Journal of Global Biosciences ISSN 2320-1355
Vol. 3(2), 2014, pp. 597-603 http://mutagens.co.in
Date of Online: 28, April- 2014

FRUCTOSYLTRANSFERASE PRODUCTION BY INDIGENOUSLY ISOLATED *SYNCEPHALASTRUM RACEMOSUM COHN*

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Abstract

A new strain of *Syncephalastrum recemosum Cohn* was isolated form rhizoshere of *Zizyphus mauritiana* which produces fructosyltransferase (Ftase) enzyme [EC: 2.4.1.9] comparable to other microorganisms. Sucrose served as a best carbon source for the enzyme production in an economic medium. Ammonium nitrate was the most preferred nitrogen source than yeast extract. Optimum pH and temperature for enzyme production were determined to be 6.0 and 30^{o}C, respectively. Static condition was favorable for enzyme production than agitation of the medium. Other physicochemical parameters have been optimized for maximum Ftase production in economic fermentation medium.

Key words: Fructosyltransferase, *Syncephalastrum recemosum Cohn*.

INTRODUCTION

Oligosaccharides are special carbohydrates which are presently in focus with reference to diet and intestinal health. Oligosaccharides are also gaining popular status as food ingredients. Apart from being good flavoring agents, fructooligosaccharides (FOS) possess properties favorable for consumers' health [1]. Fructooligosaccharides are becoming tremendously popular as they are low caloric, non cariogenic, and bifidogenic [2-4]. FOS are safe for human consumption and have been proved to be beneficial for overall health of the consumer [5, 6]. FOS is only 33% sweet as compared to sucrose. Though sweet, they are neither digested by the digestive system nor used as energy source. Hence, they find wide application as low caloric sweetener for diabetics. FOS are also shown to decrease the level of cholesterol, phospholipids and triglycerides in the blood [7].

Important classes of oligosaccharides are cyclodextrins [8], isomaltooligosaccharides [9], soybean oligosaccharides [10] and fructooligosaccharides [11-13]. Oligosaccharides can be prepared by employing enzymes of either plant or microbial origin. Microbial enzymes are preferred over plant enzymes for industrial production of oligosaccharides as they are easy to prepare. Usages of microbial enzymes make the processes cheap and convenient. Microbial Ftases have been used as a tool for production of FOS [1,7,14,15]. The detailed mechanism for FOS formation from sucrose has been reported as follows:

GF_{n+} GFn == GF_{n+1} + GF_{n-1}

Where n = < 4

G and F are glucopyranosyl and fructopyranosyl moiety in sucrose molecule respectively [12]. There are many microorganisms which can produce Ftase enzyme eg. *Aureobasidium, Penicillium, Aspergillus, Mucor and Bacillus,* etc [15].

This paper presents the observations and the work carried out on optimization of culture conditions and nutritional parameters for maximum production of Ftase from an indigenously isolated *Syncephalastrum recemosum Cohn.*

MATERIALS AND METHODS

Microorganism

Syncephalastrum recemosum Cohn species was isolated from the rhizoshere of the plant *Z. mauritiana* growing in the garden of University Department of Biochemistry, RTM Nagpur

University, Nagpur.. The purified fungus was identified by the IMT, MTCC, Chandigarh as *Syncephalastrum recemosum Cohn*. The stock cultures were maintained on PDA slants at 4°C and sub cultured on fresh PDA slants every 2 months to keep it viable and potent.

Chemicals

All chemicals were of analytical reagent grade. Distilled water was used throughout the experiments.

Cultural conditions

Unless otherwise specified Ftase production was carried out in aerobic flask cultures at 30°C for 10 days under the conditions mentioned there in. The cultivation medium contained per liter: $NaNO_3$ 3g, KH_2PO_4 1 g, $MgSO_4.7H_2O$ 0.5 g, $FeSO_4.7H_2O$ 0.01 g, and Sucrose 30 g. pH of the medium was adjusted to 5.5 before autoclaving at 121 lb per sq. inch steam pressure. Sucrose was autoclaved separately and added to the cultivation flasks before inoculation.

Preparation of inoculum

Sterile distilled water was added to the slants of *Syncephalastrum recemosum Cohn* and the spores were scrapped with sterile needle. The spore suspension was vortexed on vortex blender to break the clumps and counted for the number of spores per ml. Inoculation of the sterilized medium was carried out by transferring 1 ml spore suspension containing 10^8 spores to 100 ml medium.

Enzyme production

The inoculated flasks were incubated at 30°C for 12 days. One flask was removed after every 48 hours and assayed for the presence of Ftase. The best carbon source was selected which favored maximum production of Ftase by testing various other carbon sources including sucrose. The optimum concentration of selected carbon source was determined. To investigate the influence of initial pH of medium, fermentation was carried out by adjusting the initial pH of the cultivation medium to 3, 5, 5.5, 6 & 7. Effect of various organic and inorganic nitrogen sources was studied on Ftase production. Mineral salts were added to investigate their effect on Ftase production.

Enzyme preparation

Extracellular enzyme: After removing the mycelial mass by filtration from the fermentation medium the filtrate was centrifuged at 9886 *g* (C-24, Remi) for 20 min. to remove the debris. The clear supernatant was used as a source of crude extracellular Ftase. If required the pH of the crude enzyme was adjusted to 5.5 before assay.

Intracellular enzyme: The mycelial mat was crushed in cold distilled water using chilled pestle & mortar and suspended in the same. The suspension was left at 4°C for 2 hours for complete solubilization of the intracellular content in to the extraction medium. The contents were centrifuged at 9886 *g* (C-24, Remi) for 20 min. to remove the debris. The clear supernatant was used as a source of crude intracellular Ftase. The pH of the crude supernatant was adjusted to 5.5 before assay, if found necessary.

Enzyme assay:

The reaction mixture containing 1 ml sucrose (50%), 0.5 ml sodium citrate buffer (pH 5.5) and 0.1 ml crude enzyme was incubated for 30 min. at 55°C. Reaction was terminated by addition of 1 ml Dinitrosalicylic acid reagent. The reaction mixture was kept in boiling water bath for 10 min. After cooling to room temperature the volume was raised to 10 ml with distilled water and O.D. was recorded at 540 nm using reagent blank to adjust the instrument (Gilford, Germany). Suitable controls were run simultaneously. Liberated glucose from the sucrose was measured by using D- glucose as standard [16]. One unit of Ftase was defined as that amount which liberated 1mM glucose under experimental conditions. Protein content was measured by the method of Lowry *et. al*. (1951), using BSA as standard protein [17].

RESULTS

Effect of incubation period: Results presented in Table 1 indicate that as the period of incubation increases the mycelial mass proportionally increases up to 10 days. On 12th day although there is a rise in mycelial mass but the enzyme content was the same as that for 10th day. Same pattern was exhibited by the intracellular Ftase also. The final pH of the crude supernatant was not found to deviate from the initial pH.

Effect of Initial pH: Initial pH of the cultivation medium has a great impact on growth of the microorganism, enzyme synthesis and its release in the cultivation medium, as the ionic charges of the contents decide the diffusion of the nutrients in to the cell affecting its metabolism and synthesis of

the enzyme. In case of *Syncephalastrum recemosum Cohn* the initial pH of 5.5 appears to be best for growth and synthesis of both extra and intracellular Ftase (Table 2).

Effect of temperature: Fungi appear to be most sensitive towards the temperature w.r.t. growth. Growth of any organism has a direct impact on synthesis of enzymes and their release in to the surrounding medium. *Syncephalastrum recemosum Cohn* produced maximum Ftase at 30^{o}C with parallel rise in growth (Table 3).

Effect of shake and static cultivation: The flasks, after inoculation, were kept on the shaker in BOD incubator at 30^{o}C and the speed of the shaker was adjusted to 200 rpm. The Ftase content was recorded after 10 days. It was observed that *Syncephalastrum recemosum Cohn* did not grow in shake flask cultivation and the Ftase synthesis was too low to measure (data not shown).

Effect of inoculum built up: The spore were previously germinated for 12 hours at 30^{o}C and then transferred to the growth medium in order to know the effect of inoculum built up on production of Ftase by *Syncephalastrum recemosum Cohn*. However, it was seen that previously germinated spores did not have any effect on the growth and production of Ftase by *Syncephalastrum recemosum Cohn* after growth for 10 days (data not shown).

Effect of different carbon sources: Carbon source in the cultivation medium decides the physiology of the growing microorganism and its utilization for the growth and development as it is used for the synthesis of cellular contents. The results shown in table 4 shows that for Ftase production, in the given fermentation condition, sucrose was the most preferred carbon source by *Syncephalastrum recemosum cohn* as compared to other.

Effect of Sucrose concentration: Sucrose was included in the cultivation medium at different concentrations from 1 to 50% in order to make it available in the abundant quantity so that it does not become the limiting factor at any time for Ftase production by *Syncephalastrum recemosum Cohn* within a period of 10 days. Results presented in Table 5 show that although sucrose is the most favored carbon source for *Syncephalastrum recemosum Cohn* it supported maximum extra and intra cellular Ftase production at 3 % level only, beyond that there was no increase in the enzyme synthesis.

Effect of different organic and inorganic nitrogen sources: Nitrogen sources support the protein synthesis of the growing fungus in the fermentation medium. Enzyme synthesis is largely dependent on the growth of any organism. Table 6 shows that organic nitrogen sources such as tryptone, peptone, and yeast extract favored growth and Ftase production as compared to the inorganic sources. Amongst the inorganic sources ammonium nitrate proved to be excellent source supporting growth and production of Ftase *by Syncephalastrum recemosum Cohn*. All the nitrogen sources were included at 3 % level in the medium.

Effect of mineral salts on the growth and Ftase production: Many metal ions are known to act as cofactors in enzyme catalyzed reactions. Presence of the right metal ions in appropriate level in the growth medium governs the growth and synthesis of cell constituents by any fungus. For *Syncephalastrum recemosum Cohn* presence of NaCl led to the extracellular release of Ftase and $CaCl_2$ made the fungus to accumulate Ftase in intracellular environment when included in the cultivation medium at 0.05 % level (Table 7).

Effect of physicochemical factors on Ftase activity: The enzyme activity of Ftase was found to be measurable at 0.1 ml level in the reaction mixture. Similarly for substrate concentration 50% sucrose at 1.5 ml was sufficient for maximum activity with 0.1ml crude Ftase. When different buffers from pH 3 to 6 were included in reaction mixture sodium citrate buffer gave maximum activity at pH 5.5. The reaction mixture was exposed to different temperatures form 30 to 70^{o}C to determine the optimum temperature for maximum activity. Optimum temperature of 55^{o}C was recorded. The reaction mixture developed by using all optimum contents at pH 5.0 was exposed at 55^{o}C for different time. At the given experimental condition the enzyme showed maximum activity till 30 min. incubation time only (data not shown).

Thus the economic production medium was developed for Ftase production by *Syncephalastrum recemosum Cohn* as follows: Sucrose 3g, NH_4NO_3 0.5g, KH_2PO_4 0.1 g, $MgSO_4.7H_2O$ 0.05g, $FeSO_4.7H_2O$ 0.001g, NaCl 0.05g, pH 5.5, Distilled water 100 ml.

DISCUSSION

All the experiments were carried out in triplicate and analyzed by single linear regression using SPSS software. The cultural conditions have a great influence on enzyme synthesis and its release in to the

cultivation medium. The factors like pH, temperature, agitation and addition of different compounds in the cultivation medium has great influence on the synthesis and release of enzyme by the microorganism. *Syncephalastrum recemosum Cohn* like other fungi *P. purpurogenum* [18], *A. pullulans, A. flavus, M. meichi* are also reported to produce both extracellular and intracellular Ftase [19]. *Syncephalastrum recemosum Cohn* produced maximum quantity of Ftase after 10 days of cultivation. But *P. purpurogenum* produced maximum extracellular Ftase after 4 days and intracellular Ftase after 3 days growth in the fermentation medium [18]. The production of maximum intracellular enzyme prior to the extracellular peak has been shown by *A. flavus* and *A. oryzae* [19], but *Syncephalastrum recemosum Cohn* produced both the extracellular and intracellular Ftase in maximum quantity on the same day i.e. after 10 days of growth (Table 1). Sucrose at 3 % was found to be the best carbon source for Ftase synthesis by *Syncephalastrum recemosum Cohn* (Table 5). Similar reports w.r.t. Ftase production by *A. pullulans, A. flavus, A. oryzae* and *M. meichi* are also available [19]. Effect of different carbon sources on Ftase synthesis was also reported [20, 21]. The initial pH of the medium appears to play a key role in utilization of medium constituents and growth of the microorganism [22]. As for *Syncephalastrum recemosum Cohn* the optimum initial pH of 5.5 was noted for *P. pupurogenum* [18]. When the cultivation medium was subjected to agitation *Syncephalastrum recemosum Cohn* did not grow and thus there was no Ftase production. Similar observation was also seen for *P. purpurogenum* [18]. Enzymes being protein in nature react sharply to the surrounding pH due to the ionization of constituent amino acids. Optimum pH exhibited by *Syncephalastrum recemosum Cohn* Ftase was found to be 5.5, which was same as shown by *P. purpurogenum* [18]. Optimum temperature of Ftase activity for *Syncephalastrum recemosum Cohn* was 55^{o}C, as that for Ftase of *P. purpurogenum* [18]. However, for *A. pullulans* the optimum temperature was found to be 37^{o}C [21].

Since production of microbial Ftase is gaining importance due to their application in commercial FOS production, it is necessary to purify understand the properties of Ftase produced by *Syncephalastrum recemosum Cohn* Work on this line is in progress.

Results presented in this paper are the mean of 3 replicates. The data obtained was analysed using ANOVA technique as given by Ronald Walpole (1982). Significance was set at P<0.05. [23].

Table: 1. Effect of incubation time on production of Ftase by *Syncephalastrum recemosum Cohn*

Days of Incubation	Final pH	Mycelial weight (g)	Ext. Ftase U/ml	Int. Ftase U/ml
2	5.5	0.265	15	2
4	5.5	0.7	20	5
6	5.5	1.265	35	10
8	5.5	1.60	60	21
10	5.5	2.00	90	27
12	5.5	2.5	85	25

Ext.: Extracellular; Int.: Intracellular. P<0.05

Table: 2. Effect of initial pH on growth and production of Ftase by *Syncephalastrum recemosum Cohn*

Initial pH	Final pH	Mycelial weight (g)	Ext. Ftase U/ml	Int. Ftase U/ml
3.0	3.5	0.8	30	10
5.0	5.0	1.6	55	10
5.5	5.5	2.0	90	27
6.0	6.3	1.19	60	21
7.0	7.0	0.689	55	19

Ext.: Extracellular; Int.: Intracellular. P<0.05

Table: 3. Effect of temperature on growth and production of Ftase by *Syncephalastrum recemosum Cohn*

Temperature ^{o}C	Final pH	Mycelial weight (g)	Ext. Ftase U/ml	Int. Ftase U/ml
30	5.5	2.0	90	27
40	5.5	1.8	80	24
50	5.5	1.0	70	15
60	6.0	0.8	50	10

Ext.: Extracellular; Int.: Intracellular. P<0.05

Table: 4 Effect of different carbon sources on Ftase production by *Syncephalastrum recemosum Cohn*

Carbon source (3 %)	Final pH	Mycelial weight (g)	Ext. Ftase U/ml	Int. Ftase U/ml
Xylose	7.0	1.7	30	10
Fructose	5.5	2.0	10	5
Glucose	5.5	1.79	15	10
Lactose	5.5	1.75	5	2
Maltose	5.5	1.75	5	2
Sucrose	5.5	2.00	90	27
Inulin	7.0	1.65	15	10
Starch	8	1.5	25	8

Ext.: Extracellular; Int.: Intracellular. P<0.05

Table 5: Effect of sucrose concentration on Ftase production by *Syncephalastrum recemosum Cohn*

Sucrose Concentration %	Final pH	Mycelial weight (g)	Ext. Ftase U/ml	Int. Ftase U/ml
1	5.5	0.87	27	31
2	5.5	1.3	55	19
3	5.5	2.0	90	27
5	5.7	2.5	92	29
10	5	3.0	94	31
20	4.9	3.29	100	33
30	5.0	3.45	100	30
40	5.5	3.60	94	21
50	5.5	3.60	70	12

Ext.: Extracellular; Int.: Intracellular. P<0.05

Table 6: Effect of nitrogen sources on Ftase production by *Syncephalastrum recemosum Cohn*

Nitrogen source (3%)	Final pH	Mycelial wt. (g)	Ext. Ftase U/ml	Int. Ftase U/ml
Casaminoacid	5.5	1.7	150	80
Peptone	5.5	1.89	150	80
Tryptone	5.5	1.9	140	130
Yeast extract	5.5	2.3	210	40
$NH_4 NO_3$	5.5	2.49	200	50
$(NH_4)_2 PO_4$	2	1.49	15	30
$(NH_4)_2 SO_4$	2	0.9	15	5
$NaNO_3$	5.5	2.10	90	27
KNO_3	5.5	1.89	95	150

Ext.: Extracellular; Int.: Intracellular. P<0.05

Table 7: Effect of mineral salts on growth and Ftase production by *Syncephalastrum recemosum Cohn*

Mineral salts 0.05 %	Final pH	Mycelial weight (g)	Ext. Ftase U/ml	Int. Ftase U/ml
$MnCl_2$	5.5	1.7	40	31
$MgCl_2$	5.5	2.2	90	27
NaCl	5.5	2.20	210	40
$CaCl_2$	5.5	1.5	94	42
$ZnSO_4$	5.5	1.8	80	55

Ext.: Extracellular; Int.: Intracellular. P<0.05

ACKNOWLEDGEMENT
Authors thank, the Head, University Department of Biochemistry, RTM Nagpur University, Nagpur for Laboratory facilities and encouragement.

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