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EFFECT OF SUBSTRATE FEEDING ON PRODUCTION OF FRUCTOSYLTRANSFERASE BY *PENICILLIUM PURPUROGENUM*

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ABSTRACT

Penicillium purpurogenum was found to produce both extracellular and intracellular fructosyltransferase. The organism could also produce sucrose hydrolytic enzyme. Sucrose was found to be the best carbon source for fructosyltransferase production. Maximum intracellular and extracellular fructosyltransferase production was observed after 3rd and 4th day of cultivation, respectively. The enzyme activity was optimum at temperature of 55ºC and pH 5.5. The addition of amino acids, like leucine induced slightly the extracellular fructosyltransferase production, where as histidine and leucine had little inductive effect on intracellular fructosyltransferase production. Enhanced production of fructosyltransferase by *Penicillium purpurogenum* was observed when sucrose content was restored by additional sucrose feeding to the cultivation medium during production period.

Key words: Fructooligosaccharides, Frutosyltransferase, *Penicillium purpurogenum*

INTRODUCTION

Oligosaccharides are functional food ingredients, which have great potential to improve the quality of many foods. The important classes of oligosaccharides are Fructooligosaccharides (FOS), Galactooligosaccharides, Isomaltooligosaccharides (IMO), Inulinooligosaccharides and Soybean oligosaccharides. Microbial production of oligosaccharides has been extensively reviewed by many authors (4,19,30). Fructooligosaccharides (FOS) find important position for their favorable functionalities as they are low caloric, non-cariogenic and for acting as a growth factor for beneficial microorganisms in the intestinal flora (2,7,15,20,25,27).

Fructooligosaccharides have 1-3 fructosyl units bound to the ß, 2-1 position of sucrose. Fructooligosaccharides are commercially produced using microorganisms such as *Aspergillus niger* (10), *Aureobasidium pullulans* (29) and *Fusarium oxysporum* (17). Fructooligosaccharides [FOS] derived from sucrose using microbial enzymes have attracted special attention due to their sweet taste being very similar to that of sucrose, a traditional sweetener (30). FOS have also been commercially produced using fructosyltransferases [Fructosyltransferase, EC 2.4.1.9] obtained from various microorganisms such as *Aspergillus foetidus* (28), *Bacillus subtilis* (6), *Bacillus macerans* (12,16), *Streptococcus salivarius* (24) and *Aureobasidium pullulans* (31,32).

A *Penicillium purpurogenum* strains, isolated in our laboratory, was found to produce both extracellular and intracellular fructosyltransferases, the present paper reports the results of optimization of constituents in the culture medium and environmental parameters like cultivation time, initial pH, temperature, carbon source for maximum fructosyltransferase production. The effect of sucrose feeding to the cultivation medium when sucrose was completely exhausted was also monitored.

MATERIALS AND METHODS

Organism, maintenance and culture conditions

P. purpurogenum was maintained on potato dextrose agar (PDA), pH 5.2 to 5.8, at 4ºC. The culture was transferred to new slants at every two months to keep it viable.

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Chemicals

The amino acids and vitamins were from E Merck, Mumbai, India. The surfactants were from HiMedia, Mumbai, India. All other chemicals were of analytical grade.

Enzyme production

P. purpurogenum was cultivated aerobically in a Czapek-Dox medium used by Patil and Shastri (18). The culture medium contained (g/L of distilled water): NaCl-6, Sucrose-10, $MgSO_4 \cdot 7H_2O$-0.5, KH_2PO_4-1.5 and $NaNO_3$-25. The pH was adjusted to 5.5 with 1.0 M NaOH before autoclaving at 121ºC for 15 min. Sucrose was sterilized separately and added aseptically after cooling to the flasks containing the liquid medium to the appropriate level. The medium (50 ml in 250-ml Erlenmeyer flasks) was inoculated with 0.1 ml spore suspension of organism (10^4 spores/ml). Incubation was carried out under static condition at 30ºC.

Determination of sucrose and glucose

Sucrose concentration in the cultivation medium was determined by the method of Dubois *et al*. (5). The liberated glucose was subsequently analyzed by using dinitrosalicylic acid method as described by Miller (14).

Enzyme extraction

Intracellular enzyme: The mycelial mass was harvested by filtration, washed with distilled water, soaked between folds of filter paper and crushed in cold distilled water. The extract was centrifuged at 5,000 *g* for 20 min at 4ºC. The supernatant was used as source of intracellular enzyme.

Extracellular enzyme: The flasks were harvested after 4 days of incubation and filtered to remove the mycelial mass. The filtrate was centrifuged at 5,000 *g* for 20 min at 4ºC. The supernatant served as extracellular enzyme.

Enzyme assay

Fructosyltransferase assay was based on the procedure used by Yun *et al.* (31). 1.5 ml of 55% sucrose prepared in sodium acetate buffer 0.1M pH 5.5 was added to 0.1 ml enzyme solution. After the incubation at 55ºC for 1 h 1 ml dinitrosalicylic acid reagent was added to terminate the reaction. Suitable controls were run simultaneously. One unit of enzyme activity was defined as the amount of enzyme producing 1 mmol of glucose under experimental conditions.

Hydrolytic activity

Sucrose hydrolytic activity was measured by the method described by Sangeetha *et al*. (21). One unit of Sucrose hydrolytic activity was considered as the amount of enzyme required to produce 1 µmol of glucose under experimental conditions.

Cultural Parameters

1. Effect of incubation time

The optimum incubation time was also determined for the production of fructosyltransferase by *P. purpurogenum*. The enzyme production was measured at a regular interval of 24 h up to 10 days after inoculation.

2. Effect of different carbon sources on fructosyltransferase production

Carbohydrate utilization by *P. purpurogenum* and production of enzyme in aerobic fermentation was studied. Fructose, glucose, maltose and sucrose were added at 1% level in the cultivation medium.

3. Effect of initial pH

The effect of initial pH of the medium on fructosyltransferase production was studied by adjusting the initial pH from 3.0 to 6.0 by 0.1 N HCL.

4. Effect of agitation

The effect of agitation was studied by keeping the inoculated flasks on shaker at 120 rpm for 10 days at 30ºC.

5. Effect of substrate feeding

To monitor the effect of sucrose feeding, the original level (1% w/v) was restored after 4 days by additional transfer of sterile sucrose solution during the course of enzyme production. Mycelial mass and fructosyltransferase production were measured as described earlier.

6. Effect of addition of amino acids, vitamins and detergents

The effect of addition of amino acids, vitamins and detergents to the cultivation medium was also studied by adding them at 0.5% to the cultivation medium. Fructosyltransferase production was recorded on optimum days.

7. Effect of pH on fructosyltransferase activity and stability

The enzyme activity at pH 4.0 to 7.0 was measured using buffers prepared with sodium acetate (0.1M, pH 4.0 to 5.5) and citrate phosphate (0.1M, pH 6.0 to 7.0). For determination of stability, the enzyme was treated with different buffers in pH range from 4 to 7 for 60 min at 55ºC before the enzyme activity was measured.

8. Effect of temperature on fructosyltransferase activity and stability

The effect of temperature on fructosyltransferase activity was monitored by assaying the enzyme at 30, 40, 45, 50, 55, 60 and 70ºC under the experimental condition described earlier. The stability of enzyme was monitored by exposing the enzyme to various temperatures for 1 hour as shown above and the enzyme activity was measured.

RESULTS AND DISCUSSION

All the experiments were carried out in triplicate and the results were analyzed by single linear regression analysis. The enzyme production by microorganisms is remarkably influenced by conditions like pH, temperature, agitation and addition of different compounds to the cultivation medium. *P. purpurogenum* was found to produce both extracellular and intracellular fructosyltransferase and sucrose hydrolytic enzyme.

Some fungi like *A. pullulans, A. flavus, A. niger, M. michei* are also reported to produce both extracellular and intracellular fructosyltransferase and hydrolytic enzymes (21). Similarly *B. macerans* also produced extracellular fructosyltransferase (16). *P. purpurogenum* was grown for the period of 10 days. The maximum extracellular and intracellular fructosyltransferase production were recorded after day 4 and day 3 of cultivation, respectively (Fig. 1). The production of maximum intracellular fructosyltransferase prior to the peak extracellular fructosyltransferase was already shown for fungi like *A. oryzae* and *A. flavus* (21). There are organisms which produced maximum intracellular and extracellular enzyme on the same day. *M. michei* and *A. pullulans* showed maximum extracellular and intracellular fructosyltransferase production on day 4 and day 2 respectively (21).

The extracellular and intracellular sucrose hydrolytic enzyme production was maximum after 3 and 4 days of cultivation of *P. purpurogenum* respectively (Fig. 2). The extracellular hydrolytic enzyme level reached to its peak before intracellular. This might be due to leaking of the enzymes in external medium as soon as produced intracellularlly. The organisms like *A. pullulans, M. michei, A. oryzae* and *A. flavus* showed maximum extracellular and intracellular sucrose hydrolytic enzyme production on day 2, 4, 5 and 5 respectively.

Sucrose was the best carbon source for both extracellular and intracellular fructosyltransferase production by *P. purpurogenum* (Fig. 3). The carbohydrate source is an essential constituent in the cultivation media, being important for formation of cell constituents. The effect of different carbon sources was also studied by Yun *et al.* (31) for fructosyltransferase production by *A. pullulans* where sucrose was also found to be the preferred carbon source.

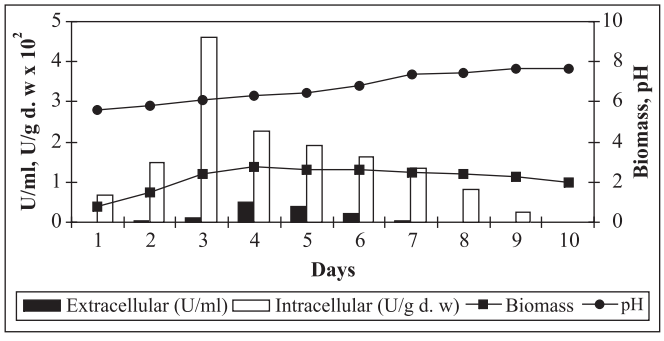

Figure 1. Fructosyltransferase production by *P. purpurogenum*.

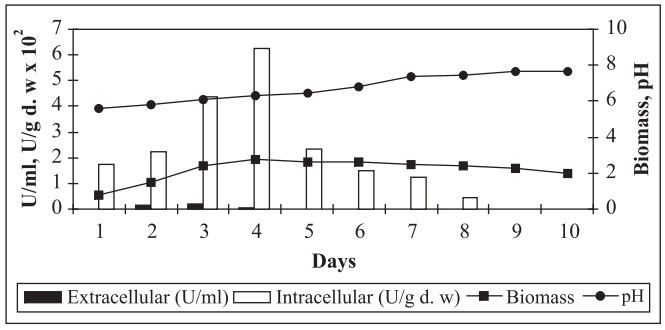

Figure 2. Sucrose-hydrolytic enzyme production by *P. purpurogenum*.

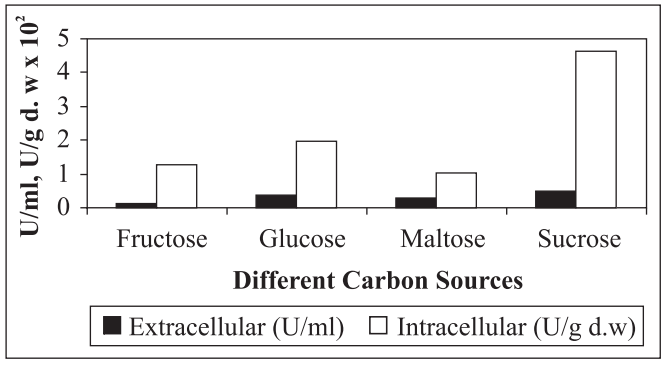

Figure 3. Effect of various carbon sources on fructosyltransferase production by *P. purpurogenum.*

The initial pH of medium plays a key role in enzyme production and in utilization of media constituents and growth of the microorganism (12). The optimum initial pH for fructosyltransferase production by *P. purpurogenum* was found to be 5.5. The influence of pH of the cultivation medium may be related directly with the stability of enzyme (26).

When the culture medium was subjected to agitation, *P. purpurogenum* failed to grow and there was little fructosyltransferase production. 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*, as reported by Yun *et al.* (31).

Enzyme show optimum activity at a particular pH. The pH of the medium to which the enzyme is exposed affects the ionization state of its amino acids, affecting its primary and secondary structure, thus controlling its activity (8). The intracellular and extracellular fructosyltransferase showed stability in the pH range

of 4.5 to 7.0, with optimum activity at pH 5.5 (Fig. 4). Yun *et al.* (32) also obtained same pH optimum for fructosyltransferase produced by *A. pullulans*. Song *et al.* (24) detected optimum activity at pH 6 for *S. salivarius* fructosyltransferase.

The intracellular and extracellular fructosyltransferase produced by *P. purpurogenum* has optimum activity of 55ºC (Fig. 5). Yun *et al.* (32) reported the same optimum temperature for fructosyltransferase from *A. pullulans*. Song *et al.* (24) reported 37ºC as optimum temperature for fructosyltransferase produced by *S. salivarius*.

P. purpurogenum produced maximum extracellular and intracellular fructosyltransferase after 4 and 3 days of fermentation, respectively (Fig. 1). Sucrose concentration in the cultivation medium reached zero after four days of incubation. When sucrose concentration was restored by fresh sucrose solution at 1%, there was increase in biomass and fructosyltransferase production both intracellular and extracellular fructosyltransferase (Fig. 6,7,8). The increase might be due to the availability of fresh substrate and an enhanced mass transfer effect in substrate feeding operation. Yun *et al.* (32) also detected enhancement in fructosyltransferase and glucosyltransferase production from *A. pullulans* by fresh substrate feeding. After 10 days of cultivation the pH of the medium was found to be 7.64 in additional sucrose nonfed flasks (control) which might be due to the accumulation of alkaline end products in the medium, where as in sucrose supplemented flasks there was also an increase in pH but it was less as compared to the control and found to be 6.6. This may be due to the addition of fresh substrate to the cultivation medium after 4 days during the growth of *P. purpurogenum*.

The addition of different compounds in cultivation medium affected the fructosyltransferase production in different ways.

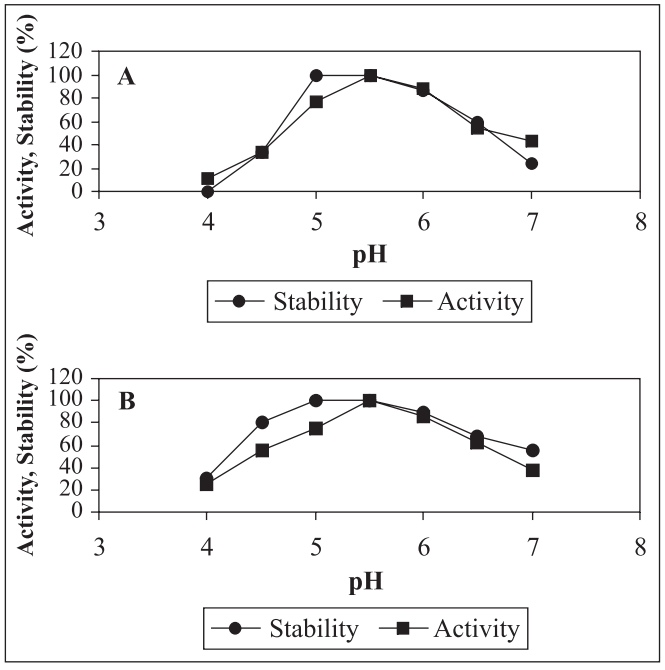

Figure 4a: Effect of pH on activity and stability of extracellular fructosyltransferase from *P. purpurogenum.* **Figure 4b:** Effect of pH on activity and stability of intracellular fructosyltransferase from *P. purpurogenum.*

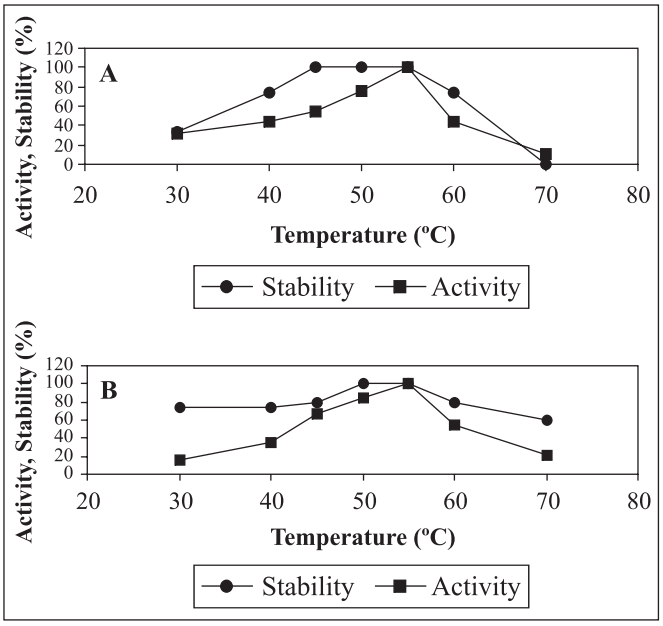

Figure 5a*:* Effect of temperature on activity and stability from extracellular fructosyltransferase from *P. purpurogenum.* **Figure 5b:** Effect of temperature on activity and stability of intracellular fructosyltransferase from *P. purpurogenum.*

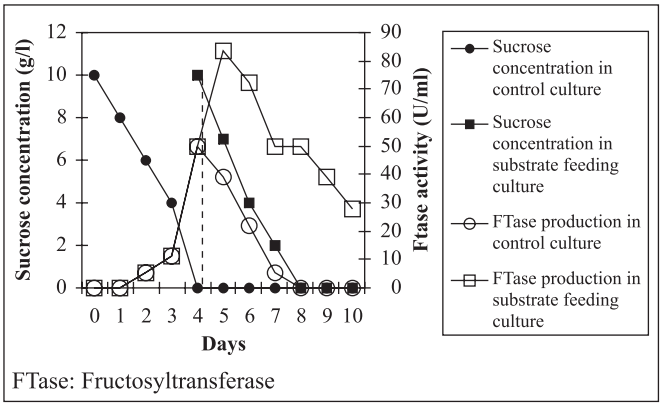

Figure 6. Effect of substrate feeding on production of extracellular fructosyltransferase by *P. purpurogenum.*

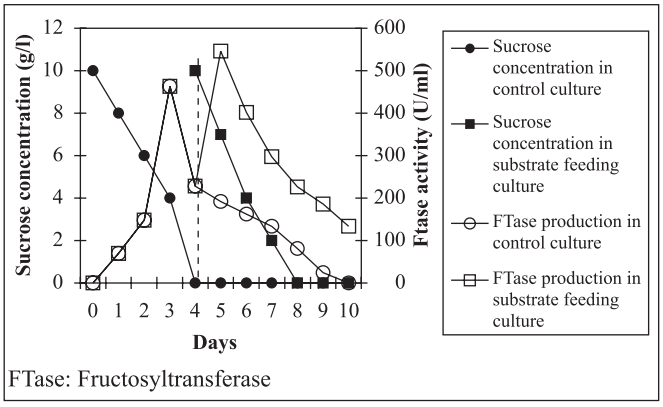

Figure 7. Effect of substrate feeding on production of intracellular fructosyltransferase by *P. purpurogenum.*

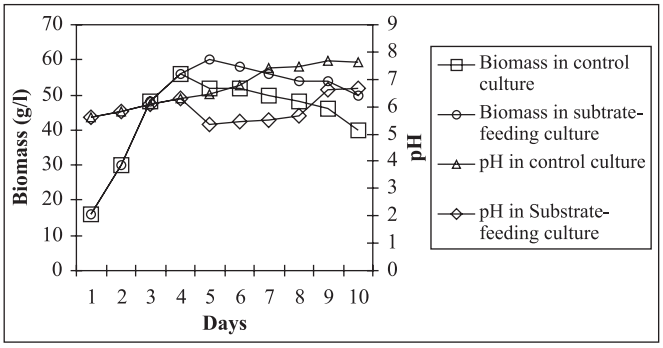

Figure 8. Effect of substrate feeding on biomass and pH of the medium during fermentation.

Table 1. Effect of additives on production of fructosyltransferase by *P. purpurogenum.*

Additive (0.5%)	Fructosyltransferase (Extracellular) (U/ml)	Fructosyltransferase (Intracellular) (U/g d. w)
Control	50	463
Alanine	44	434
Aspartic acid	—	—
Cysteine	39	411
Cystine	28	353
Glutamine	22	294
Histidine	33	492
Leucine	61	507
Lysine	39	404
Norvaline	44	441
Proline	—	—
Serine	39	397
Threonine	44	441
Tryptophan	33	367
Tyrosine	50	463
Valine	28	338
Ascorbic acid	39	404
Thiamine	50	456
SDS	—	—
TritonX100	—	—
Tween 20	—	—
Tween 80	—	—

U /ml - units per ml; U /g d. w - units per gram mycelium dry weight.

Addition of amino acids like leucine had slight induction effect on extacellular fructosyltransferase production, where as addition of histidine and leucine showed slight induction in intracellular fructosyltransferase production (Table 1). The influence of addition of amino acids and vitamins was studied by Sapre *et al.* (22) on xylanase production where enhancement in enzyme production in presence of amino acids like cystine and leucine was observed. Amino acids have also been reported to stimulate the production of other enzyme such as a-amylase (Zhang *et al.* (33)) and xylanase (Gupta *et al*. (9); Beg *et al.* (1)).

The addition of vitamins did not affect the enzyme production (Table 1). The vitamins had no influence on xylanase production by *S. recemosum* also (22). The induction in enzyme production by vitamins was reported by Kapoor and Kuhad, 2002 in case of polygalacturonase from *Bacillus* sp (11).

The effect of surfactant addition on enzyme production has also been widely investigated (13,23). It has been reported that stimulatory effect of these additives resulted from efficient spore dispersion, rheological properties of the medium, availability of nutrients and oxygen and physiological functions of the cells (3). The addition of surfactants like SDS, Triton X-100, Tween 20 and Tween 80 completely inhibited the growth of *P. purpurogenum* hence no enzyme production was observed (Table 1). In this case the surfactant may have solubilized the cell membrane, causing in no mycelial growth. The addition of surfactant has also reported to increase enzyme production for polygalacturonase from *Bacillus* sp (11).

Since the production and application of fructosyltransferase has gained tremendous commercial importance for synthesis of fructooligosaccharides, it is worth to purify and understand the properties of fructosyltransferase produced by *P. purpurogenum.* Work on this line is still in progress.

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RESUMO

Efeito da adição de substrato sobre a produção de frutosiltransferase pelo *Penicillium purpurogenum*

Penicillium purpurogenum foi identificado como produtor extracellular e intracelular de frutosiltransferase. O microrganismo também é capaz de produzir uma enzima hidrolítica de sacarose. Sacarose foi identificada como a melhor fonte de carbono para a produção de frutosiltransferase. A produção máxima de frutosiltransferase extracelular e intracelular foi observada após o 3º e 4º dia de cultivo, respectivamente. Atividade ótima da enzima foi observada na temperatura de 55º C e pH 5,5. A adição de amino ácidos, como leucina, induziu ligeiro aumento na produção extracelular de frutosiltransferase, enquanto que histidina e leucina induziram um pequeno aumento na produção da frutosiltransferase intracelular. Observou-se aumento na produção de frutosiltransferase por *Penicillium purpurogenum* quando a quantidade de sacarose era restaurada por adição do carboidrato ao meio de cultura durante o período de produção.

Palavras chave: frutooligossacarídeos, frutosiltransferase, *Penicillium purpurogenum*

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