

Original Research Article

Kinetic evidence for improved invertase activity from a novel mutant of *Candida fermentati* DMS-4

Sikander Ali^{1*}, M. Umer Hayyat², Rashid Mehmood²

¹Institute of Industrial Biotechnology (IIB), GC University Lahore, Pakistan

²Sustainable Development Study Centre (SDSC), GC University Lahore, Pakistan

***Corresponding Author:**

Sikander Ali

Email: alisbiotech@yahoo.com

Abstract: *Candida fermentati* SA3-7, a haploid yeast strain was treated by ultraviolet (UV) radiations and Dimethyl sulphonate (DMS) for enhanced invertase (EC 3.2.1.26) production. The UV induced mutagenesis did not produce any stable mutant with improved enzyme production. The culture was subjected to DMS treatment. The mutant DMS-4 obtained after exposure with 0.06 mg/ml of DMS for 45 min gave many fold increased enzyme production (34.12 U/ml) compared to the wild. After optimization of incubation time (48 h), sucrose concentration (5 g/l), initial pH (6.0) and inoculum size (2 % v/v, 16 h old), the enzyme production reached 45.65 U/ml with a remarkable >40 fold enhancement. On the basis of kinetic parameters markedly Q_p (0.872 U/g/h), $Y_{p/s}$ (2.403 U/g) and q_p (0.209 U/g yeast cells/h), it was concluded that the mutant was a hyper producer of enzyme and has a faster growth rate. The rate of volumetric production was 25 fold improved over the parental strain. The enhancement in the enzyme production is highly significant (HS, LSD 0.045, $p \leq 0.05$).

Keywords: *Candida fermentati*, invertase, random mutagenesis, UV, DMS, kinetic study.

INTRODUCTION

Invertase (EC 3.2.1.26) is a glycoenzyme with mannose being the major component of the carbohydrate moiety. The enzyme attacks β -D-fructofuranoside viz. raffinose, stachyose or sucrose. It is useful in the production of confectionery with liquid or soft centres and in the aid for fermentation of cane molasses into ethanol[1]. Its demand is increasing with the development of confectionery industry[2]. The design and formulation of the medium is based on the fundamental knowledge of the microorganism, substrate and the production process. *Saccharomyces cerevisiae* is a documented microorganism for enzyme production because of its characteristic higher sucrose fermentability[3]. Biosynthesis of enzyme in yeast is controlled by the addition of proper carbon source in to the culture medium. An extracellular enzyme was secreted by *C. fermentati* when grown on a medium containing the β -fructofuranosides sucrose or raffinose, indicating that synthesis was subjected to induction by the substrate[4]. Strain development is critical for a biotechnological process and it is based on a number of factors such as physiological stability, yield consistency, incubation time required for the maximum enzyme production as well as the tolerance to temperature, aeration and shear stress. Traditional methods for strain improvement, such as ultraviolet (UV) radiations or use of alkylating agents like dimethyl sulphonate (DMS) to obtain mutants have

been proved successful [5], followed by suitable selection and screening of the survivors. The use of enzyme is somewhat limited due to its high price, thus optimization of production process become important so as to make the process economical and feasible. Therefore work is needed on the mutagenesis of a novel *Candida* spp. for invertase production by submerged fermentation on kinetic basis.

MATERIALS AND METHODS

Microorganism

In the present study, a haploid strain of *Candida fermentati* SA3-7 was obtained from culture collection of Institute of Industrial Biotechnology, GC University Lahore. Enriched medium containing (YPS, % w/v) was 0.3 yeast extract, 0.5 peptone, 3 sucrose was used for culture maintenance.

Mutation and screening

a) UV irradiation: The yeast cells were suspended in 5 ml of sterilized 0.5 % (w/v) sucrose acetate buffer, pH 4.5 and washed twice. The cells were resuspended in 50 ml of the buffer. Five millilitre each of this suspension was transferred to the individual sterile petriplates and exposed to UV light for different time intervals (10-60 min) at a distance of 6 cm (dose 1.2×10^2 J/m²/S). Approximately 0.1 ml of the irradiated cell suspension was transferred to the petriplates containing YPS agar medium containing sucrose (12 %).

b) DMS treatment: Two millilitres of different DMS concentrations (0.02-0.10 mg/ml) was added to the individual centrifuge tubes containing the washed yeast cells and shaken till homogenous suspension. In the control, 5 ml acetate buffer instead of DMS solution was added. After specific time interval (20-60 min), the yeast cells were centrifuged and washed twice in phosphate buffer, pH 7 [6]. The DMS treated yeast cells were resuspended in 5 ml of phosphate buffer.

After mutagenesis and expression, variants on YPS agar medium containing 12 % (w/v) sucrose were plated. The colonies were spread on the medium. Faster growing but thicker colonies were picked and then i) took each colony (stabbing selection module, 30 colonies/plate) with sterile toothpick and stabbed on two plates having same numbers. Kept one plate as the master to select the variant, ii) after growth of the colonies, flooded each colony independently with glucose measuring kit solution. The colonies having larger pinkish zones (as master culture) were picked.

Mutant strain was cultured overnight in YPS 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, agar 20 and 2-deoxy-Dglucose 0.02-0.10. Raffinose was used replacing sucrose because sucrose hydrolysis by yeast invertase results into glucose [7]. Colonies appearing between 3 to 5 days were subcultured on the same medium and those growing vigorously were tested for stability in enzyme production by shake flask fermentation. Samples were drawn periodically, washed and plated on medium to select the strains resistant to 2dg. The master mutant culture was preserved in liquid paraffin oil.

Assay methods

1. Dry cell mass: Yeast dry cell mass was determined by centrifugation of the fermented broth at 9,000 rpm (8,331/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.

2. Sugar consumption: Sugar was estimated by DNS method [8]. Sugar concentration in the supernatant was determined by taking 1 ml of supernatant along with 1.5 ml of DNS reagent in test tubes. Blank containing 1 ml of distilled water along with 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 (40 %, w/v). The test tubes were cooled at room temperature and % transmittance was noted at 575 nm using UV/Vis double beam scanning spectrophotometer (Model: Cecil CE 100, UK). Sugar concentration was determined from the standard.

3. Invertase activity: Enzyme in the form of β -D-fructofuranosidase activity was determined after Akgol *et al*[9]. "One active enzyme unit is defined as the amount of enzyme, which releases 1 mg of inverted sugar in 5 min at 35°C and pH 5.5". For activity, 2.5 ml acetate buffer (50 mM, pH 5.5) and 0.1 ml sucrose (300 mM) was added into the individual test tubes. The tubes were pre-incubated at 35°C for 5 min. After the addition of 0.1 ml of appropriately diluted enzyme solution, incubation was continued for another 5 min. The reaction mixture was placed in a boiling water bath for 5 min to stop the reaction and allowed to cool at room temperature. A blank was also run parallel replacing the enzyme solution with distilled water. To 1 ml of each reaction mixture 1 ml of DNS was added and placed the tubes in boiling water for 5 min. After cooling to an ambient temperature, volume was raised up to 10 ml. Transmittance was measured at 546 nm using spectrophotometer.

Kinetic study

The kinetic parameters were studied according to the procedures of Pirt [10]. 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 production coefficient ($Y_{x/s}$) was calculated as the dry cell mass of saccharide utilized from the test substrate following fermentation. The product production 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 enzyme 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 $q_s = \mu \times Y_{s/x}$, respectively. The specific rate for cell mass formation (q_x) was, however, calculated by multiplying the specific growth rate (μ) with the growth production coefficient ($Y_{x/s}$).

Statistical analysis

Treatment effects were compared by the method of Snedecor and Cochran [11]. 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 ($p \leq 0.05$) values.

RESULTS AND DISCUSSION

The present study deals with the mutagenesis of a haploid strain of *Candida fermentati* for invertase production. The strain was used for isolation of recessive mutations in diploid as thought to be difficult due to the presence of two sets of genes [12]. After exposure to UV radiation ($1.2 \times 10^2 \text{ J/m}^2/\text{S}$, 10-60 min)

for different time intervals, 25 mutant strains were obtained. The UV irradiated mutants were screened for enzyme production, however, it was noted that UV treatment did not induce any stable mutant that might lead to the increased enzyme production, while most of the viables produced slightly higher enzyme as compared to the wild culture. Therefore, chemical mutation using DMS (0.02-0.10 mg/ml, 20-60 min) was undertaken. Eighteen isolates were obtained after DMS treatment from the petriplates having at least 90 % death rate. Among the mutants, the best enzyme mutant strain of *C. fermentati* (34.12 U/ml) was coded to as DMS-4.

The strain DMS-4 was cultured on the medium containing 2-deoxy D-glucose (2dg) and its stability for invertase production was determined at various 2dg levels. Initially, high producing 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 grown on the medium containing different concentrations of 2dg. The concentration of 0.04 mg/ml was found optimal, as at this level DMS-4 gave consistent enzyme production.

In batch wise invertase fermentation, the enzyme production started after a lag phase of 8 h and reached maximum at the onset of stationary phase. Afterwards, enzyme activity declined due to decrease in nutrients availability in the medium, or carbon catabolite repression, as the expression of enzyme in *Saccharomyces* is checked by the presence of monosaccharides like glucose and fructose [13]. Thus proper incubation time is critical for optimal enzyme production. The time course profiles for enzyme production by the wild *C. fermentati* SA3-7 and 2dg-stabilized mutant DMS-4 have been shown in Fig. 1. Enzyme activity was estimated after different time intervals (8-72 h). Maximum enzyme production (34.72 U/ml with 17.05 g/l sugar consumption & 7.85 g/l dry cell mass) was observed after 48 h of incubation by the mutant DMS-4. Thus, over all the rate of volumetric production was 31.43 fold improved over the parental strain. Further increase in the incubation period did not enhance enzyme production. It might be due to decrease in amount of available nitrogen in fermentation medium, age of organism, addition of inhibitors produced by yeast itself and the protease production characteristic of decline phase. Other workers have reported enzyme production by *C. fermentati* incubated for 72-96 h.

Effect of sucrose concentrations (1-10 g/l) on invertase production by the mutant *C. fermentati* DMS-4 was studied (Fig. 2). Maximum enzyme activity (35.56 U/ml) was obtained at sucrose concentration of 5 g/l. Sucrose concentration more than 5 g/l caused an

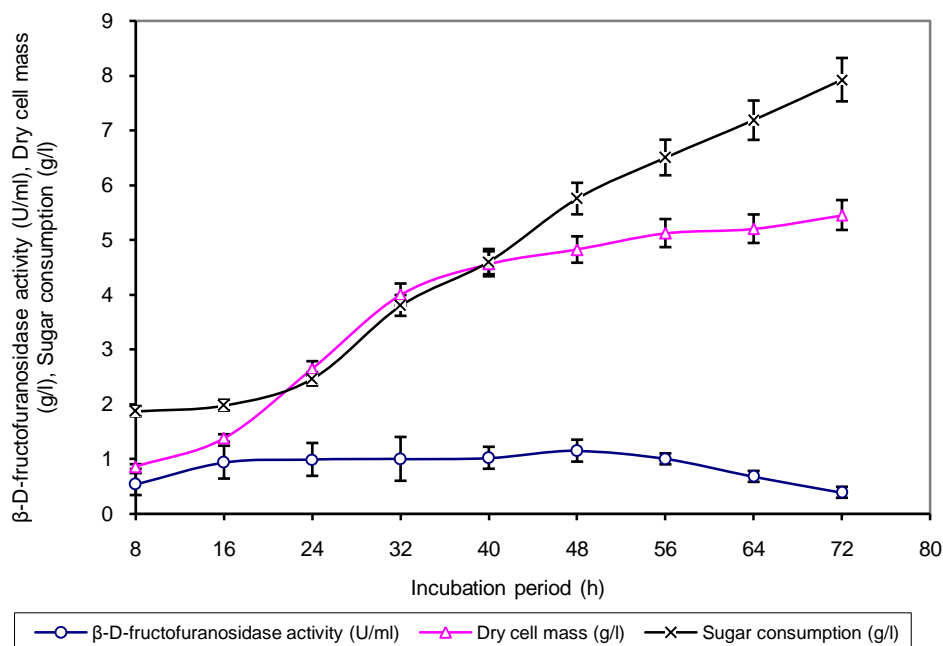
increase in the sugar consumption and dry cell mass, however, there was no net increase in enzyme production. The reason might be generation of higher concentration of inverted sugar in the medium resulting in glucose-induced repression of enzyme [14]. Fig. 3 shows the effect of initial pH on enzyme production by the mutant *C. fermentati* DMS-4. Maximum production of enzyme was obtained when initial pH was adjusted to 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. Less enzyme activity, accompanied by a decrease in dry cell mass and sugar consumption, was noticed at pH other than the optimal. Workers have reported similar results [15]. It was noted that during submerged fermentation of *C. fermentati*, final pH of the reaction mixture was less than initial pH; besides, extent of the decrease in pH was proportional to the enzyme activity. It may be due to the fact pH affected enzyme production by altering the tertiary and/or quaternary structures of the protein.

Among the factors that determine morphology and general course of yeast fermentations, size and age of inoculum are of prime importance. Early attempts have been made to standardize the inocula for invertase production in shaking culture [16]. In the present investigation, a 16-h-old vegetative inoculum was optimal for maximum enzyme production (45.65 ± 1.6 U/ml) in shake flasks when added at a level of 2 % (v/v). An inoculum greater or smaller than 2 % (v/v) resulted in the reduction of enzyme production (Fig. 4). It might be due to the fact that at a lower concentration, not sufficient yeast cells were available in the medium to convert more substrate into the enzyme. Bokosa [17] optimized 3 % (v/v) vegetative inoculum for enzyme production. In contrast to our studies, Roitsch *et al.* [1] found that 48 h old cells are as good as those a 72 to 96 h old slant culture for enzyme production, which suggested that the age of yeast cells may not have a bearing on the enzyme production. The lag associated with inoculum from the stationary phase of a culture may be attributed to the reorganization necessary in the cell to reverse the changes caused by cessation of growth.

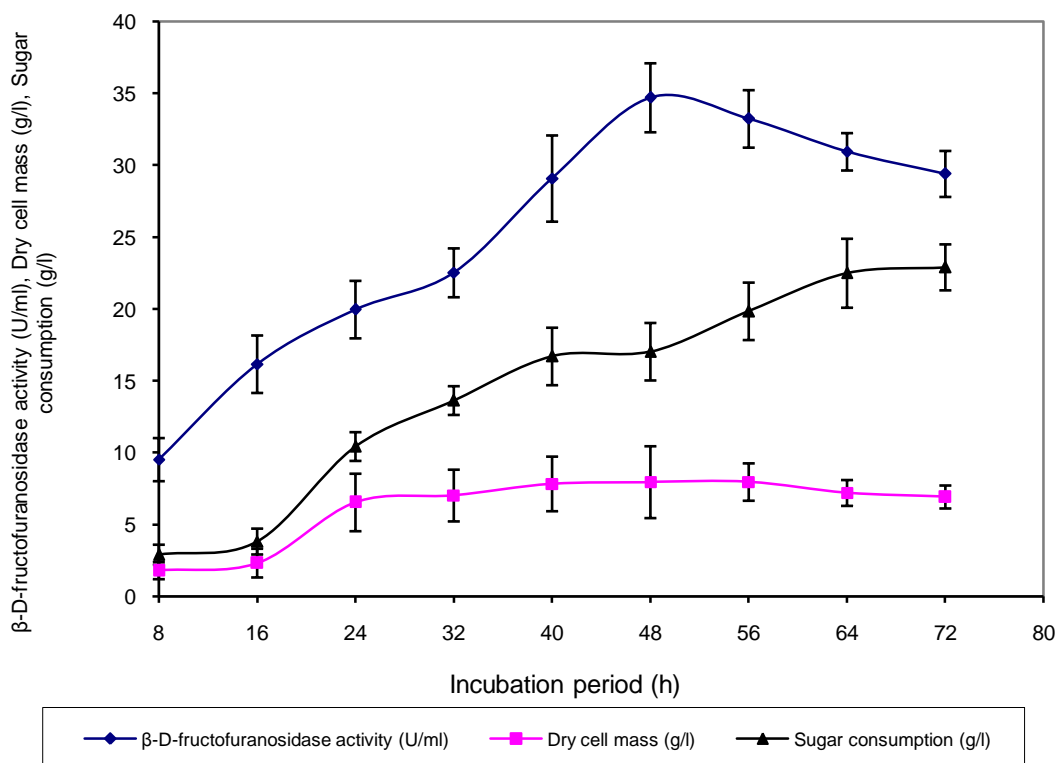
In the present study, the comparison of Q_s (g cells/l/h) for invertase production demonstrated that the mutant DMS-4 has a higher value for volumetric rate of substrate consumption ($Q_s = 0.235$ g/l/h) than the wild-culture SA3-7. Several fold improvement in terms of volumetric enzyme production was noticed with the mutant DMS-4 on all the rates examined (Table 1). Although wild-type SA3-7 achieved a higher value ($Y_{x/s} = 0.479$ g yeast cells/g) than the mutant, the mutant DMS-4, however, demonstrated a several fold improvement in terms of volumetric rate of product formation. In addition, when both of the cultures were monitored for comparison of specific rate constant, the mutant DMS-4 gave higher values for q_p (>25 fold improvement). Therefore, on the basis of kinetic

variables, it was found that the mutant DMS-4 showed 2-6 fold improved values for Q_p , $Y_{p/x}$, $Y_{p/s}$ and q_p over the parental strain (LSD 0.045). Similar kinds of findings have also been reported by Pirt (1975). Neto *et*

al. [3] however, found that the aeration rate and substrate moisture content influenced the substrate consumption rate, specific growth rate and subsequent enzyme production.



a) SA3-7



b) DMS-4

Fig. 1: Time course profile of invertase production in submerged culture by wild strain of *C. fermentati* SA3-7*
 *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.

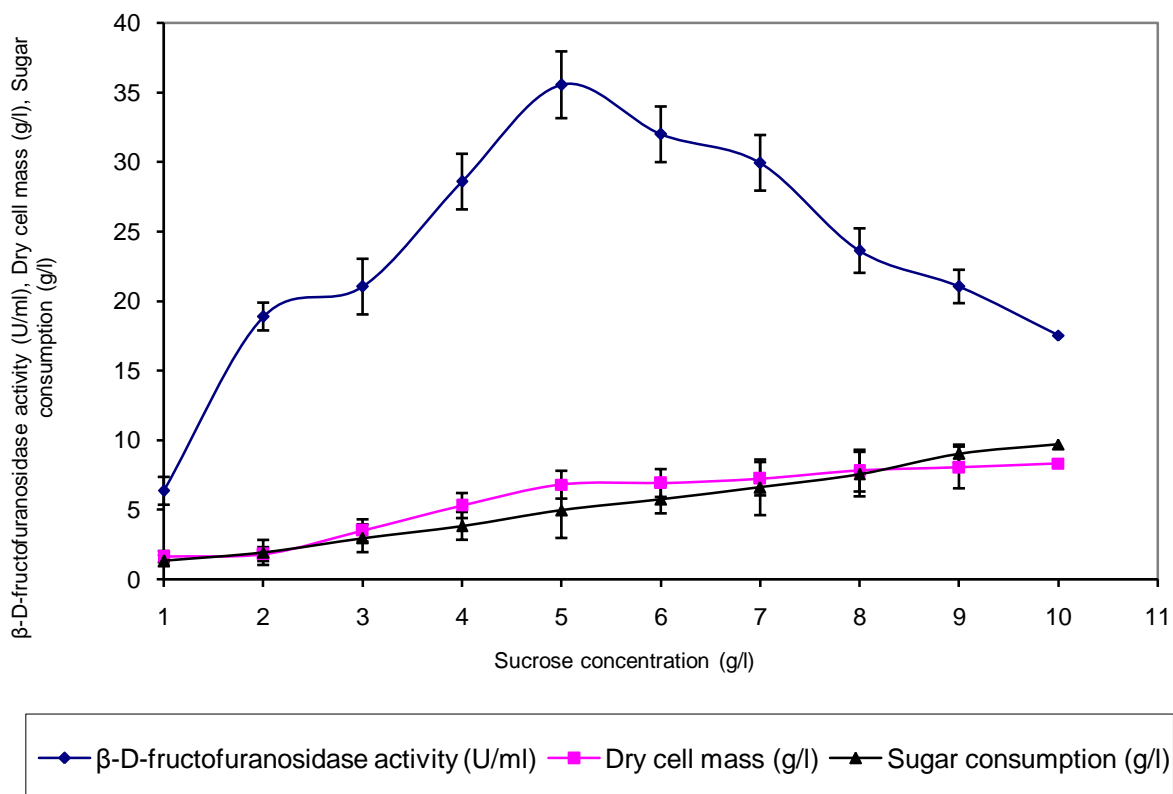


Fig. 2: Effect of sucrose concentration on the invertase production in submerged culture by the mutant *C. fermentati* DMS-4*

*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.

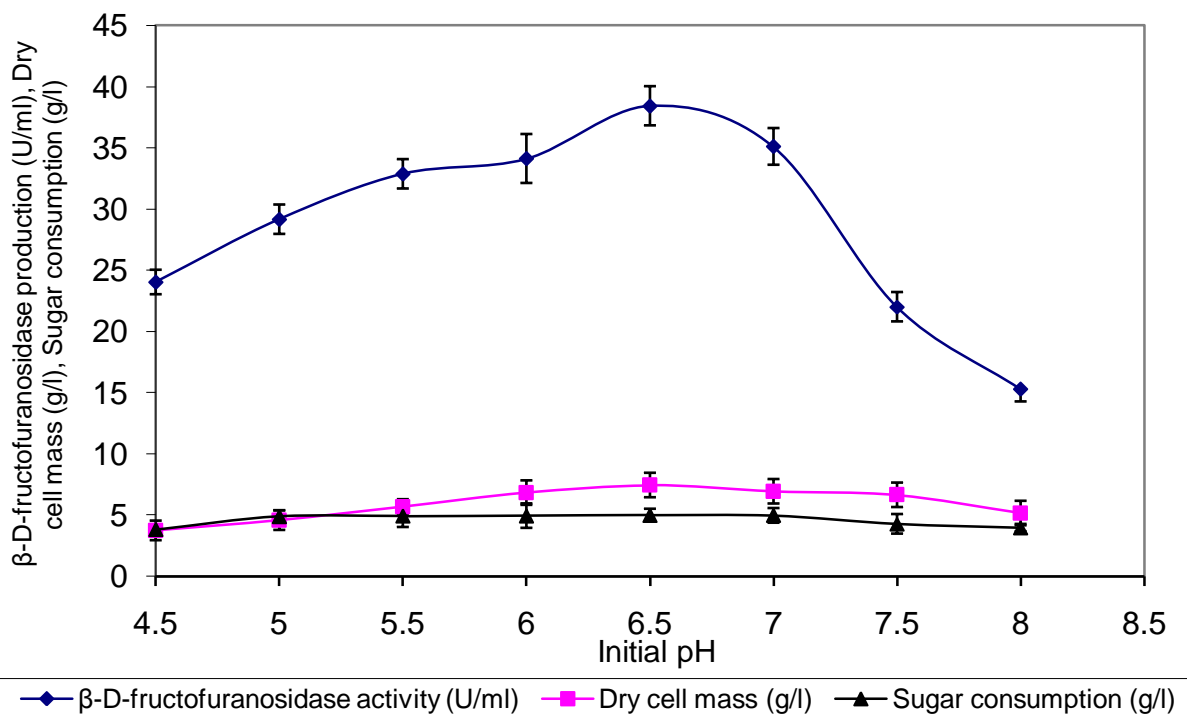


Fig. 3: Effect of initial pH on the invertase production in submerged culture by the mutant *C. fermentati* DMS-4*

*Incubation period 48 h, sucrose concentration 5 g/l, temperature 30°C, agitation rate 200 rev/min.

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

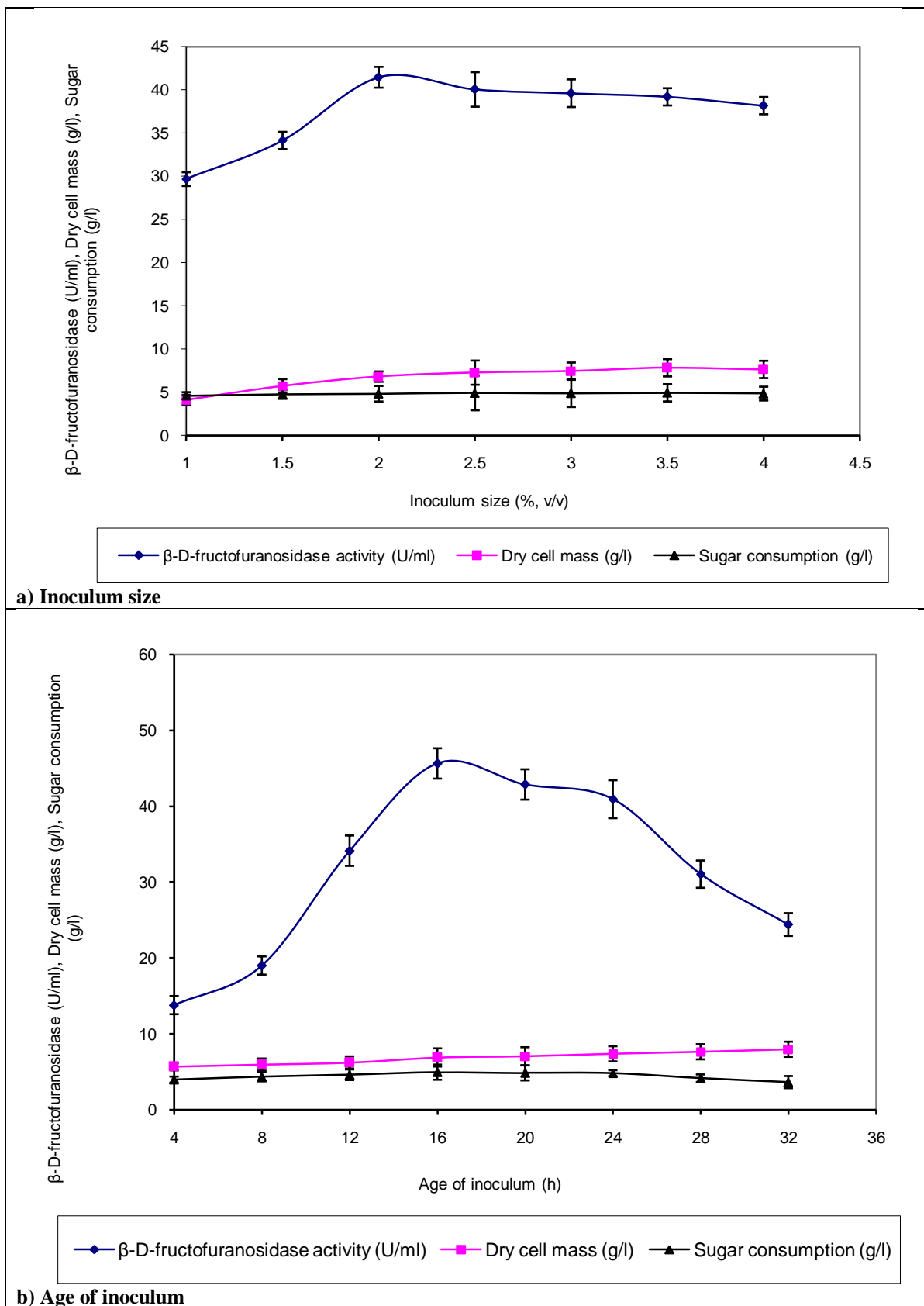


Fig. 4: Effect of inoculum on the invertase production in submerged culture by the mutant *C. fermentati* DMS-4*
 *Incubation period 48 h, sucrose concentration 5 g/l, temperature 30°C, agitation rate 200 rev/min. *Y-error bars indicate standard deviation among the three parallel replicates.

Table 1: Comparison of kinetic variables for invertase activity by *C. fermentati* in shake flask 48 h after the incubation*

| Kinetic variables | Enzyme activity (U/ml) | |
|------------------------------------|------------------------|----------------|
| | SA3-7 (Wild-culture) | DMS-4 (mutant) |
| Product formation parameters | | |
| Q _p (U/h/h) | 0.302 | 0.872 |
| Y _{p/s} (U/g) | 0.519 | 2.403 |
| Y _{p/x} (U/g) | 0.323 | 4.635 |
| q _p (U/g yeast cells/h) | 0.104 | 0.209 |
| Substrate consumption parameters | | |
| Y _{x/s} (g yeast cells/g) | 0.479 | 0.146 |
| Q _s (g/l/h) | 0.112 | 0.235 |
| q _s (g/g yeast cells/h) | 0.202 | 0.104 |
| Q _x (g yeast cells/l/h) | 0.109 | 0.217 |
| Least significant difference (LSD) | 0.051 | 0.045 |
| Significance level <p> | S | HS |

HS is for the 'highly significant' while S for 'significant' values.

CONCLUSION

In the present study, a 2dg resistant mutant strain of *C. fermentati* DMS-4 was developed with a 10 fold increase in invertase production under optimal conditions (5 g/l sucrose, 2 inoculum, 48 h incubation period and pH 5.0). The reason for this enhancement was attributed to sucrose in the medium which after hydrolysis produced glucose and fructose and if not fully utilized induced carbon catabolite repression of enzyme. Further work on improvement of culture by ethyl methane sulphonate and enzyme characterization is in progress.

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