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Solid state fermentation for the synthesis of inulinase from *Staphylococcus* sp. and *Kluyveromyces marxianus*

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Abstract

Solid state fermentation was carried out for the production of inulinase from *Staphylococcus* sp. RRL-1 and *Kluyveromyces marxianus* ATCC 52466. Wheat bran, rice bran, coconut oil cake and corn flour, individually or in combinations were tested for their efficiency to be used as the solid substrate. Both cultures grew well in a wheat bran medium. Although the yeast culture produced relatively higher enzyme yields, the bacterial culture took a relatively shorter time to attain maximal yield. Under optimized conditions, the extra-cellular enzyme concentration reached a peak in 48 h with *Staphylococcus* sp. (107.64 U of inulinase per gram dry fermented substrate-gds) and in 72 h with *K. marxianus* (122.88 U/gds). This is the first report on inulinase production in SSF.

Keywords: Solid state fermentation; Inulinase; *Staphylococcus* sp.; *K. marxianus*

1. Introduction

Solid state fermentation (SSF) is distinguished from submerged cultures by the fact that microbial growth and product formation occurs at or near the surfaces of solid materials with low moisture contents. Substrates traditionally fermented in the solid state include a variety of agricultural products, including rice, wheat, millet, barely, corn and soybeans [1–4]. In this process, the solid substrate not only supplies the nutrients to the culture but also serves as an anchorage for the microbial cells. Cost and availability are important considerations and therefore the selection of an appropriate solid substrate plays an important role in the development of efficient SSF processes.

Inulin, a polyfructan, occurs as a reserve carbohydrate in the roots and tubers of a number of plants including Jerusalem artichoke, Chicory and Dahlia. It consists of linear β-2, 1-linked polyfructose chain, terminated by a glucose residue attached through a sucrose type linkage [5]. Such inulin sources have recently received attention as potential feed stocks for fuel ethanol [6,7] and fructose syrup production [8,9]. Hydrolysis of inulin requires inulinase (2, 1-β-D-fructan fructanohydrolase, EC-3.2.1.7) and several inulinases have been described in the literature as originating from micro-organisms. Microbial inulinases have been described in fungi, e.g. *Penicillium* sp. TN88 [10], *Aspergillus niger* 817 [11], *A. ficuum* [12], yeast, e.g. *Kluyveromyces marxianus* CDBB-L-278 [13], *Candida kefyr* [14] and bacteria, e.g. *Bacillus circulans* [15], *Clostridium acetobutylicum* ABKn8 [16], *Pseudomonas* sp. [17]. However, there are few reports on bacterial inulinases.

In the present study, we optimized some of the critical factors affecting inulinase production by a new bacterial isolate (*Staphylococcus* sp. RRL1) and a yeast strain, (*Kluyveromyces marxians* ATCC 52466) in solid state fermentation. This is the first report on inulinase production in SSF.

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2. Materials and method

2.1. Micro-organism

A new strain of *Staphylococcus* sp. [18] designated as RRL1, and a strain of *Kluyveromyces marxianus* (ATCC 52466) were used in this study. Both cultures were maintained on Yeast extract–Peptone–Inulin agar, subcultured fortnightly and stored at 4°C for short-term preservation.

2.2. Inoculum preparation

Inocula were prepared in a medium containing (g/l) yeast extract 10, Bacto peptone 20 and inulin 10. A loopful of cells from the slant were transferred into 250 ml conical flasks containing 20 ml culture media and incubated for 24 h on a rotary shaker operating at 150 rpm at 30°C for the yeast culture and 37°C for the bacterial culture.

Table 1
Inulinase synthesis by *Staphylococcus* sp. and *K. marianus* on different solid substrates

Solid substrate	Inulinase activity (U/gds) *Staphylococcus* sp.	Inulinase activity (U/gds) *K. marianus*
Wheat bran (coarse)	82.12	106.72
Wheat bran (fine)	96.77	97.74
Rice bran	62.95	88.14
Copra waste	67.16	83.18
Corn flour	16.11	21.23
Wheat bran (coarse)+corn flour, 9:1, w/w	85.04	101.41
Wheat bran (fine)+corn flour, 9:1, w/w	87.95	98.14
Wheat bran (coarse+fine), 1:1, w/w	77.15	93.70
Wheat bran (coarse+fine)+corn four, 4.5:4.5:1, w/w	85.00	94.32
Rice bran+corn flour, 9:1, w/w	62.61	88.17
Copra waste+corn flour, 9:1, w/w	69.99	81.23

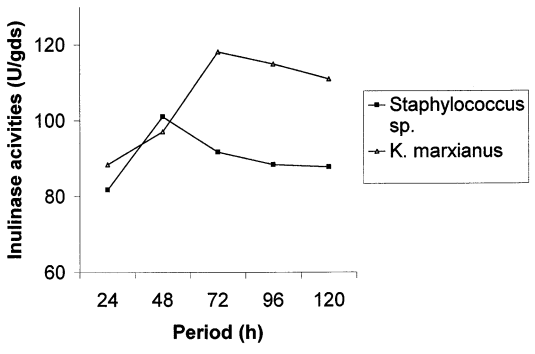

Fig. 1. Time course of cultivation for inulinase synthesis of *Staphylococcus* sp. (37°C) and *K. marxianus* (30°C) (60% moisture, 120 h).

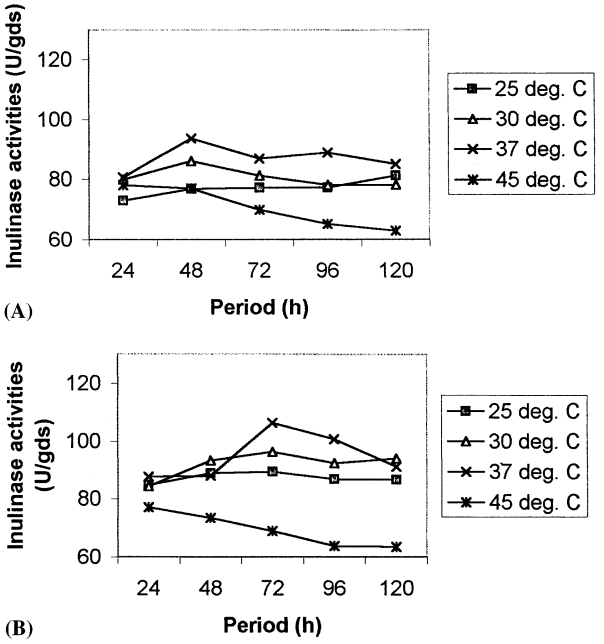

Fig. 2. (A) Effect of incubation at different temperature on inulinase synthesis by *Staphylococcus* sp. (60% moisture, 120 h); (B) Effect of incubation at different temperature on inulinase synthesis by *K. marxianus* (60% moisture, 120 h).

2.3. Solid state fermentation

Commercial quality wheat bran, rice bran, coconut oil cake (copra waste) and corn flour were procured from the local market and used as the solid substrate. The substrates were supplemented with an acidified mineral solution containing 3.5 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$, 2.8 mg $MnSO_4.7H_2O$, 8.7 mg $FeSO_4.7H_2O$, 2.5 mg $ZnSO_4.7H_2O$ and 3.5 mg $CaCl_2$ for 100 g dry substrate. Initial pH and moisture of the substrates were set at 5.5–6.0 and 60%, respectively. All substrates were autoclaved at 15 psi for 20 min.

Fermentation was carried out with 25-g autoclaved substrate in 250-ml conical flasks. Each flask was inoculated with 4% (v/w) cell suspension and was incubated at 30 or 37°C for stipulated period for the yeast and the bacterial culture, respectively. The samples, as whole flasks in duplicate, were withdrawn after each 24 h or as mentioned elsewhere. The results reported are the average of three sets of the experiments.

2.4. Extraction of enzyme

A weighed quantity 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

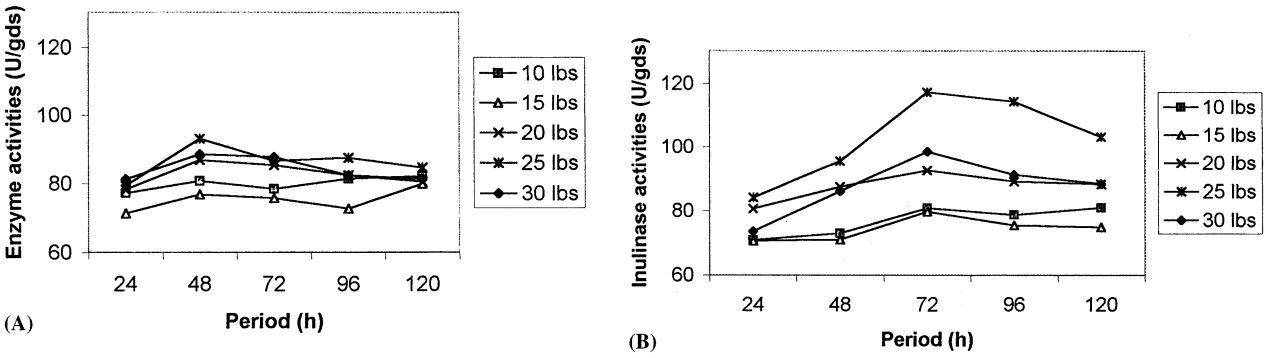

Fig. 3. (A) Effect of steam cooking of the substrate at different pressure on inulinase synthesis by *Staphylococcus* sp. (60% moisture, 37°C, 120 h); (B) Effect of steam cooking of the substrate at different pressure on inulinase synthesis by *K. marxianus* (60% moisture, 37°C, 120 h).

again treated with another 50 ml of distilled water, mixed in the same way and filtered. Both filtrates were combined.

2.5. Enzyme assay

Inulinase assay was carried out by the following procedure. To 2-ml 0.2% inulin, 2-ml acetate buffer (pH 4.6) and 0.5 ml enzyme (supernatant culture, diluted, if necessary) were added and incubated at 50°C for 20 min. After incubation, the tubes were held 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 [19] by reading the absorbance at 575 nm on a spectrophotometer (Shimadzu 160A, Japan). A calibration curve was prepared with fructose solutions of known strength and blanks were run simultaneously with enzyme and substrate solutions. One unit of inulinase activity was defined as the amount of enzyme, which produced 1 μmol of fructose under the assay conditions.

2.6. Moisture analysis

Moisture contents of the solid substrate were determined by drying at 80°C for 24 h.

3. Results and discussion

3.1. Selection of the substrate

In SSF, the selection of a suitable solid substrate for a fermentation process is a critical factor and thus involves the screening of a number of agro-industrial materials for microbial growth and product formation. In the present studies, four substrates, *viz*. Wheat bran (fine and coarse variety), rice bran, copra waste and corn flour were used to prepare eleven sets of substrates (Table 1) for growth and inulinase production by the *Staphylococcus* sp. and *K. marxianus* in 96 h. All the substrates supported growth and enzyme formation by both the cultures, although wheat bran media proved superior to rice bran, copra waste or corn flour. While the highest extra-cellular enzyme activity for the bacterial strain (96.77 U/gds) was obtained in a medium containing wheat bran (fine) alone as the substrate, for the yeast culture, the medium with coarse quality wheat bran proved the best, giving 106.72 U enzyme/gds. The order of substrate suitability was wheat bran > copra waste > rice bran > corn flour.

Recently, Pandey et al. [20] reviewed industrial enzyme production in SSF and concluded that although

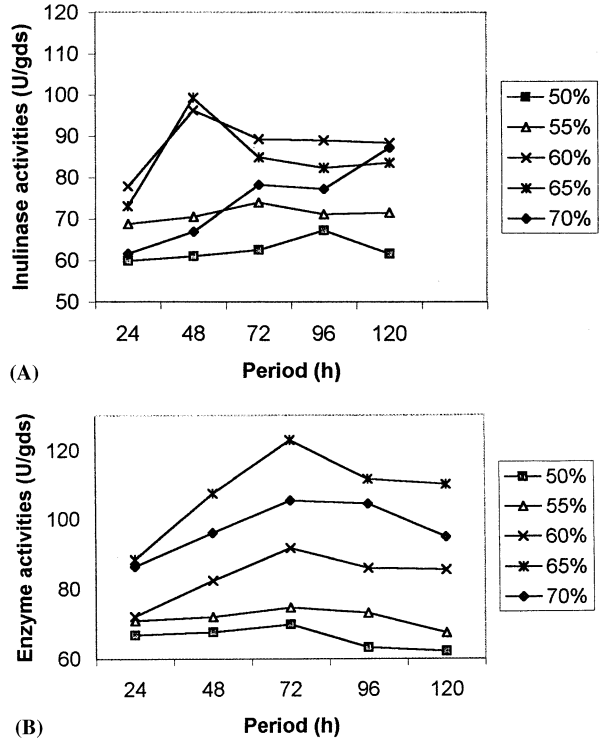

Fig. 4. (A) Effect of initial moisture of the substrate on inulinase synthesis by *Staphylococcus* sp. (37°C, 120 h); (B) Effect of initial moisture of the substrate on inulinase synthesis by *K. marxianus* (37°C, 120 h).

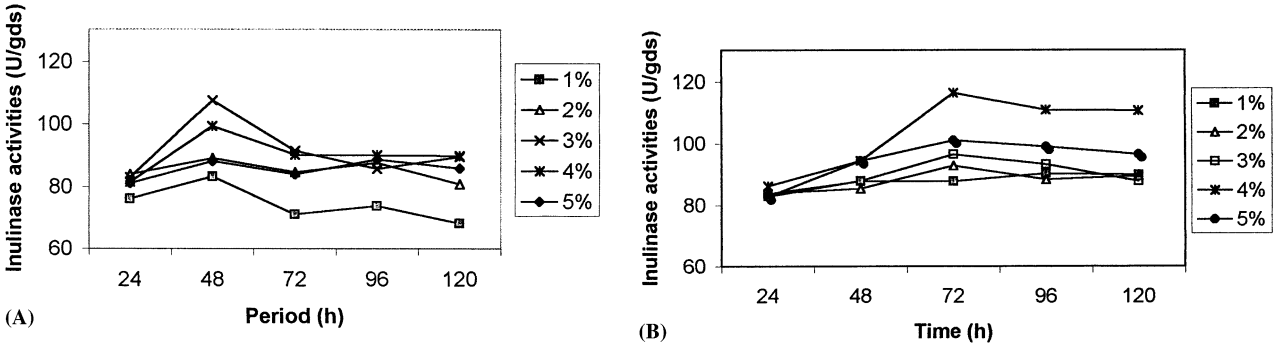

Fig. 5. (A) Effect of the size of inoculum on inulinase synthesis by *Staphylococcus* sp. (65% moisture, 37°C, 120 h); (B) Effect of the size of inoculum on inulinase synthesis by *K. marxianus* (65% moisture, 37°C, 120 h).

there are a number of agro-industrial residues used as substrates for enzyme production in SSF, wheat bran was the most significant. The present study is also a pointer to this conclusion. In subsequent experiments, therefore, wheat bran of fine and coarse variety was used as the substrate for the bacterial and yeast strains, respectively.

3.2. Time course of fermentation

Fig. 1 shows the time course of inulinase synthesis by the strains of *Staphylococcus* sp. and *K. marxianus*. Fermentation was carried out for 120 h. Accumulation of maximal extra-cellular inulinase (101.45 U/gds) was observed after 48-h growth of *Staphylococcus* sp. *K. marxianus* took a longer time for maximum enzyme yield but the yield was about 14% higher than that of the bacterial culture (118.14 U/gds in 72 h). Cultivation for longer period showed a decrease in enzyme titres.

3.3. Effect of incubation temperature

Four different incubation temperatures, viz. 25, 30, 37 and 45°C were used to cultivate the cultures for inulinase synthesis. Fermentation was carried out for 120 h. Both cultures showed best inulinase synthesis when incubated at 37°C. In contrast to this, when the yeast strain was cultivated in liquid culture (SmF), it showed optimum activity at 30°C (Selvakumar and Pandey, Unpublished results). Maximal inulinase titres in the present study were 90.53 U/gds in 48 h for *Staphylococcus* sp. and 106.37 U/gds for *K. marxianus* in 72 h (Fig. 2(A–B).

3.4. Effect of steam cooking pressure of solid substrate

In order to study if heat treatment (steam cooking) made the solid substrate more accessible for microbial growth and activity by bringing out desirable changes in its physical structure and chemical properties, the substrate was steam cooked under different pressures for 20 min. Five different cooking pressures, viz. 10, 15, 20, 25, 30 psi (which correspond to 116, 121, 127, 131 and 134°C, respectively) were chosen for cooking of the solid substrate. Fermentation was carried out at 37°C for 120 h and results obtained are shown in Fig. 3(A–B). Substrate cooking at 25 psi proved to be best for both the cultures. Substrate cooking at higher pressures (i.e. 30 psi) had no useful impact on enzyme production.

3.5. Effect of initial moisture content

Five different initial moisture levels, viz. 50, 55, 60, 65, and 70% were established in the substrates and the fermentation was carried out for 120 h. Results obtained are shown in Fig. 4(A) for the bacterial culture and in Fig. 4(B) for the yeast culture. Significantly, extra-cellular inulinase synthesis reached at a peak in those substrates with 65% initial moisture for both cultures. Substrate moisture is a critical factor in SSF and its importance for enzyme production has been well established [3,21]. It is reported that low substrate moistures in SSF resulted in suboptimal product formation due to reduced mass transfer process such as diffusion of solutes and gas to cell during fermentation. The decrease in moisture level is advantageous since the chance of contamination of fermentation medium is reduced [22].

3.6. Effect of inoculum density

Fig. 5(A–B) shows the effect of inoculum density on inulinase production by *Staphylococcus* sp. and *K. marxianus*, respectively. Five different inoculum densities (1, 2, 3, 4 and 5%, 24 h old culture) were tested. The results showed that extra-cellular inulinase synthesis reached its maximum value of 107.64 U/gds with 3% inoculum at 48-h growth from the bacterial strain whereas in yeast, it was 116.43 U/gds with 4% inoculum at 72-h growth. It is important to provide an optimal inoculum size in fermentation process as a lower inoculum density may give insufficient biomass and permit the growth of undesirable organisms whereas a higher

inoculum density may produce too much biomass and deplete the substrate of nutrients necessary for product formation [1].

In conclusion, although the yeast strain, *K. marxianus*, showed superiority over the bacterial strain, *Staphylococcus* sp., for the production of extra-cellular inulinase, the study has shown the feasibility of SSF for the first time for inulinase production utilizing both bacterial and yeast cultures. It would be of interest to explore further both of these microbial strains in SSF for enzyme production.

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