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β-D-Fructofuranosidase Production by a 2-Deoxy-D-Glucose Stabilized Mutant Strain of *Saccharomyces cerevisiae* on Kinetic Basis

IKRAM-UL-HAQ, SIKANDER ALI, AAFIA ASLAM AND M.A. QADEER
Biotechnology Research Centre, Department of Botany, Government College University, Lahore, Pakistan

Abstract: The present study deals with the β-D-fructofuranosidase production by a 2-deoxy-D-glucose resistant mutant strain of *Saccharomyces cerevisiae* on kinetic basis. For this purpose, the wild-culture (*Saccharomyces cerevisiae* IS-14) was developed by combined treatments of radiations and chemicals (UV/MNNG, UV/EMS, MNNG/EMS) for different time intervals (10-30 min). The best β-D-fructofuranosidase mutant strain of *Saccharomyces cerevisiae* (34.12 U/ml) was coded to as UME-2. This mutant strain was cultured on the medium containing 2-deoxy D-glucose (2dg) and its stability for β-D-fructofuranosidase productivity was determined at various 2dg levels. The 2dg concentration of 0.04 mg/ml was found optimal, as at this level UME-2 gave consistent β-D-fructofuranosidase yield. After optimization of incubation time (48 h), sucrose concentration (5.0 g/l), initial pH (6.0) and 16 h old inoculum (size 2.0 %, v/v), enzyme production reached 45.65 U/ml with a noticeable ≈ 40-fold increase compared to the original wild-culture (IS-14). On the basis of kinetic parameters notably Q_p (0.723 U/g/h), $Y_{p/s}$ (2.036 U/g) and q_p (0.091 U/g yeast cells/h), it was concluded that the mutant (UME-2) is a hyper producer of β-D-fructofuranosidase and has a faster growth rate. The over all rate of volumetric productivity is 31.43 fold improved over the parental strain and economically highly significant (LSD 0.054, $p<0.05$).

Keywords: β-D-fructofuranosidase, *Saccharomyces cerevisiae*, sucrose salt media, random mutation, 2-deoxy-D-glucose.

INTRODUCTION

The enzyme β-D-fructofuranosidase (EC 3.2.1.26) attacks β-D-fructofuranoside (raffinose, stachyose or sucrose) from the fructose end. This enzyme is produced by a large number of organisms such as *Neurospora crassa, Candida utilis, Fusarium oxysporum, Phytophthora meganosperma, Aspergillus niger, Saccharomyces cerevisiae, Schizosaccharomyces pombe* and *Schwanniomyces occidentalis* (Lothe *et al*., 1999). However, *Saccharomyces cerevisiae* is well documented as an organism of choice for its production because of its characteristic high sucrose fermentability (Neto *et al.*, 1996). β-D-fructofuranosidase is useful in the production of confectionery with liquid or soft centres and in the aid for fermentation of cane molasses into ethanol. Its demand is increasing with the development of confectionery industry. Microbial β-D-fructofuranosidase is used in calf feed preparation and also for the manufacture of inverted sugars (Sanchez *et al.,* 2001).

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The provision of appropriate fermentation conditions and induction of mutation is required for proper growth and high yield of the desired product. Basal medium composition may be yeast-extract, salt, carbohydrates, peptone or vitamin solution (Roitsch *et al*., 2003). Industrial molasses media with ethanol and NaCl have also been used for β-D-fructofuranosidase production (Zech and Georisch, 1995). Biosynthesis of β-D-fructofuranosidase in yeast is controlled by the addition of proper carbon source into the culture medium (Bokosa *et al*., 1992). The use of β-D-fructofuranosidase is somewhat limited due to its high price, thus optimization of production process is important so as to make the process economical. The present study is concerned with the improvement of *Saccharomyces cerevisiae* strain after random mutation for β-D-fructofuranosidase overproduction by submerged fermentation in shake flask and its kinetic basis.

MATERIALS AND METHODS

Microorganism

The parental strain of *Saccharomyces cerevisiae* IS-14 was obtained from the available

culture collection of Biotechnology Research Centre, GCU Lahore and maintained on YPSA medium containing (g/l): yeast extract 3.0, peptone 5.0, sucrose 20.0 and agar 20.0 (pH 6.0). The slant cultures were incubated at 30ºC for 2-3 days and preserved in sterile liquid paraffin at 4°C in a lab cool (SANYO, Japan).

Preparation of vegetative inoculum

Vegetative inoculum of *Saccharomyces cerevisiae* was used in the present study. Fifty millilitre of the YPSA medium (without agar) was transferred to the individual 250 ml Erlenmeyer flasks. The flasks were cotton plugged and autoclaved at 15 lbs/in^2 pressure (121°C) for 15 min and cooled to ambient temperature. Cell suspension was prepared from a 2-3 day old slant culture by adding 10 ml of sterilized distilled water and shaking vigorously. One millilitre of the cell suspension (1.2×10^6 cells/ml) was aseptically transferred into the flask and incubated at 30°C in a rotary shaking incubator (Gallenkamp, UK) at 200 rpm for 24 h.

Batch culture technique

Fifty millilitre of the YPS medium (pH 6.0) was transferred to the individual 250 ml Erlenmeyer flasks. The vegetative inoculum was transferred to the production medium at a level of 4.0% (v/v). Flasks were incubated in a rotary shaking incubator at 30°C for 48 h. The agitation rate was kept at 200 rev/min. The flasks were run parallel in triplicates.

Strain development by random mutation

The combined effects of radiations and chemical mutagens by random mutation were undertaken for different time intervals (10-30 min) following the schematic relations,

Scheme-I: UV (1.2×10^6 J/m^2/S) / MNNG (0.06 mg/ml)
Scheme-II: UV (1.2×10^6 J/m^2/S) / EMS (100 µl/ml)
Scheme-III: MNNG (0.06 mg/ml) / EMS (100 µl/ml)

Culture stability by 2-deoxy D-glucose

Mutant strain (UME-2) was cultured overnight in the YPSA medium, harvested during the exponential phase of growth (1.2×10^6 cells/ml), washed with sterilized distilled water and plated on the (2dg-YPRA) medium containing (mg/ml): yeast extract 3.0, peptone 5.0, raffinose 20.0, agar 20.0 and 2-deoxy-Dglucose 0.02-0.10. Raffinose was used replacing sucrose because sucrose hydrolysis by yeast β-D-fructofuranosidase results into glucose, which competes the toxin (Rincon *et al.,* 2001). Colonies appearing between 2-5 days were subcultured on the same medium and those still growing vigorously were tested for stability in β-D-fructofuranosidase production by shake flask fermentation. Samples were taken periodically, washed and plated on the medium to select the strains resistant to 2dg.

Assay methods

Dry cell mass

Dry cell mass was determined by centrifugation of the fermented broth at 9,000 rev min^{-1} (8,831/g) for 15 min using preweighed centrifuge tubes. After decanting off the supernatant, cell mass was washed twice with distilled water. The tubes containing cell mass were oven dried at 105°C for 1 h.

Sugar consumption

Sugar was estimated by DNS method (Miller 1959). Sugar concentration in the supernatant was determined by taking 1.0 ml of supernatant alongwith 1.5 ml of DNS reagent in a test tube. Blank containing 1.0 ml of distilled water and 1.5 ml of DNS was also run parallel. The test tubes were heated in a boiling water bath for 15 min with subsequent addition of 0.5 ml Rochelle salt solution (40%, w/v). Cooled the test tubes at room temperature and the transmittance was noted at 575 nm using UV/VIS double beam scanning spectrophotometer (Cecil CE 100, UK). Sugar concentration was determined from the standard.

β-D-fructofuranosidase activity

β-D-fructofuranosidase activity was determined after Rouwenhorst *et al.* (1991). "One β-D-fructofuranosidase unit is defined as the amount of enzyme, which releases 1.0 mg of inverted sugar in 5 min at 20°C, pH 4.5". One millilitre of the

fermented broth, after centrifugation, was transferred to 5.0 ml of 6.5 % (w/v) sucrose-acetate buffer having pH 4.5 in a test tube. Reaction mixture was incubated at 20°C in a thermostat bath. After 5 min, added 5.0 ml of 0.1 N NaOH. Inverted sugar was determined in 1.0 ml aliquot by DNS method. Milligrams of inverted sugar multiplied by 11 gave β-D-fructofuranosidase units per millilitre of fermented broth.

Kinetic parameters

The kinetic parameters were studied according to the procedures of Pirt (1975). The values for specific growth rate *i.e.*, μ (h^{-1}) were calculated from the plots of ln(X) vs. time of fermentation. The growth yield coefficient ($Y_{x/s}$) was calculated as the dry cell mass of saccharide utilized from the test substrate following fermentation. The product yield coefficients namely $Y_{p/s}$ and $Y_{p/x}$ were determined by using the relationships *i.e.*, $Y_{p/s}$ = dP/dS and $Y_{p/x}$ = dP/dX, respectively. The volumetric rates for substrate utilization (Q_s) and product formation (Q_p) were determined from the maximum slopes in plots of substrate utilized and β-D-fructofuranosidase produced each vs. the time of fermentation. The volumetric rate for biomass formation (Q_x) was calculated from the maximum slope in plot of cell mass formation vs. the incubation time period. The specific rate constants for product formation (q_p) and substrate utilization (q_s) were determined by the equations *i.e.*, $q_p = \mu \times Y_{p/x}$ and $qs = \mu \times Y_{s/x}$, respectively. The specific rate for cell mass formation (q_x) was, however, calculated by multiplying the specific growth rate (μ) with the growth yield coefficient ($Y_{x/s}$).

Statistical analysis

Treatment effects were compared after Snedecor and Cochran (1980). Duncan's multiple range tests (Spss-10, version 4.0) were applied under one-way ANOVA. Significance has been presented in the form of probability ($p<0.05$) values.

RESULTS AND DISCUSSION

The data of Table I shows the range of β-D-fructofuranosidase activity of mutant strains of *Saccharomyces cerevisiae* developed after the combined treatments of radiations and chemicals (UV/MNNG, UV/EMS, MNNG/EMS). Among the mutants, the best β-D-fructofuranosidase mutant strain of *Saccharomyces cerevisiae* (34.12 U/ml) was coded to as UME-2. This mutant strain was cultured on the medium containing 2-deoxy D-glucose (2dg) and its stability for β-D-fructofuranosidase productivity was determined at various 2dg levels. Initially, high yielding colonies were obtained at 2dg concentration of 0.02 mg/ml, however, these cultures lost stability after a couple of weeks. The reason may be the development of resistance in yeast cells after few generations that allowed a few unstable mutants to thrive. To eradicate this problem, these cultures were again

Table I.- Range of β-D-fructofuranosidase activity of mutant strains of *Saccharomyces cerevisiae* IS-14 developed after the combined treatments of radiations and chemicals*

Number of mutant strains	Range of β-D-fructofuranosidase activity (U/ml)
Combined effect of UV and MNNG	
2	0 - 5.0
4	5.1 - 10.0
3	10.1 - 15.0
2	15.1 - 20.0
1	> 20.0
Combined effect of UV and EMS	
2	5.1 - 10.0
1	10.1 - 15.0
5	15.1 - 20.0
3	20.1 - 25.0
1	> 25.0
Combined effect of MNNG and EMS	
2	15.1 - 20.0
3	20.1 - 25.0
2	25.1 - 30.0
1	> 30.0

*Sucrose concentration 5.0 g/l, incubation period 48 h, temperature 30°C, initial pH 6.0, agitation rate 200 rev/min.

* The mutants were picked up in comparison with the control from the petriplates having at least 90 % death rate. The best β-D-fructofuranosidase mutant strain of *Saccharomyces cerevisiae* (34.12 U/ml) was coded UME-2 and selected for cultural and nutritional studies in shake flask on kinetic basis.

grown on the medium containing different levels of 2dg. The concentration of 0.04 mg/ml was found optimal, as at this level UME-2 gave consistent β-D-fructofuranosidase yield (Randez *et al.,* 1995).

In batch wise β-D-fructofuranosidase fermentation, the enzyme production started after a lag phase of 8 h and reached maximum at the onset of stationery phase. Afterwards, enzyme activity declined due to the decreased nutrient availability in the medium, or carbon catabolite repression, as the expression of β-D-fructofuranosidase in *Saccharomyces* is checked by the presence of monosaccharides like glucose and fructose (Herwig *et al.*, 2001). Maximum β-D-fructofuranosidase production (34.72 U/ml with 17.05 g/l sugar consumption and 7.85 g/l dry cell mass) was observed 48 h after incubation by the mutant UME-2 (Figs. 1-2). Thus, over all the rate of volumetric productivity is 31.43 fold improved over the wild-culture. Further increase in the incubation period did not enhance β-D-fructofuranosidase production. It might be due to the decreased amount of available nitrogen in fermentation medium, the age of organism, the addition of inhibitors produced by yeast itself and the proteolytic characteristic of decline phase.

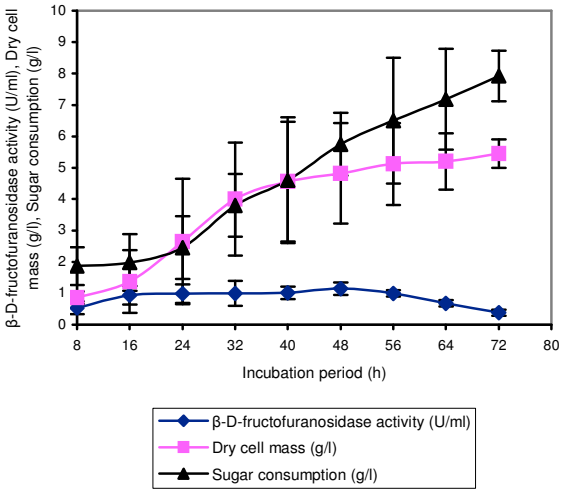

Fig 1. Time course profile of β-D-fructofuranosidase production in submerged culture by the wild strain *Saccharomyces cerevisiae* IS-14*
*Sucrose concentration 30 g/l, temperature 30°C, initial pH 6.0, agitation rate 200 rev/min.
*Y-error bars indicate standard deviation among the three parallel replicates.

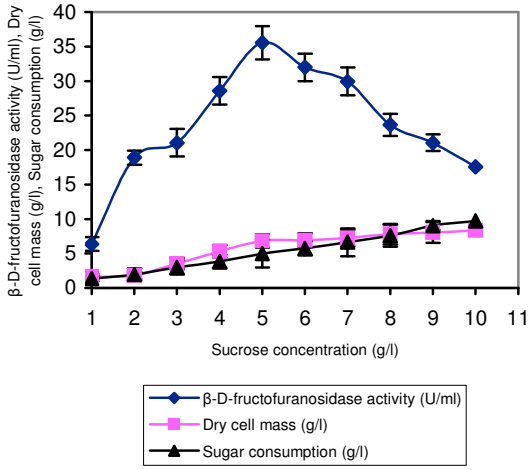

Fig 2. Time course profile of β-D-fructofuranosidase production in submerged culture by 2dg-stabilized mutant strain of *Saccharomyces cerevisiae* UME-2*
*Sucrose concentration 30 g/l, temperature 30°C, initial pH 6.0, agitation rate 200 rev/min.
*Y-error bars indicate standard deviation among the three parallel replicates.

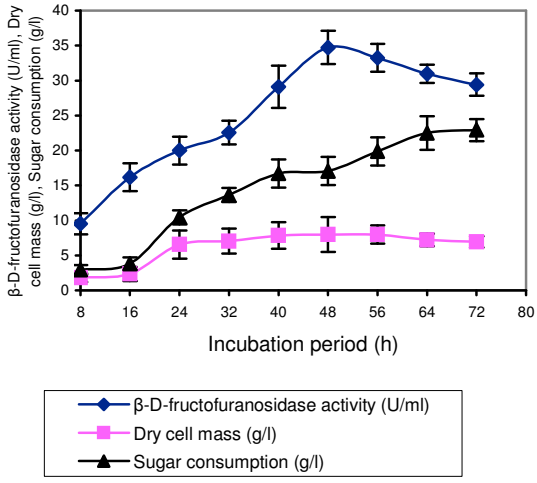

Fig 3. Effect of sucrose concentration on the β-D-fructofuranosidase production in submerged culture by 2dg-stabilized mutant strain of *Saccharomyces cerevisiae* UME-2*
*Incubation period 48 h, temperature 30°C, initial pH 6.0, agitation rate 200 rev/min.
*Y-error bars indicate standard deviation among the three parallel replicates.

Effect of sucrose concentration (1.0-10.0 g/l) on β-D-fructofuranosidase production by the mutant *Saccharomyces cerevisiae* UME-2 was studied (Fig. 3). Maximum enzyme activity (35.56 U/ml) was obtained at sucrose concentration of 5.0 g/l. Sucrose concentration more than 5.0 g/l caused an increase in the sugar consumption and cell biomass, however, there was no net increase in β-D-fructofuranosidase production. The reason might be generation of higher concentration of inverted sugar in the medium, which resulted in glucose-induced repression of β-D-fructofuranosidase. At concentrations of sucrose less than optimal, enzyme production was significantly less. As sucrose is carbon source in the medium, lower concentrations might limit proper growth of yeast, resulting in a lesser yield of β-D-fructofuranosidase.

The production of β-D-fructofuranosidase is largely dependent on initial pH. Figure 4 shows the effect of initial pH on enzyme production by the mutant *Saccharomyces cerevisiae* UME-2. Maximum production of β-D-fructofuranosidase was obtained when initial pH was kept at 6.5. Similarly, dry cell mass and sugar consumption were maximal at pH 6.5 i.e., 7.43 and 4.99 g/l, respectively. Final pH of the medium was 6.7. Less enzyme activity, accompanied by decreased dry cell mass and sugar consumption, was noticed at pH other than the optimal (Lothe *et al.*, 1999). It was noted that during submerged fermentation of *S. cerevisiae*, final pH of reaction mixture was more than initial pH; besides, extent of the increase in pH was proportional to β-D-fructofuranosidase activity. It may be due to the fact that β-D-fructofuranosidase production accompanies secretion of some anions and basic proteins, or selective uptake of cations.

Some attempts have been made to standardize the inocula for β-D-fructofuranosidase production in shaking culture (Gancedo 1998). In the present investigation, 16 h old vegetative inoculum was optimised for the maximum β-D-fructofuranosidase production (45.65 U/ml) in shake flasks (Fig 5-6) when added at a level of 2.0 % (v/v). When the size of inoculum was greater or smaller than 2.0 % (v/v), it resulted in the reduction of β-D-fructofuranosidase production. It might be due to the fact that at a lower concentration, amount of mycelium formed was not well enough to convert more substrate into enzyme.

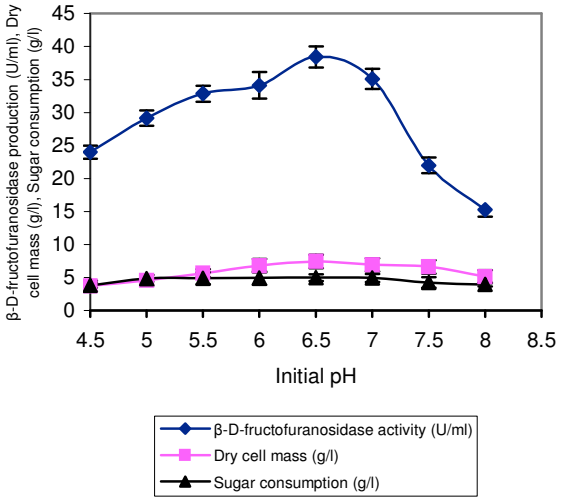

Fig 4. Effect of initial pH on the β-D-fructofuranosidase production in submerged culture by 2dg-stabilized mutant strain of *Saccharomyces cerevisiae* UME-2*
*Incubation period 48 h, sucrose concentration 5.0 g/l, temperature 30°C, agitation rate 200 rev/min.
*Y-error bars indicate standard deviation among the three parallel replicates.

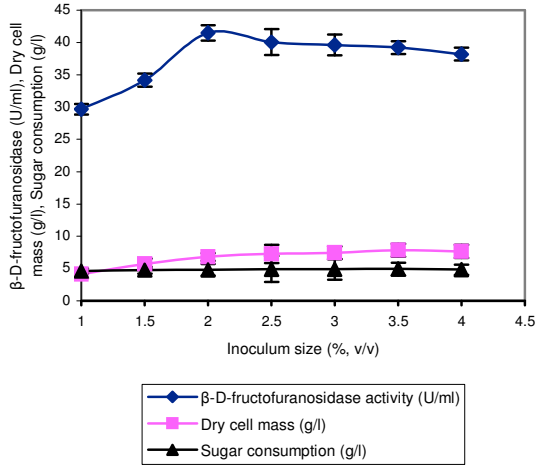

Fig 5. Effect of inoculum size on the β-D-fructofuranosidase production in submerged culture by 2dg-stabilized mutant strain of *Saccharomyces cerevisiae* UME-2*
*Incubation period 48 h, sucrose concentration 5.0 g/l, temperature 30°C, agitation rate 200 rev/min.
*Y-error bars indicate standard deviation among the three parallel replicates.

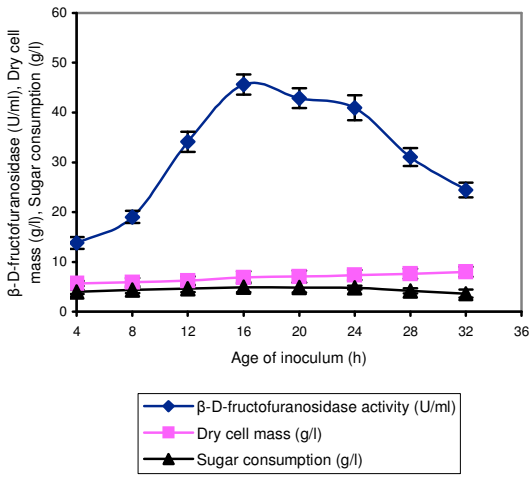

Fig. 6. Effect of age of inoculum on the β-D-fructofuranosidase production in submerged culture by 2dg-stabilized mutant strain of *Saccharomyces cerevisiae* UME-2*

*Incubation period 48 h, sucrose concentration 5.0 g/l, temperature 30°C, agitation rate 200 rev/min.

*Y-error bars indicate standard deviation among the three parallel replicates.

At higher concentration of vegetative inoculum, the viscosity of fermentation medium increased which caused diffusional problems and hence, decreased β-D-fructofuranosidase production (Roitsch *et al.,* 2003).

In the present study, the comparison of Q_s (g cells/l/h) for β-D-fructofuranosidase fermentation depicts that mutant strain (UME-2) has a higher value for volumetric rate of substrate consumption (Q_s = 0.355 g/l/h) than the wild-culture IS-14 (Pirt 1975). Several folds improvement in terms of volumetric β-D-fructofuranosidase productivity was noticed with the mutant UME-2 on all the rates examined (Table II, Figs. 1-2). Although wild-culture IS-14 has got a higher value ($Y_{x/s}$ = 0.793 g yeast cells/g) than the mutant, mutant UME-2, however, has about 31.43 fold improvement in terms of volumetric rate of product formation. In addition, when both of these two cultures were monitored for comparison in specific rate constant, the mutant UME-2 gave higher values for q_p (>18 fold improvement). So, on the basis of kinetic parameters, it was found that the mutant UME-2 showed many folds improved values for Q_p, $Y_{p/x}$, $Y_{p/s}$ and q_p over the parental strain. Neto et al (1996) investigated that the aeration rate and substrate moisture content influences the substrate consumption rate, specific growth rate and enzyme productivity.

Table II. Comparison of kinetic parameters for β-D-fructofuranosidase activity by *Saccharomyces cerevisiae* (wild-culture and 2dg-stabilized mutant strain) in shake flask 48 h after the incubation*

Kinetic parameters	β-D-fructofuranosidase activity (U/ml)	
	IS-14 (Wild-culture)	UME-2 (mutant)
Specific growth rate		
μ (h^{-1})	0.092	0.166
β-D-fructofuranosidase formation parameters		
Q_p (U/h/h)	0.023	0.723
$Y_{p/s}$ (U/g)	0.190	2.036
$Y_{p/x}$ (U/g)	0.239	4.356
q_p (U/g yeast cells/h)	0.005	0.091
Substrate consumption parameters		
$Y_{x/s}$ (g yeast cells/g)	0.793	0.467
Q_s (g/l/h)	0.121	0.355
q_s (g/g yeast cells/h)	0.026	0.045
Q_x (g yeast cells/l/h)	0.096	0.174
Least significant difference (LSD)	0.013	0.054
Significance level <p>	S	HS

Kinetic parameters: μ (h^{-1}) = specific growth rate, Q_p = β-D-fructofuranosidase units/g/h, $Y_{p/s}$ = β-D-fructofuranosidase units/g substrate consumed, $Y_{p/x}$ = β-D-fructofuranosidase units/g yeast cells formed, q_p = β-D-fructofuranosidase units/g yeast cells/h, $Y_{x/s}$ = g yeast cells/g substrate utilized, Q_s = g substrate consumed/l/h, q_s = g substrate consumed/g yeast cells/h, Q_x = g yeast cells formed/litre/h.
HS is for the 'highly significant', while S is for 'significant' values.

CONCLUSIONS

In present study, the best β-D-fructofuranosidase mutant strain of *Saccharomyces cerevisiae* (34.12 U/ml) was coded to as UME-2, developed after random mutation (UV/MNNG, UV/EMS, MNNG/EMS). This mutant strain was cultured on the medium containing 2-deoxy D-glucose (2dg) and its stability for β-D-

fructofuranosidase productivity was determined at various 2dg levels. The 2dg concentration of 0.04 mg/ml was found optimal, as at this level UME-2 gave consistent β-D-fructofuranosidase yield. The cultural and nutritional requirements such as rate of β-D-fructofuranosidase fermentation (48 h), sucrose concentration (5.0 g/l), initial pH (6.5) and 16 h old vegetative inoculum (2.0 %, v/v) were optimized. Several fold improvement in terms of volumetric β-D-fructofuranosidase productivity was noticed with the mutant UME-2 over to that of wild-culture.

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