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# Optimization of Medium Composition and Cultivation Parameters for Fructosyltransferase Production by *Penicillium aurantiogriseum* AUMC 5605

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**Abstract** Fructooligosaccharides have been mainly produced by microbial fructosyltransferases (FTase) enzymes. The present work focuses on the optimization of medium composition and cultivation parameters affecting FTase produced by *Penicillium aurantiogriseum* AUMC 5605 in shake flask cultivation. FTase production was optimized in two steps using DeMeo's fractional factorial design. A 1.46-fold increase in FTase production (105.4 U/mL) was achieved using the optimized culture medium consisting of (g/L): sucrose, 600; yeast extract, 10; $K_2HPO_4$, 5; $MgSO_4 \cdot 7H_2O$, 0.5; $(NH_4)_2SO_4$, 1.0 and KCl, 0.5. The obtained results showed that the maximum FTase enzyme activity was produced at initial cultivation pH values ranging from 6.0–6.5, at agitation speed of 200 rpm and using vegetative fungal cells as inoculum. Moreover, results showed that optimization of medium composition and some cultivation parameters resulted in an increase of about 93.7% in the enzyme activity than the non-optimized cultivation conditions after 96 h of cultivation. Additionally, maximum production and specific production rates recorded 2340 U/L/h and 102 U/L/h/g cells, respectively.

**Keywords** DeMeo's fractional factorial design · fructosyltransferases · growth kinetics · medium optimization · *Penicillium aurantiogriseum*

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## Introduction

Microbial fructooligosaccharides (FOS) have attracted special attention and are attributed to the expansion of the sugar market due to mass production is not complicated and the similarity of sweet taste to sucrose, a traditional sweetener. FOS are calorie-free and noncariogenic sweeteners, stimulate the growth of bifidobacteria, and have been claimed to contribute towards the prevention of colon cancer and to reduce cholesterol, phospholipids and triglyceride levels in serum (Yun, 1996). The enzymes catalyzing the production of FOS are classified as fructosyltransferases (FTase, E.C. 2.4.1.9), or β-fructofuranosidases (FFase, E.C. 3.2.1.26) (Maiorano et al., 2008). FTase catalyzes the transfer of a fructosyl group to a molecule of sucrose or a fructooligosaccharide when a FOS chain which has one fructosyl unit more, is formed. The enzyme shows a little affinity towards water as an acceptor, due to its very low hydrolase activity. The yields of FOS prepared by the action of the FTases were generally high even in dilute substrate solutions (Antošová and Polakoviè, 2001).

A number of fructosyltransferic microorganisms have been reported, including fungi belonging to genera *Aspergillus* (*Aspergillus niger*, *Aspergillus oryzae*, *Aspergillus foetidus*, *Aspergillus phoenicis*); *Fusarium* (*Fusarium oxysporum*); *Penicillium* (*Penicillium citrinum*, *Penicillium rugulosum,*

*Penicillium purpurogenum*) and *Aureobasidium* (*Aureobasidium pullulans*) (Sangeetha et al., 2004; Maiorano et al., 2008; Ottoni et al., 2012). Investigations of the effect of the cultivation media composition and cultivation conditions on the FTase production by different strains have been performed by several authors (Vandáková et al., 2004; Lim et al., 2006; Wang and Zhou, 2006). The comparison of the effect of carbon source at the cultivation of different microorganisms showed that sucrose was by far the best inducer of FTase production (Hayashi et al., 1992; Chen and Liu, 1996). Cell growth and enzyme production are also influenced by the nitrogen source. Complex nitrogen sources, such as yeast extract and corn steep liquor, enhance the production of FTase but other nitrogen sources such as urea, $(NH_4)_2SO_4$, $NaNO_3$ and $NH_4NO_3$ can limit the enzyme production (Balasubramaniem et al., 2001). Similarly, yeast extract was declared to be the best nitrogen source (Hayashi et al., 1992; Chen and Liu, 1996). Another common subject of investigation was the effect of minerals, mainly phosphorus, magnesium, iron, and calcium, on the FTase production and cell growth (Sangeetha et al., 2005; Dhake and Patil, 2007).

However, there is no report on the transfructosylating enzyme activity of *Penicillium aurantiogriseum*. The aim of this study is to optimize the media components and cultivation parameters affecting the production of FTase by a locally isolated fungus, *Penicillium aurantiogriseum* AUMC 5605, in shake-flask cultivation.

## Materials and Methods

**Microorganism, maintenance and cultivation parameters.** A fungal strain was isolated from Egyptian soil sample taken from Alfayoum region and was identified as *Penicillium aurantiogriseum* AUMC 5605 (Assiut University, Mycological Center, Faculty of Science, Egypt) according to the methods of Moubasher (1993) and Samson et al. (2000). The producing strain was regularly maintained on yeast sucrose agar medium containing (g/L): yeast extract, 2; sucrose, 50; agar, 20 and distilled water 1 L. The pH of the medium was adjusted to 6.5 prior to sterilization.

**Inoculum preparation.** A vegetative growth medium was used to prepare the inoculums for the enzyme production experiments. This medium contained (g/L): Yeast extract, 10; $K_2HPO_4$, 5.0; $NaNO_3$, 10.0; $MgSO_4 \cdot 7H_2O$, 0.5 and sucrose 100; distilled water 1 L (Yun et al., 1997). The pH was adjusted to 6.5 before sterilization. Spores of the producing strain from 6–7 day old agar slants (approximately $1\times10^6$ spore/mL) were used to inoculate 50 mL of the vegetative growth medium and then incubated at 28±2°C in an incubator shaker at 200 rpm for 24 hours (approximately $1\times10^4$ cells/mL). Cells count was measured by the dilution plate count method (Parkinson et al., 1971).

**Production medium and batch cultivation.** Unless otherwise stated, batch fermentation was conducted in 250 mL Erlenmeyer flasks containing 50 mL of production medium (Yun et al., 1997) (g/L): sucrose, 200; yeast, 10.0; $K_2HPO_4$, 5.0; $MgSO_4 \cdot 7H_2O$, 0.5; $(NH_4)_2SO_4$, 1.0; KCl, 0.5. The pH of the medium was adjusted to 6.0 before autoclaving at 121°C for 20 min. Sucrose was sterilized separately and added aseptically after cooling the flasks containing the production medium. Unless otherwise stated, vegetative growing cells (10 mL) of *P. aurantiogriseum* AUMC 5605 of 24 h old were used to inoculate production medium (50 mL in 250 mL Erlenmeyer flasks). The flasks were then incubated on a rotary shaker at 200 rpm at 28±2°C. After 144 h, the cultures were harvested and the necessary analyses were done.

**Table 1** The De Meo's design of experimental runs used for FTase production

| Expr. run | Constituents | | | | | | |
|---|---|---|---|---|---|---|---|
| | A | B | C | D | E | F | G |
| 1 | L | L | L | H | H | H | L |
| 2 | H | L | L | L | L | H | H |
| 3 | L | H | L | L | H | L | H |
| 4 | H | H | L | H | L | L | L |
| 5 | L | L | H | H | L | L | H |
| 6 | H | L | H | L | H | L | L |
| 7 | L | H | H | L | L | H | L |
| 8 | H | H | H | H | H | H | H |

H, high level value; L, low level value; A, sucrose; B, Yeast extract. C, $K_2HPO_4$; D, $MgSO_4.7\ H_2O$; E $NaNO_3$; F, $(NH_4)_2SO_4$; G, KCl.

**Medium optimization.** A fractional factorial experimental design described by De Meo et al. (1985) was used for the optimization of production medium for FTase production in submerged fermentation by *P. aurantiogriseum* AUMC 5605. In the present study, seven different components namely: sucrose (A), yeast extract (B), $K_2HPO_4$ (C), $MgSO_4 \cdot 7H_2O$ (D), $NaNO_3$ (E), $(NH_4)_2SO_4$ (F), and KCl (G) were taken into consideration in eight experiments. In Table 1, the rows represent eight different experiments while columns represent different components. For each component, high (H) and low (L) concentrations were tested. For evaluation of the effect of each constituent, the coefficients fructosyltransferase activity (Cp), biomass dry weight (Cx) and specific activity (Cs) were calculated as follows. If P is the FTase activity (U/L), X is the biomass dry weight (g/L) and S is the specific activity of FTase (U/mg proteins), the coefficients Cp, Cx and Cs relating to each of the seven constituents are given by:

$$C_{pj} = 1/8\ [\Sigma_{i=1}^{8}\ Aj \times P_i] \quad (1)$$

$$C_{xj} = 1/8\ [\Sigma_{i=1}^{8}\ Aj \times X_i] \quad (2)$$

$$C_{sj} = 1/8\ [\Sigma_{i=1}^{8}\ Aj \times S_i] \quad (3)$$

Here, Aj means either H or L level in experimental run i. If a calculated coefficient has a positive value, it means that the particular constituent has positive effect at its high level. P refers to FTase activity, X to biomass dry weight and S to specific activity of FTase. Media optimization was carried out in two steps. The first step was to determine the effect of each constituent on production of FTase, while the second step was to further adjust the levels of constituents for higher production of the enzyme.

**Determination of FTase activity.** Fructosyltransferase activity in

culture filtrate was measured by estimating the liberated reducing sugar released from sucrose as described by Duan et al. (1993) using glucose as a standard. The assay mixture contained appropriately 0.1 mL of the enzyme solution, (0.15 M Mcllvaine buffer pH 5.5) and 0.4 mL of 50% (W/V) sucrose solution. The reaction mixture was incubated at 55°C for one h. At the end of the incubation time, the reaction was stopped by adding copper reagent. The amount of reducing sugar liberated was determined spectrophotometrically at 520 nm using glucose as a standard according to Somogyi (1945). One unit of the enzyme activity was defined as the amount that produced 1 μmol of reduced sugar per minute under the above assay conditions.

**Determination of extracellular protein.** The protein content of the crude enzyme source was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

**Determination of cell dry weight.** The cell dry weight (CDW) was determined by harvesting the fungal mycelia through filtration using Whatman paper No. 1, washed twice with distilled water and dried overnight at 70°C in an oven and weighed until a constant dry weight was obtained.

**Data analysis.** Treatment effects were analyzed and the mean comparison was performed by ANOVA One-Way analysis of variance using computer software Minitab 16 and the average values were reported. Significant differences among the replicates have been presented at the 95% confidence level ($p \leq 0.05$).

## Results and Discussion

In batch fermentation, *P. aurantiogriseum* AUMC 5605 grow in the production medium (Yun et al., 1997) with initial pH 6.0 at 30°C for 144 h and 200 rpm produced a maximum activity of about 72 U/mL.

De Meo's fractional factorial design is a valuable tool for rapid evaluation of the effects of different media constituents. This design is a preliminary technique, which tests only two levels of each medium component, but cannot determine the exact quantity of each constituent required for best performance in the medium. However, the method suggests the importance of each constituent. This design is as efficient as that of Placket-Burman design for fermentation media optimization for production of aroma, nuclease P1 and β-fructofuranosidase (Ashokkumar et al., 2001).

**First optimization step.** The different levels of constituents used in first optimization step are given in Table 2. FTase activity, specific activity and enzyme yield from the fermentation trails are given in Table 3. The calculated coefficient values for FTase activity ($C_p$), biomass dry weight ($C_x$) and specific activity ($C_{s)}$ are also shown in Table 4. In all the eight runs, FTase activity varied from 70.26 to101 U/mL with a mean of 86.7 U/mL while its specific activity ranged between 9.7 and 13.7 U/mg proteins with a mean of about 11.4 U/mg proteins. Similarly, there were wide variations in the amount of the soluble protein (7.2–9.7 mg/mL) and biomass yield (27.9–42.5 g/L) from the different fermentation trials. In the first step, constituents such as sucrose, yeast extract and $K_2HPO_4$ showed positive coefficients for FTase activity whereas $MgSO_4 \cdot 7H_2O$, $NaNO_3$, $(NH_4)_2SO_4$ and KCl showed negative coefficients (Table 4). However, all constituents except $MgSO_4$, $NaNO_3$ and KCl showed positive coefficients for specific activity. On the other hand, all constituents except sucrose, yeast extract and $(NH_4)_2SO_4$ had positive coefficients on the growth and cell biomass production.

**Second optimization step.** In the second optimization step, the levels of the constituents were adjusted based on the results of the first optimization step. As sodium nitrate, $(NH_4)_2SO_4$, KCl, and $MgSO_4 \cdot 7H_2O$ exhibited negative effects on both FTase activity and its specific activity in the first step of media optimization, their levels were reduced to levels below to their low levels.

**Table 2** Different levels of constituents used in first and second optimization steps

| Components | First step | | Second step | |
|---|---|---|---|---|
| | Low level (g $L^{-1}$) | High level (g $L^{-1}$) | Low level (g $L^{-1}$) | High level (g $L^{-1}$) |
| Sucrose | 200 | 600 | 400 | 600 |
| Yeast extract | 5 | 10 | 5 | 10 |
| $K_2HPO_4$ | 2 | 5 | 1 | 2 |
| $MgSO_4 \cdot 7H_2O$ | 0.5 | 1 | 0.2 | 0.5 |
| $NaNO_3$ | 5 | 10 | 2 | 6 |
| $(NH_4)_2SO_4$ | 2 | 10 | 0.2 | 1 |
| KCl | 0.1 | 2 | 0.1 | 0.5 |

**Table 3** FTase activity, specific activity and yield for first and second optimization steps

| Run | First step | | |
|---|---|---|---|
| | FTase activity (U $mL^{-1}$) | Specific Activity (U $mg^{-1}$ protein) | Yield (U g $cells^{-1}$) |
| 1 | 70.26±3.04$^{d}$ | 9.77±1.85 | 1824.14±113.08 |
| 2 | 94.81±3.98$^{ab}$ | 9.75±0.69 | 2960.85±337.05 |
| 3 | 76.56±0.16$^{cd}$ | 10.55±0.25 | 1989.53±16.55 |
| 4 | 96.27±1.59$^{ab}$ | 12.25±0.94 | 3449.43±101.93 |
| 5 | 74.09±0.48$^{cd}$ | 9.98±0.51 | 1743.19±40.25 |
| 6 | 101.00±1.27$^{a}$ | 11.53±0.66 | 3101.20±100.49 |
| 7 | 85.35±5.89b$^{c}$ | 13.71±1.74 | 2157.29±190.47 |
| 8 | 95.48±2.07$^{ab}$ | 13.21±0.89 | 2624.28±119.77 |
| | Second step | | |
| | FTase activity (U $mL^{-1}$) | Specific Activity (U $mg^{-1}$ protein) | Yield (U g $cells^{-1}$) |
| 1 | 90.53±7.49$^{b}$ | 12.88±1.50 | 2149.88±295.75 |
| 2 | 103.14±1.11$^{ab}$ | 22.44±0.24 | 2855.47±129.33 |
| 3 | 93.12±0.0$^{ab}$ | 12.60±0.0 | 2300.89±94.15 |
| 4 | 103.36±0.48$^{a}$ | 11.13±1.36 | 2550.96±77.29 |
| 5 | 93.79±2.23$^{ab}$ | 15.91±2.56 | 2434.91±51.23 |
| 6 | 103.25±1.27$^{ab}$ | 11.97±1.66 | 2633.58±156.74 |
| 7 | 96.83±4.30$^{ab}$ | 20.34±0.30 | 2621.37±18.07 |
| 8 | 105.39±0.80$^{a}$ | 22.61±2.00 | 3060.25±338.75 |

Means followed by different letters within each column differ significantly at $p \leq 0.05$; ± indicates standard deviation among replicates.

**Table 4** Coefficient for FTase activity (*Cp*) and specific activity (*Cs*) and cell dry weight (*Cx*) for each constituent

| Coefficient | Medium components | | | | | | |
|---|---|---|---|---|---|---|---|
| | Sucrose | Yeast extract | $K_2HPO_4$ | $MgSO_4$ | $NaNO_3$ | $(NH_4)_2SO_4$ | KCl |
| | First optimization step | | | | | | |
| $C_p$ | 10.16 | 1.69 | 2.25 | -2.7 | -0.9 | -21.59 | -1.49 |
| $C_s$ | 0.34 | 1.09 | 0.76 | -0.04 | -0.08 | -3.16 | -0.47 |
| $C_x$ | -3.77 | -0.41 | 1.76 | 0.33 | 0.49 | -9.26 | 1.35 |
| | Second optimization step | | | | | | |
| $C_p$ | 5.11 | 1 | 1.14 | -0.41 | -0.61 | -23.91 | 0.18 |
| $C_s$ | 0.8 | 0.43 | 1.47 | -0.6 | -1.22 | -1.75 | 2.16 |
| $C_x$ | -0.97 | -0.45 | -1.26 | 0.36 | 0.52 | -10.37 | -1.15 |

Similarly, the concentration of $K_2HPO_4$ was also decreased whereas; the low level of sucrose was raised to 400 g/L. The level of yeast extract was set at its concentration of the first step science yeast extract contains abundant nitrogen compounds as well as many growth factors its addition can stimulate FTase production by *P. aurantiogriseum* AUMC 5605. The optimal concentration of yeast extract for FFase production was between 1.5 to 3% (W/V) (Hayashi et al., 1992; Chen, 1995). The concentration of each constituent for the second optimization step is shown in Table (2). In all eight runs (Table 3), the FTase activity varied from 90.5 to 105.4 U/mL with a mean of 98.7 U/mL which is higher than the mean of FTase activity in the first step (86.7 U/mL) representing about 1.14-fold higher than in the first step. The maximum FTase activity in the eighth run of second step was 1.46-fold higher than that of enzyme activity before optimization (72.2 U/mL). Similarly the specific activity of FTase in the second optimization step varied from 11.1 to 22.6 U/mg protein and its mean value (16.24 U/mg) higher than that of the first step (11.4 U/mg). The calculated coefficient values for the second step suggested that $MgSO_4$ and $NaNO_3$, showing again a negative effect for both FTase and its specific activity, while the other constituents exhibited positive effect on both factors (Table 4). Therefore, sodium nitrate was omitted and low level of $MgSO_4$ in the second optimization step was taken in designing optimized medium.

Fructosyltransferase production is significantly influenced by carbon source. Sucrose was the best carbon source to FTase production and growth although Wang and Rakshit (1999) showed that maltose at 10 g/L (W/V) was the best carbon source for transferase enzyme production by *A. foetidus* NRRL 337. Chen and Liu (1996) reported that the optimal enzyme production was achieved with 250 g/L (W/V) sucrose but maximum cell growth occurred at 100 g/L (W/V) sucrose. The authors suggested that below 100 g/L (W/V) sucrose the large portion of the carbon source was used for cell growth, while high sucrose concentrations resulted in higher enzyme induction but cell growth inhibition. In this work, it is observed that no suppression of FTase production by increasing sucrose concentration (400–600 g/L W/V) was detected, which is contrary to the results of Hayashi et al. (2000). The authors found the largest amount of FTase produced at 100-200 g/L (W/V) of sucrose by *Aureobasidium pullulans* CCY 27-1-1194 did not show this tendency. The amount of biomass of *Aureobasidium pullulans* obtained after 4 days of cultivation was almost the same, 14–16 g/L (W/V), with sucrose ranging from 50 to 350 g/L. The highest FTase activity was achieved with sucrose at 350 g/L (W/V) and the ratio of intracellular and extracellular activities was independent of sucrose amounts (Antošová et al., 2002).The optimal sucrose concentration for the FTase production shows a large variation for *A. japonicas* and *A. niger*. High FTase production by *A. japonicus* MU-2 (Antošová et al., 2002) and by *A. japonicus* JN19 (Wang and Zhou, 2006) was reached at 200 and 150–180 g/L (W/V) sucrose, respectively. Differently, FTase production by *A. japonicus* FCL 119T (Dorta et al., 2006) and by *A. niger* ATCC 20611 (Cruz et al., 1998) was higher at 30 and 25 g/L (W/V) sucrose, respectively. A low sucrose concentration, from 10 to 100 g/L (W/V), has been employed by several authors for transfructosylation enzyme production (Cuervo et al., 2004; Nguyen et al., 2005; Sangeetha et al., 2005).

The effects of inorganic salts are also reported by various authors. $K_2HPO_4$ is described as a microelement source for cell growth, as well as a buffering reagent. Its optimal concentration ranges from 4 g/L (W/V) (Sangeetha et al., 2005) to 5 g/L (W/V) (Vandáková et al., 2004; Lim et al., 2006), $Mg^{2+}$ affects the permeability of the cell wall for *A. pullulans* (Vandáková et al., 2004) and its optimal concentration varies from 0.3 g/L (W/V) (Sangeetha et al., 2005) to 2 g/L (W/V) (Balasubramaniem et al., 2001). $NaNO_3$ is the most common source of inorganic nitrogen in FTase production by some fungi and can be employed from 2 g/L (W/V) (Lim et al., 2006) to 25 g/L (W/V) (Dhake and Patil, 2007). The highest FTase production by *Aureobasidium pululans* was achieved using a culture medium containing (g/L): $K_2HPO_4$, 5; $NaNO_3$, 10 and $MgSO_4 \cdot 7H_2O$, 0.5 (Vandáková et al., 2004). Those authors noted an increase in the release of FTase into the medium when $MgSO_4 \cdot 7H_2O$ and $K_2HPO_4$ contents were decreased in the medium. On the other hand, high FTase production and growth stimulation in *A. japonicus* JN19 was observed after $MgSO_4$ and $K_2HPO_4$ addition (Wang and Zhou, 2006). Previous workers have not noticed such effects for *A. japonicus* TIT 90076. It has been reported that addition of adequate amounts of $MgSO_4$ and $K_2HPO_4$ in the culture medium would have appositive effect on enzyme production. However, sodium nitrate was found to have no influence on fructofuranosidase (FFase) production from *A. japonicus* TIT 90076 (Chen, 1995).

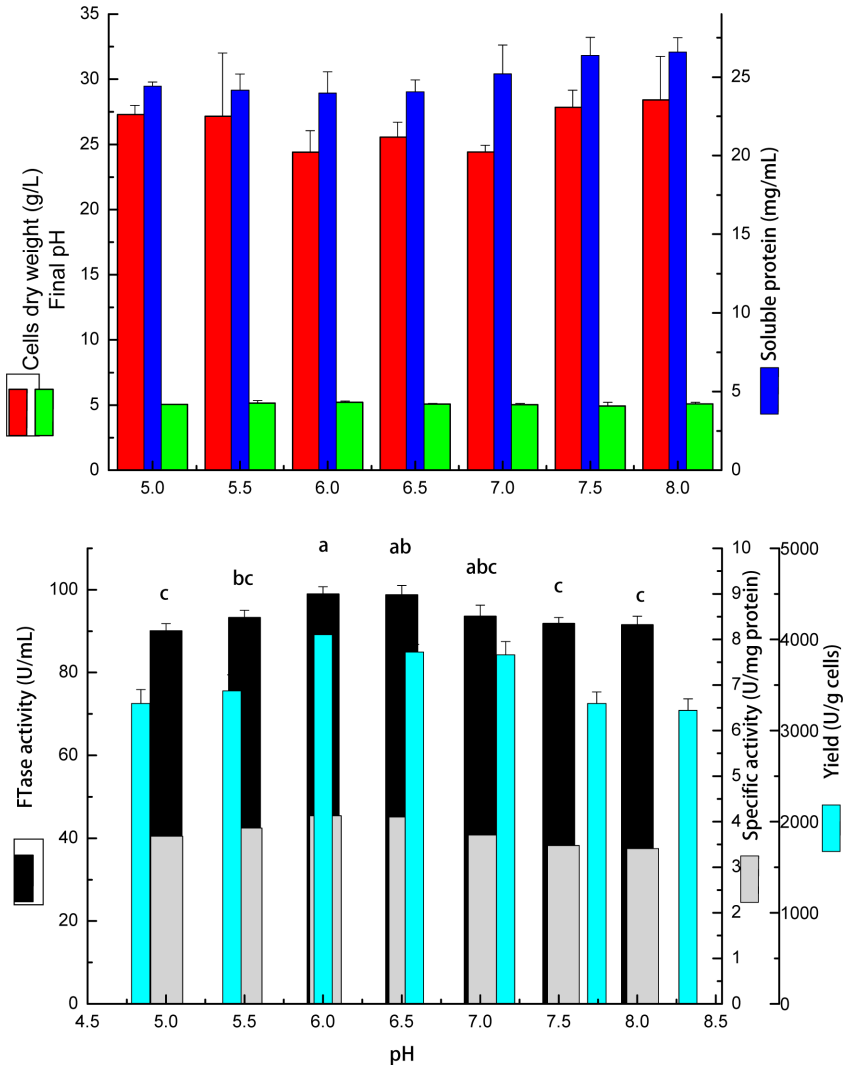


**Fig. 1** Effect of initial medium pH on cell growth and FTase production by *P. aurantiogriseum* AUMC 5605 in a shake-flask system. Y-error bars indicate the standard deviation (±SD) among the replicates. Means followed by different letters within FTase activity column differ significantly at $p \leq 0.05$.

Based on the results obtained in the second optimization step, composition of the optimized medium for production of FTase by *P. aurantiogriseum* AUMC 5605 was formulated to contain (g/L): sucrose, 600; yeast extract, 10; $K_2HPO_4$, 5; $MgSO_4 \cdot 7H_2O$, 0.5; $(NH_4)_2SO_4$, 1.0 and KCl, 0.5.

**Effect of initial pH value on the production of FTase.** Results presented in Fig. 1 show that initial pH of the fermentation medium was an important factor for the production of fructosyltransferase enzyme. Maximum enzyme level (about 100 U/mL was obtained when the fungal strain was grown on the fermentation medium initially adjusted within the range of 6.0 to 6.5. This was correlated with maximum specific activity (4.1 U/mg) and yield coefficient of about 4054.5 and 3862.5 U/g cells/L, respectively. A notable decrease in the enzyme activity was observed upon using media initially adjusted to pH values higher or lower than the suitable range. However, the final pH values of all fermentation media ranged between pH 5.0 to 5.2. Concerning the cell dry mass, it was observed at pH 6–7 the cells dry mass was almost constant. At lower or higher initial pH values a noticeable increase of the cell dry mass was recorded. The amount of soluble protein in the fermentation medium increased with the increase of the initial pH value. For higher FTase production in flasks by *P. purpurogenum* (Chen, 1995), *Aspergillus oryzae* CFR202 (Sangeetha et al., 2005) and by *A. japonicus* JN19 (Wang and Zhou, 2006) the best initial pH found was 5.5. The pH effect on FTase production in bioreactor by *A. foetidus* NRRL 337 was also studied (Wang and Rakshit, 1999). The pH was controlled at 4.0, 5.0 and 6.0 and for comparison an uncontrolled pH condition with initial pH of 6.0 was run. The results indicated that uncontrolled pH fermentation produced the highest transferase activity.

**Effect of aeration on the production of FTase.** Microorganisms vary in their oxygen requirements. In particular, oxygen acts as a terminal electron acceptor for oxidative reactions to provide energy for cellular activities. The variation in the agitation speed

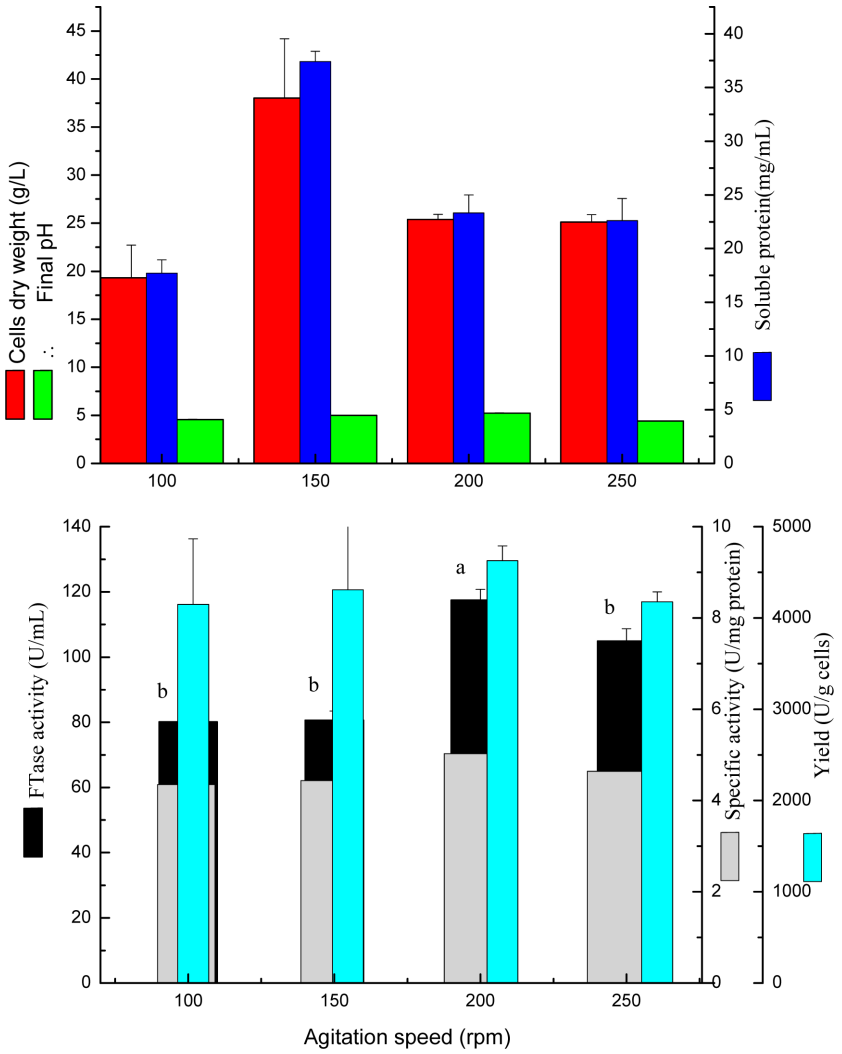


**Fig. 2** Effect of agitation speed (rpm) on cell growth and FTase production by *P. aurantiogriseum* AUMC 5605 in a shake-flask system. Y-error bars indicate the standard deviation (±SD) among the replicates. Means followed by different letters within FTase activity column differ significantly at $p \leq 0.05$.

has been found to influence the extent of mixing in the shake flasks or the bioreactor, and also affect the nutrient availability (Nascimento and Martins, 2004).

The effect of aeration was studied through investigating different agitation speeds and different volumes of cultivation medium (Fig. 2). The relation between cell growth, enzyme production as a function of different agitation speeds (100, 150, 200, and 250 rpm) revealed that increasing the agitation speed increases cell dry weight, soluble protein and FTase production. It was noticed that at agitation speed of 200 rpm maximum enzyme production (117.5 U/mL), cell dry weight (25.38 g/L) and soluble protein (23.3 mg/mL) were obtained (Fig. 2). It is seen that there is no obvious difference in cell dry weight and soluble protein when the fermentation process was carried out at 200 and 250 rpm. It is clear that higher shaking speed resulted in higher enzyme activity. These results may be attributed to the fact that increasing the agitation speed from 100 to 200 rpm tend to increase the amount of dissolved oxygen available to the culture (Bartholomew et al., 1950; Venkatadri and Irvine, 1990). But the increase of enzyme activity slows down from 117.47 to 104.94 U/mL when shake speed was higher than 200 rpm. The low enzyme activities observed at 100 and 150 rpm are most probably caused by poor oxygenation, despite the control of the dissolved oxygen concentration, especially at higher sucrose concentration (600 g/L) and also may be due to the inadequate mixing of the broth towards the later stages of growth affected the enzyme synthesis . When the culture medium was subjected to agitation, *P. purpurogenum* failed to grow and there was little fructosyltransferase production (Dhake and Patil, 2007). Agitation has been shown to influence enzyme production in many organisms. Agitation of medium was found to be effective for fructosyltransferase production by *A. pullulans* (Yun et al., 1997). Investigation the relationship between the morphology and rheology properties of *P. citrinum* to improve neo-fructosyltransferase in a 2.5 L bioreactor was also studied

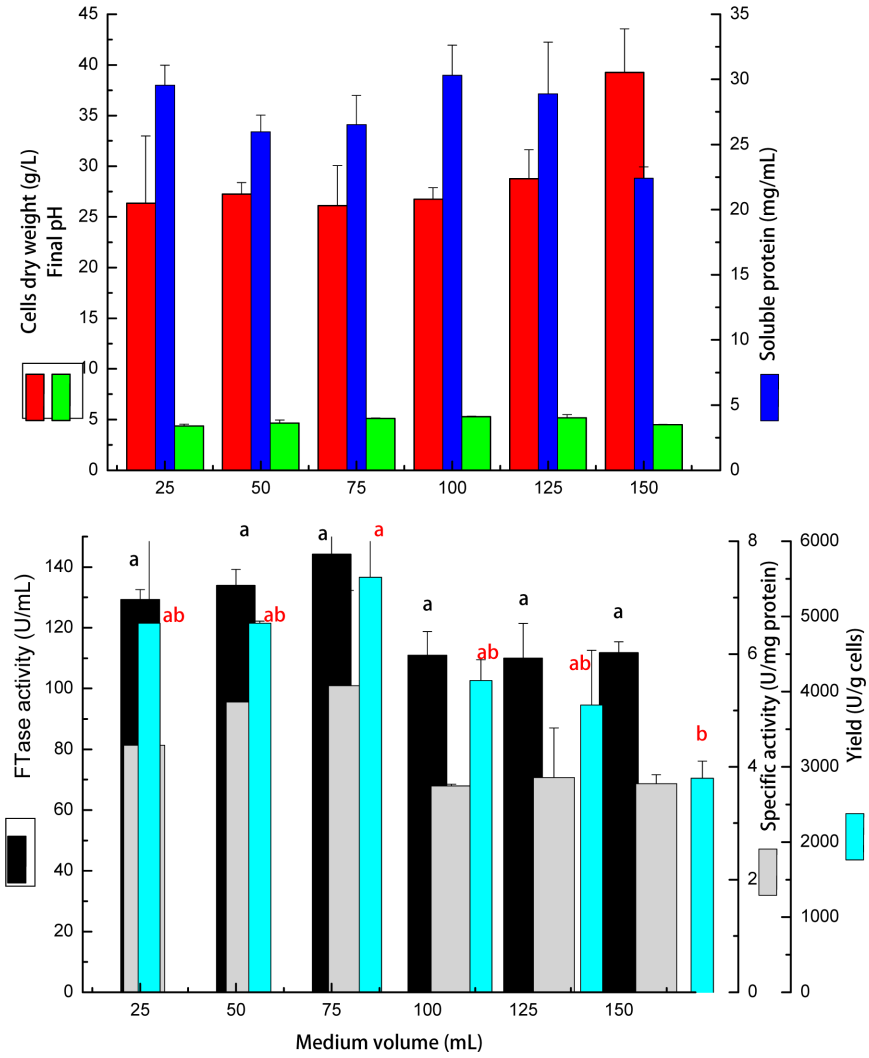


**Fig. 3** Effect of different medium volumes on cell growth and FTase production by *P. aurantiogriseum* AUMC 5605 in a shake-flask system. Y-error bars indicate the standard deviation (±SD) among the replicates. Means followed by different letters within FTase activity and yield column differ significantly at $p \leq 0.05$.

(Lim et al., 2006). They mentioned that pellets formation varied during fermentation process due to fermentation parameters (such as nutritional effect, mechanical agitation and aeration effect), lost their rigidity and broke up. These changes in pellet morphology might facilitate enzyme secretion. On the other hand, oxygen dependence at the lower agitation rate varied with the substrate type and its concentration (Lejeune and Baron, 1995). In much fungal fermentation, the high apparent viscosities and the non-Newtonian behavior of the broths necessitate the use of high agitation speeds to provide adequate mixing and oxygen transfer (Amanullah et al., 1999). However, mycelia damage at higher stirring speeds can limit the acceptable range of speeds. The results in Fig. 3 also demonstrate that upon increasing medium volumes, from 25 to 150 mL/flask, the FTase activity gradually increased and reached its maximal values of 144.2 U/mL in case of 75 mL medium volume. Also, results showed that cultivation volume more than 75 mL/flask has a negative effect on the production of FTase. However, the cell dry weight and the soluble protein increased with increasing medium volume.

**Effect of inoculum type and size on the production of FTase.** The finite volume of a culture medium means that it can only contain limited nutrients for the micro-organism. Furthermore, the consumption of the nutrients is largely dependent on the population and type of microorganism. To ensure a high production of enzyme in the limited volume of medium, the microorganism inoculum size should therefore be controlled.

The present experiment was undertaken to investigate the effect of inoculum type and size on the production of FTase from *P. aurantiogriseum* AUMC 5605. Different volumes of the prepared vegetative cell inoculums (5, 10, 15, 20, and 25 mL), prepared as mentioned in materials and methods, were transferred to the optimized medium of final volume of 50 mL/flask (inoculum+ fermentation medium) with initial pH of 6.5 and incubated at 30°C on a rotary shaker at 200 rpm for 144 h. Another sets of the

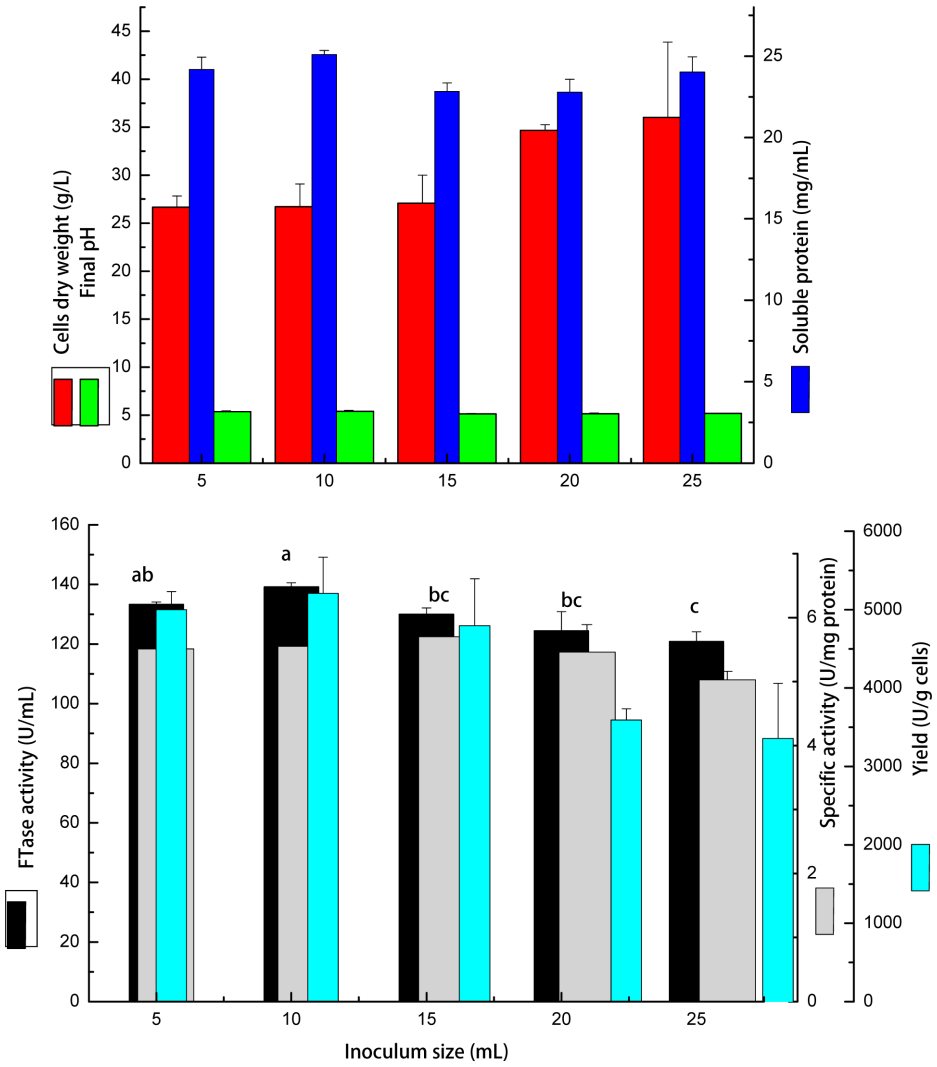


**Fig. 4** Effect of different inoculum sizes on cell growth and FTase production by *P. aurantiogriseum* AUMC 5605 in a shake-flask system. Y-error bars indicate the standard deviation (±SD) among the replicates. Means followed by different letters within FTase activity column differ significantly at $p \leq 0.05$.

fermentation medium each containing 50 mL/flask were inoculated with a spore suspension of one slant of 5 days old and then incubated as previously described. The obtained results (Fig. 4) reveal that highest FTase level was obtained when the culture was inoculated with vegetative cells (10 mL/50 mL culture) of 24 h old. Furthermore, the results showed that increasing the inoculums size more than 10 mL/50 mL culture, resulted in a gradual decrease in the enzyme level. However, it was noticed that increasing the inoculum size from 15–25 mL/50 mL culture was accompanied with increase in cell dry mass. Therefore, high inoculum sizes do not necessarily give higher FTase yield. The increase in the production of FTase using small inoculum sizes was suggested to be due to the higher surface area to volume ratio, which resulted in the increased production of FTase. In addition, an improved distribution of dissolve oxygen and more effective uptake of nutrient also contributed to a higher FTase production. If the inoculum sizes are too small, insufficient number of fungi would then lead to a reduced amount of secreted FTase (Frost and Moss, 1987). On the other hand, inoculation with spore suspension of 5–7 days old results in a lower FTase level.

**Growth curve kinetics for the production of FTase by *P. aurantiogriseum* AUMC 5605.** Based on the data from previous results, the growth kinetics of batch cultivation were investigated. The results illustrated in Fig. 4 show that the cell growth, soluble protein and enzyme activity of FTase were influenced by the fermentation time. The fungus grew well during the fermentation time and both mycelia growth and protein content increased with the increase of fermentation time. As shown, there was no lag phase observed because the inoculum was in the form of an activated cell mass previously grown for 24 h in vegetative growth medium. The fungal cell dry weight increased exponentially from about 17 g/L at 24 h to reach its maximum value of 36.1 g/L after 168 h. However, the volumetric FTase production increased exponentially from 24 to 96 h where it reached a maximal value

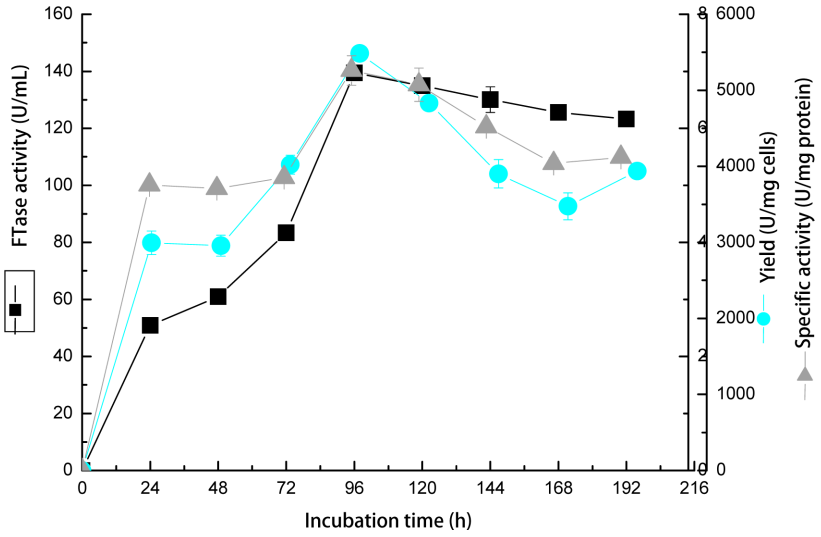


**Fig. 5** Growth curve parameters for FTase production of *Penicillium aurantiogriseum* AUMC 5605 at different time intervals in a shake-flask system. Y-error bars indicate the standard deviation (±SD) among the replicates.

of 139.5 U/mL (about 36.5% higher than the value of volumetric FTase produced in the batch culture before optimization). After that, the activity remained more or less constant for another 72 h, and began to decrease to reach a minimum value of 123.2 U/mL after 192 h. The maximal specific enzyme activity and maximal yield coefficient were recorded at 96 h, where they reached 7.01 U/mg and 5483.2 U/g cells/L, respectively. Results in Fig. 5 showed that during the whole cultivation process, the cells grew with a growth rate ranging from 0.1 to 0.23 g cells/L/h until 168 h, then the cell growth was stopped and at 192 h they started to die with a death rate of −0.2 g cells/L/h. Concerning the production and specific production rates, both rates increased gradually with time, till reaching their maximal values at 96 h (2340 U/L/h and 102 U/L/h/g cells, respectively). Then, both rates were sharply decreased recording −187.7 U/L/h and −7.05 U/L/h/g cells at 120 h, respectively. Thereafter, the produced enzyme was degraded with a degradation rate ranging from −96.1 to −202.7 U/L/h.

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