

Culture Conditions for Production of 2-1- β -D-Fructan-fructanohydrolase in Solid Culturing on Chicory (*Cichorium intybus*) Roots

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ABSTRACT

Investigations were carried out to optimize the culture conditions for the production of **b**-D-fructan-fructanohydrolase by an indigenously isolated bacterial culture of *Staphylococcus* sp. RRL8. Experiments were carried out in solid culturing using chicory roots (powdered) as the source of carbon, which was supplemented, with corn steep liquor and potassium dihydrogen phosphate. A number of process parameters, like period of cultivation, initial moisture content in the substrate and temperature of incubation were optimized. Maximum extra-cellular enzyme was produced when fermentation was carried out at 30°C for 24 h using chicory roots with 60% initial moisture. Supplementation of the substrate with additional carbon source (except with sucrose) resulted decreased enzyme titres, which indicated that the strain was partially depressive. Addition of external nitrogen sources (in addition to corn steep liquor) also failed to stimulate enzyme formation; rather exerted harmful impact on bacterial culture of *Staphylococcus* sp. RRL8.

Key words: Fructan-fructanohydrolase, Inulinase, Solid state fermentation, *Staphylococcus* sp., Chicory

INTRODUCTION

The microbial enzyme, 2-1- β -D-fructan-fructanohydrolase (inulinase, EC - 3.2.1.7) hydrolyses the plant polymer inulin into practically pure fructose. As such, inulinase offers interesting perspectives in view of the growing need for production of pure fructose syrups or may present an alternative way to produce the so called "Ultra High Fructose-Glucose Syrups" (UHFGS) - not from starch, but from inulin. Conventional fructose production from starch needs at least three enzymatic steps, including α -amylase, amyloglucosidase and glucose isomerase action, yielding maximum only ~ 45% fructose solutions at the best. Fructose formation from inulin is a single enzymatic step reaction and yields up to

95% fructose is easily obtainable. Fructose is more soluble than sucrose, displays a greater sweetening power, and can mask the bitter 'after-taste' of saccharin. Another timely application of inulinase consists of the direct fermentation of inulin into ethanol, using a yeast culture having inulinase activity also.

Chemical (acid) hydrolysis of inulin to fructose displays several drawbacks. Acid hydrolysis always resulted in an undesirable colouring of the inulin hydrolyse. Changes in taste and aroma are minimal upon enzymatic hydrolysis. Acid hydrolysis also results in formation of ~ 5% difructose anhydride, which has practically no sweetening properties. This compound can not be further hydrolysed enzymatically into fructose. Based on this, a forced interest has developed

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towards the microbial inulinase enzyme and its production. (Vandamme *et al*, 1981).

Solid-state Fermentation (SSF) is generally defined as the growth of the micro-organisms on moist solid substrates in the absence or near absence of free water (Pandey 1992a, 1994). Solid substrates employed in SSF processes are insoluble in water and act as a source of carbon, nitrogen, minerals as well as growth factors. They have the capacity to absorb water and it is this water that meets the vital requirement of water by micro-organisms. The solid substrate also provides anchorage to the microbial cells.

Literature survey showed that although a number of microbial cultures belonging to yeast and fungi have been used for the production of inulinases, not much work has been done on bacterial inulinases (Pandey *et al.* 1998a). In the present study, we report the production of extra-cellular inulinase in solid cultures by a strain of *Staphylococcus* sp.

MATERIALS AND METHODS

Micro-organism: A bacterial strain of *Staphylococcus* sp. RRL8, a RRL isolates (Selvakumar and Pandey, 1998 a, b), was used for this study. It was maintained on Yeast extract-Peptone-Inulin agar, sub-cultured fortnightly and stored at 4°C.

Inoculum preparation: The medium contained (g/L): Yeast extract 10, Bacto peptone 20 and inulin 10 as a sole carbon source (pH 6.5). It was autoclaved at 121°C for 20 min. A loopful of cells were transferred into 250-ml conical flask containing 20-ml medium. The culture was incubated at 30°C for 24 h on a rotary shaker operating at 150 rpm.

Solid state fermentation: Chicory root was procured from the local market, washed well, chopped into small pieces and dried at 80°C in a hot air oven. The dried root was powdered and

used as solid substrate. A weighed quantity (5 g, dry wt.) of chicory root powder was supplemented with 1.0 g of corn steep liquor (Sigma, USA) and 0.5 g of KH₂PO₄ and mixed well. Initial pH and moisture of the substrate were set at 5.5 and 50%, respectively. The wet substrate was autoclaved at 15 psi for 20 min.

Fermentation was carried out with 7.5-g moist substrate in Petri dish (internal diameter, 9.0 cm). Each Petri dish was inoculated with 5% (v/w) cell suspension and was incubated at 30°C for stipulated period of time. Petri dishes were withdrawn in duplicate as samples. Results reported are the average of three sets of the experiments.

Extraction of enzyme: A weighed quantity (5 g) of the fermented matter was treated with 50 ml-distilled water and mixed thoroughly on a magnetic stirrer for 30 min. The whole contents were filtered through muslin cloth. The residue was again treated with another 50 ml of distilled water, mixed in the same way and filtered. Both the filtrates were combined.

Enzyme assay: Enzyme assay was carried out by the following procedure. To 2-ml 0.2% inulin, 2-ml 0.01 mol/L acetate buffer (pH 4.6) was added. Culture filtrate (0.5-ml, diluted, if necessary) were added to this and incubated at 50°C for 20 min. After incubation, the tubes were kept in a boiling water bath for 10 min. to inactivate the enzyme and then cooled to room temperature. The reaction mixture was assayed for reducing sugars as fructose by DNS method (Miller, 1959) by reading the absorbance at 575 nm on a spectrophotometer (Shimadzu 160A, Japan). Calibration curve was prepared with a fructose solution of known strength. Blanks were run simultaneously with the enzyme and substrate solutions. One unit of inulinase activity was defined as the amount of enzyme, which produced one micro mol of fructose under the assay conditions.

Moisture analysis: Moisture content of the solid substrate was determined by drying it at 80°C for 24 h.

RESULTS

Fig.1 shows inulinase production on chicory root powder by *Staphylococcus* sp. RRL8 over a period of 72 h to determine the optimal period of cultivation. Fermentation was carried out at 30°C

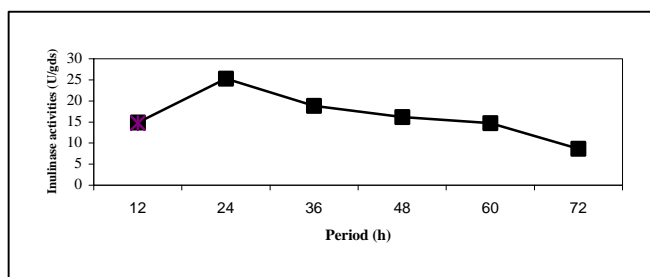


Fig. 1. Inulinase synthesis in SSF (72h)

and samples were withdrawn each 12 h. Evidently, maximum accumulation of extra-cellular inulinase in the fermentation medium by the bacterial strain occurred at 24 h, which was 25.27 U/gram dry (fermented) substrate (gds). With increased time of cultivation, there was decrease in enzyme titres and at 72 h, enzyme activities were only about 30% of the optimal level. Table 1 shows the pH of the substrate.

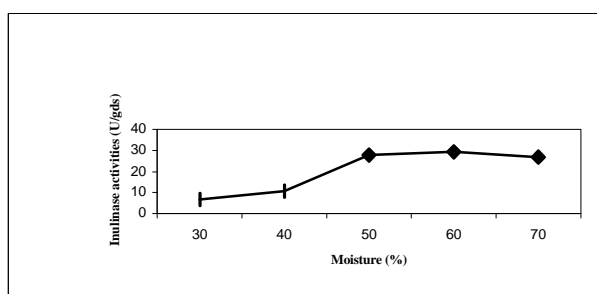


Fig. 2. Effect of substrate moisture on inulinase synthesis in SSF

In order to study the effect of initial substrate moisture on enzyme production by the culture, five different initial moisture level, viz. 30, 40, 50, 60, and 70% were established in the substrate. All the Petri dishes were cultivated for 24 h at 30°C and the results obtained are presented

in Fig.2. As is evident, maximum extra-cellular enzyme activity was obtained with the substrate having initial moisture as 60% (29.86 U/gds). At 50% moisture level too reasonably good amounts of the enzymes were produced.

Fig. 3 shows the effect of temperature of incubation on extra-cellular inulinase synthesis by the bacterial strain. Four different incubation temperatures, viz. 25, 30, of 35 and 40°C were tested. Fermentation was carried out for 24 h with the substrate having 60% initial moisture. Maximum inulinase activity (27.99 U/gds) was observed at 30°C. Higher incubation temperature resulted decreased yields of the enzyme.

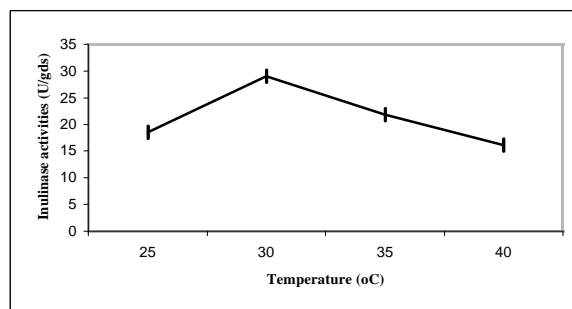


Fig. 3. Effect of incubation temperature on inulinase synthesis in SSF

Effect of addition of carbon sources on inulinase production

Experiments were also carried out to study the effect of addition of different carbon sources in the fermentation medium on the growth and activity of the bacterial strain. Fermentation was carried out at 30°C with the substrate having 60% initial moisture for 48 h and samples were withdrawn at 24 h. Six different carbon sources, glucose, sucrose, maltose, starch, glycerol and lactose, at 1.0 % (w/w) concentration were tested individually. Results obtained are shown in the Table 2. While glucose, maltose, starch, glycerol and lactose inhibited the bacterial activity of enzyme synthesis, sucrose resulted a significant increase in the inulinase titres. Highest enzyme activities were 37.74 U/gds after 24 h of fermentation. However, there was a marked deterioration in the enzyme yields on longer cultivation period (cf. Table 2).

Table 1. pH profile of fermenting substrate during the course of solid state fermentation

Period (h)	0	12	24	36	48	60	72
pH	5.50	5.52	5.56	5.57	5.78	5.87	5.89

Table 2. Effect of carbon sources on inulinase synthesis in SSF

C-source	Inulinase activity (U/gds)	
	24h	48h
Glucose	23.30	21.92
Sucrose	37.74	23.49
Maltose	19.50	18.03
Lactose	28.31	22.95
Starch	20.10	19.41
Glycerol	25.98	20.56
Control	29.31	17.10

Effect of addition of nitrogen sources on inulinase production

Studies were performed to test the efficacy of different organic and inorganic nitrogen sources on enzyme synthesis by *Staphylococcus* sp. when added in the fermenting medium. Fermentation was carried out at 30°C with initial moisture of substrate as 60% for 48 h. Samples were withdrawn at 24 h. The results are recorded in Fig. 4. Six nitrogenous sources of organic and

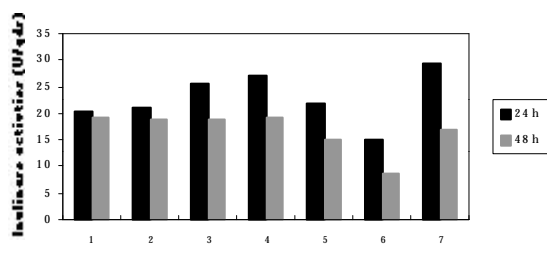


Fig. 4. Effect of nitrogen source on inulinase synthesis in SSF

inorganic nature, viz. NaNO_3 (1), NH_4Cl (2), $(\text{NH}_4)_2\text{SO}_4$ (3), urea (4), beef extract (5), and yeast extract (6) at 0.5 % (w/w) concentration were added individually in the medium. As is evident from the Fig. 4, none of the tested compound proved useful for bacterial activity of inulinase synthesis as in all cases the enzyme titres was less than the control experiment (7). Among all the compounds, while urea proved to be the least

harmful, yeast extract exerted most harmful impact with the result that inulinase activities were reduced to almost 50% of the control experiment.

DISCUSSION

Solid state fermentation is being increasingly used in microbial processes, and microbial enzyme production is an area where SSF has particularly established its credential (Pandey *et al.* 1998b). There are a number of process parameters, which need to be optimized for any individual strain for the production of a given enzyme. Among these, cultivation period, moisture of the substrate, and temperature of incubation are the critical factors. In the present studies, a period of 24 h was found to be optimum for enzyme synthesis by the bacterial culture; cultivation beyond this period resulted loss of the product. Inulinase is a primary metabolite, which is secreted by the micro-organism during its growth phase.

Moisture has been termed as one of the most important aspect, which controls microbial growth and activity in SSF (Pandey, 1992b). For this reason, generally moulds are the preferred choice in SSF. But in the present studies, we successfully cultivated a bacterial strain of *Staphylococcus* sp. RRL8, which produced maximum enzyme with a substrate having 60% initial moisture. Low substrate moistures in SSF resulted in suboptimal product formation due to the reduced mass transfer process such as diffusion of solutes and gas to cell during the fermentation. The decrease in moisture level is advantageous since the chances of contamination of fermentation medium are reduced.

Temperature is yet another important aspect, which affects SSF processes largely. It was reported that during microbial cultivation in SSF, temperature of fermenting bed risen which exerted harmful impact on the microbial strain (Pandey, 1990). In the present studies, 30°C temperature proved to be the best for inulinase synthesis by microbial culture. Higher temperatures affected bacterial activity harmfully. During SSF, a large amount of heat is generated which is directly proportional to the metabolic activities of the micro-organisms (Mudgett, 1986). In the early phases of SSF, the temperature and concentration of oxygen remain uniform throughout the substrate bed but as the fermentation progresses, oxygen transfer takes place resulting the generation of heat (Pandey, 1994).

For the optimum growth of micro-organisms, apart from above physical parameters, a number of other factors (chemical and biochemical parameters) and some times, inducers are required to be added in the formulation of growth and/or fermentation medium. With this view, we added a number of carbon and nitrogen sources in the fermentation medium to see their impact. As is apparent from the Table 2 and Fig. 4, the added compounds largely were unfavourable for the growth and activity of the bacterial culture. All the carbon sources, except sucrose, retarded enzyme synthesis, which indicated that the strain was probably repressive. All the nitrogenous compounds, irrespective of their nature, organic or inorganic, also retarded enzyme synthesis which indicated that with the addition of corn steep liquor, a reasonably good C/N ratio was established and no further nitrogen was needed in the medium. When additional nitrogen was supplemented, it resulted imbalanced C/N ratio, which interfered with the bacterial activity of inulinase synthesis.

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RESUMO

Estudos foram realizados com o objetivo de otimizar as condições de cultura para a produção de β -D-fructan-fructanohidrolase (inulinase) com uma cepa selvagem de *Estafilococos* sp. RRL8. Os experimentos foram realizados em meio sólido utilizando raiz de chicória moída como única fonte de carbono e suplementada com licor de milho e KH_2PO_4 p. Um certo número de parâmetros, tais como tempo de cultivo, umidade inicial e temperatura de incubação foram otimizadas. A produção máxima de enzima extracelular foi obtida quando a fermentação foi realizada à temperatura de 30°C por um período de 24 horas e um teor umidade de 60%. A suplementação do substrato com uma fonte de carbono adicional (exceto com sacarose) resultou no decréscimo da produção de enzima, o que indica que a cepa foi parcialmente depressiva. A adição de uma fonte de nitrogênio externa também não resultou em maior produção da enzima, ao contrário um impacto nocivo na cultura de *Estafilococos* sp. RRL8.

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