutation causing constitutive synthesis of high level invertase was previously isolated as a suppression of SNF1 gene. The SSn6 mutation completely suppressed the defects in de-repression of invertase by snf, snf3, snf4, and snf6 end each double mutant.

The main aim of this work was to isolate wild type yeast cells, and perform UV mutagenesis to conform the glucose repression of invertase constitutive mutants and to optimize the fermentation conditions, for the excess production of invertase in *S. cerevisiae* mutant type-2 strain.

## MATERIALS AND METHODS

### Screening of High Sucrose Fermenting Yeast Strain

Wild type baker's yeast was isolated from contaminated grape juice. 0.1 ml of grape juice was spread on the YEP (Yeast extract peptone) rich medium and incubated at 30°C for 30 h. Large white colonies of cells were observed. These cells were identified and confirmed as yeast through microscopical examination. Isolated single colonies were subcultured in YEPD (Yeast extract peptone dextrose) agar medium, containing 10gm of yeast extract, 20gm of peptone, 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 and slants. YEPS (Yeast extract peptone sucrose) agar medium containing 10gm of yeast extract, 20gm of peptone, 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 fermenting sucrose. Sucrose fermentation is an indication, by the change in color of the medium from purple to yellow.

### Invertase Assay

Invertase assay was performed by Nelson-Somagy's method (1944), 1 mg/ml of standard glucose stock solution was prepared. From this, different concentrations (10, 20, 30, 40, and 100 µg) 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-2 yeasts. Both 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 µl of the culture supernatant was used for invertase assay.

**Table 1: The Following Reaction Mixtures Used in 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 µl | 50 µl | 50 µl |
| **Total** | **2 ml** | **2 ml** | **2 ml** | **2 ml** | **2 ml** |

These mixtures were incubated at 37° C in a water bath for exactly 15 min, and color intensity was measured at 520 nm.

Protein concentration in enzyme sample was estimated by Bradford's (Bradford, M. M. 1976) 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 µg. The final volume was made up to 500 µl 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 (Palanivalu, 1992).

### UV Mutagenesis

Isolated culture (50 ml) was prepared, as described previously using antibiotics in medium. The 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 was aliquoted into 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 (5, 10, and 30 min), to provide varying UV dosage under the laminar flow hood and UV source for 48cm. 100 µl of each sample were used, to spread on YEP glucose plate.

### Filter Assay of Invertase Activity

This method was used to screen large numbers of mutants, for detecting invertase activity in yeast grown in glucose medium (Robert J. Trumbly, 1986). Yeast colonies grown in YEPD 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$^{o}$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. Invertase positive colonies were named as mutant type-2 strain which was used in further studies.

### Effect of Various Concentration of Glucose

Mutant type-2 yeast was inoculated in 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 for 30 h and the invertase activity was assayed

### Effect of Various pH

The YEPD broth was prepared at various pH such as 3, 4, 5, 6, 7, 8, and 9. Mutant type-2 yeast culture was inoculated in to the medium and allowed for incubation, and then the invertase activity was analyzed.

### Effect of Various Incubation Periods

The YEPD broth was prepared and the mutant type-2 yeast culture was inoculated in to the medium and kept for incubation at various time intervals such as 20, 30, 40, 50, and 60 h. After incubation, the culture was assayed for the invertase activity.

### Effect of Different Temperature

The YEPD broth was prepared and the mutant type-2 yeast culture was inoculated. The cultured medium was incubated at various temperatures such as 10, 20, 30, 40, 50, and 60°C and maintained in a water bath shaker. After 30 h of incubation, the invertase activity was assayed and the results were tabulated.

### Effect of Oxygen on the Invertase Assay

The YEPD broth was prepared in conical flask and screw cap tube (20 ml). The mutant type-2 yeast culture was inoculated in both medium. Inoculated conical flask was incubated with rotating shaker and screw cap tube medium was purged with nitrogen gas and incubated. After 30 h, the invertase activity was assayed.

### Statistical Analysis

All the data point represents the mean of three independent assays. Statistical implication was determined by standard deviation. The data are represented as the mean±standard error (Figures 1-6).

## RESULTS AND DISCUSSIONS

Wild type baker's yeast was isolated from contaminated grape juice. After incubation, large white colonies were observed. These colonies were identified and confirmed as yeast through microscopical examination. Under the microscope, the morphological and budding features were studied. Isolated single colonies were subcultured in YEPD medium along with streptomycin 100 µg/ml and ampicillin 50 µg/ml containing plates.

### Screening of High Sucrose Fermenting Yeast

Purified yeast culture was spread on indicator media. After incubation, yellow halos were observed around certain colonies indicating sucrose fermentation by yeast.

After UV mutagenesis, several colonies were obtained on YEP glucose medium which is used for further analysis.

### Filter Assay of Invertase Activity

The red color spots were developed in the filter paper indicating the positive colonies of invertase. (Robert J. Trumbly in 1986), previously reported the same results. This indicates the ability of the mutated culture, to produce invertase in YEP glucose medium. These positive colonies were used in further studies.

### Invertase Assay for Wild and Mutant Type-2 Yeast

The isolated mutant type-2 yeast cells were grown in YEP glucose and YEP sucrose medium. The invertase activities in glucose, sucrose, and wild yeast were 32.0, 24.3, and 25.0 Units/ml, respectively. Invertase activity of mutant grown in glucose medium was gradually increased from 3.1 to 32.0 Units/ml as compared to the wild type. But the activity exhibited by the mutant in sucrose medium was similar as that of wild type. Therefore, the mutant type-2 yeast isolated could be a constitutive mutant for the production of invertase (Table 2-3, Figure: 1).

### Effect of Various Concentration of Glucose on Invertase Assay

Enzyme activity of the mutant type-2 yeast at different glucose concentration was studied. The maximum enzyme activities were observed at 2% (28.3 Units/ml) and at 5% (28.8 Units/ml) of glucose. Beyond 5% glucose, the enzyme activity was reduced to almost 10% (Figure: 2).

### Effect of Various pH on the Invertase Activity

Enzyme activity at different pH levels was studied. The enzyme activity was gradually increased up to pH 5.0. Beyond pH 8, sudden decrease in the enzyme activity was observed (Figure: 3). Similar observation has been reported previously with pH 3.5-5.0 (Andi 2010).

### Effect of Various Incubation Periods

The enzyme activity was increased gradually with the time of incubation (Figure: 4). It clearly shows that the maximum enzyme activity was observed at 30 h (21.0 Units/ml) of incubation. Further increase in the incubation period, significantly reduces the enzyme activity.

## Effect of Various Temperatures on the Invertase Activity

This result clearly indicates that the optimum temperature for enzyme activity was 30°C (27.1 Units/ml). Further increase or decrease in the temperature, significantly reduces the enzyme activity (Figure: 5). Optimum 70°C observation have been reported previously (da selva 2011).

## Effect of Oxygen on the Invertase Activity

The enzyme activity of the mutant grown under aerobic and anaerobic conditions was studied. In aerobic condition, the enzyme activity was 30.2 Units/ml but under anaerobic condition the activity was 28.9 Units/ml (Figure: 6).

**Table: 2: 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 3: 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

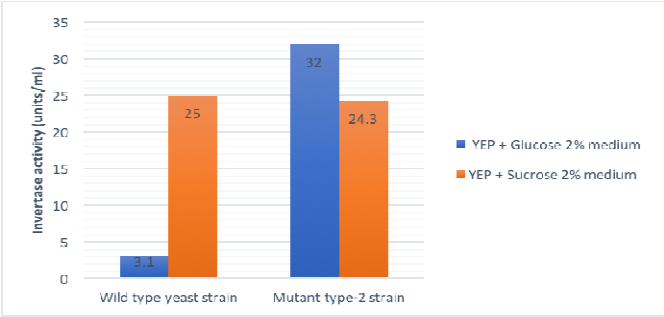


**Figure 1: Invertase Activity for Wild and Mutant Type-2 Yeast Strain**

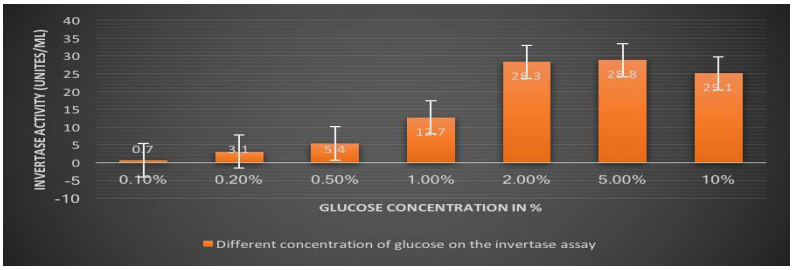


**Figure 2: Different Glucose Concentrations in Mutant Type-2 Growth Medium on Invertase Assay**

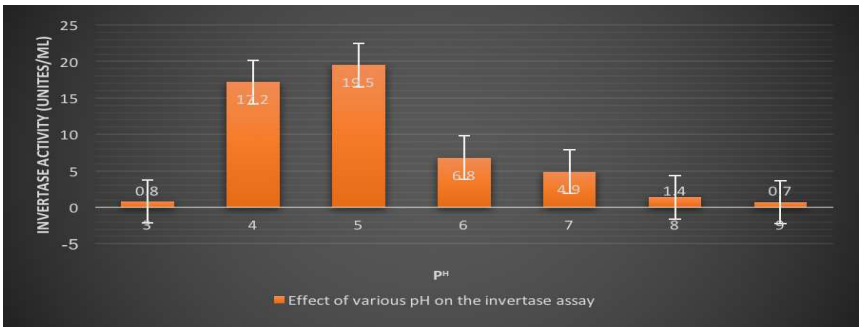


**Figure 3: Effect of Various pH of Mutant Type-2 Strain on the Invertase Assay**

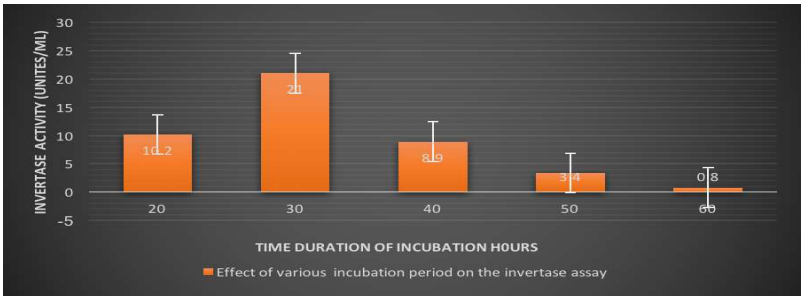


**Figure 4: Different Incubation Periods for Mutant Type-2 Strain on the Invertase Assay**

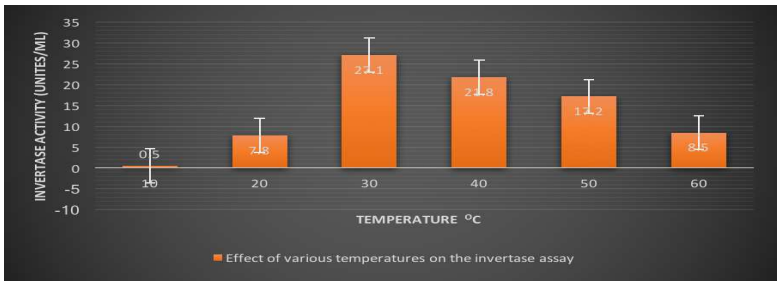


**Figure 5: Effect of Various Temperature on Mutant Type-2 Strain on Invertase Assay**

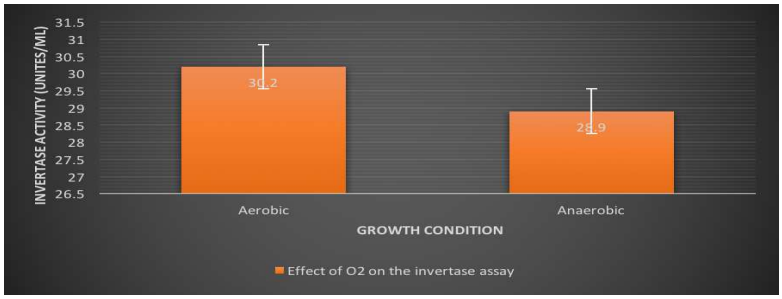


**Figure 6: Aerobic and Anaerobic Growth Conditions of Mutant Type-2 Strain on Invertase Assay**

## CONCLUSIONS

The main objective of this work is to overcome the glucose repression of the secreted invertase through mutation. Invertase activity of mutant grown in glucose medium was gradually increased from 3.1 to 32.0 UNITS/ml as compared to the wild type. This mutation may have occurred in any glucose repressible genes such as SSn, snf1, snf3, snf4, snf6, and including the SUC2 structural gene. This single or double mutant leads to de-repress the repressible gene. This mutation causes constitutive synthesis of invertase. This isolated mutant type-2 yeast strains were optimized under various fermentation conditions such as aerobic condition for growth, 2-5% of glucose concentration, pH 4-5 of medium, and incubation at 30°C for 30 h. Therefore, the above condition is valuable for the excess production of invertase and to speed up the industrial fermentation processes. However, these data can be used in further studies including pathway analysis, transcriptome, and proteome analysis to determine the biology of the eukaryotic cells function.

### Conflict of Interest

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

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