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Microbial production of levanase for specific hydrolysis of levan

Imen Dahech[a,*], Hanen Ben Ayed[a], Karima Srih Belghith[a], Hafedh Belghith[b], Hafedh Mejdoub[a]

[a] Biochemistry Laboratory, Faculty of Sciences of Sfax, PB 802, 3018 Sfax, Tunisia
[b] Biotechnology Center of Sfax BP "1177" 3018-Sfax, Tunisia, University of Sfax, Tunisia

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ABSTRACT

A newly isolated bacterial strain from Tunisian thermal source was selected for its ability to produce extracellular levanase when grown on levan substrate. The optimization of carbon source, nitrogen source, temperature and initial pH of the growth medium in submerged liquid cultures were investigated. In fact, levan was found to be a good inducer of levanase enzymes. The optimal temperature and pH of the levanase activity were 40 °C and 6.4, respectively. This enzyme exhibited a remarkable stability and retained 75% of its original activity at 55 °C for more than 1 h at pH 6.4. Crude enzyme of the strain rich in levanase was established for the hydrolysis of levan in order to produce fructooligosaccharides with variable degrees of polymerization which could be used in important fields such medicine, food-processing industry and cosmetic. The extracellular levanase of the strain was then, partially purified as determined by SDS-PAGE. The purification was achieved by ammonium sulfate precipitation, gel filtration and DEAE cellulose chromatographies.

1. Introduction

Levan, known as a promising material for the production of fructooligosaccharides, is composed of a β-2,6-fructan main chain and β-2,1-linked side chains and functions as a reserve carbohydrate of monocotyledons [1]. It is also produced by several kinds of bacteria during their assimilation of sucrose by the action of levansucrase (EC 2.4.1.10) [2]. Bacterial levans can be used as a raw material for the production of levanoligosaccharides by using a levan-degrading enzyme (levanase, 2,6-β-D fructan fructohydrolase, EC 3.2.1.65) [3]. Several studies on the production of levanoligosaccharides by microbial levanases have been carried out; however their reaction products are heterogeneous with various degrees of polymerization.

Oligosaccharides are considered to have several important functions in nutrition (energy source), material properties (protection from aging, lowering of water activity), and physiology (plant differentiation, growth promoting activities for some enteric bacteria, and stimulating effects on the immune system). They are selectively hydrolyzed by *Bifidobacterium* which is predominant in the intestinal tract of animals including human and provide a benefit to its host by inhibiting proliferation of harmful bacteria [4]. They are also potential noncaloric sweeteners. An attempt to synthesize a large number of novel oligosaccharides has been made by using sugar hydrolases or sugar transferases.

There have been several reports about levanases which hydrolyze the β-2,6-linked main chain of levan to fructose or various levanoligosaccharides [5]. Such levanase activities have been found in yeasts [6], in filamentous fungi [7], and also in some bacteria, among them *Streptococcus mutans* [8], *Clostridium acetobutylicum* [9] and Bacillus *subtilis* [10].

Enzymes involved in the hydrolysis of polyfructans are of interest both for fundamental studies and for industrial applications.

As a part of our studies for the development of useful oligosaccharides from such unused natural fructans as levan, we tried to isolate new strain which could digest levan into levanoligosaccharides of limited length by the microbial polysaccharide-degrading enzymes. Then, the optimization of some parameters of production of this extracellular enzyme was carried and the enzyme was partially purified.

2. Materials and methods

2.1. Microorganism

Several samples were collected from Tunisian thermal source and different thermophilic bacteria were screened for their levanase activity.

* Corresponding author at: Biochemistry Laboratory, Department of Life Science, Faculty of Science, University of Sfax, PB 802, 3018 Sfax, Tunisia. Tel.: +216 98 656 228; fax: +216 74 274 437.
E-mail address: imenbiologie@yahoo.fr (I. Dahech).

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2.2. Medium and growth conditions

The medium for growth and enzyme production contained K_2HPO_4 (1%), $MgSO_4$ (0.05%), $MnCl_2$, levan (0.2%), yeast extract (0.3%); pH 6.4 distilled water to 1l. The incubation was carried out in a rotary shaker (200 rpm) at 30 °C for 36 h. The inoculum was grown in the same medium at 40 °C in an incubator shaker (200 rpm) for 24 h. The media were first autoclaved at 121 °C for 20 min and then cooled in test tubes before the inoculation.

2.3. Fermentation conditions

Crude levanase was obtained by harvesting the culture medium after 36 h. A step-by-step optimization procedure regarding the effect of important parameters (carbon, nitrogen source and initial growth pH and temperature) on enzyme production was employed. Levan, maltose and sucrose, at 2% (w/v) each, were the carbon sources investigated. Under these conditions, initial nitrogen source (yeast extract 3.0 g/l) and an initial pH medium (pH 6.4) were remained fixed. Different concentrations of levan were then used. The pH was adjusted to 6.4 and the medium was incubated for 36 h in a rotary shaker (200 rpm) at 30 °C.

Yeast extract, sodium nitrate, trypton, Urea, ammonium sulfate and combinations were the nitrogen sources examined at different concentrations. The based medium used in each experiment consisted of the enzyme production medium with one of the nitrogen sources cited above.

2.4. Substrate

Levan was synthesized from sucrose by levansucrase from *Bacillus licheniformis* isolated in our laboratory [11].

2.5. Analytical methods

2.5.1. Enzyme extraction

After suitable time intervals, aliquots were withdrawn and centrifuged at 10.000 rpm for 10 min to remove cells and the clear supernatant obtained was used for all subsequent analyses.

2.5.2. Bacterial growth

The cells centrifuged from 5 ml culture broth were washed twice with distilled water and the cellular was determined by measuring optical density at 600 nm.

2.5.3. Enzyme assay

The enzyme activity was assayed under the standard conditions [12] with modifications as follows. The reaction mixture contained 20 mM phosphate buffer (pH 6.5) and 250 μl of levan 2% and the enzyme solution was diluted appropriately in a total volume of 1 ml. The mixture was incubated at 40 °C for 20 min and then heated in boiling water for 5 min to stop the enzyme reaction. Next to check the presence of the levanase activity, sugars in the reaction mixture were analyzed qualitatively by TLC. After that, for quantitative determination of enzyme activity, the reducing sugars in the reaction mixtures were assayed by the DNS method [13]. One unit of enzyme activity was defined as the amount of the enzyme that produced 1 mmol of reducing sugars as fructose per min under these assay conditions. The protein concentration was determined by the methods of Lowry et al. [14].

2.5.4. Thin-layer chromatography (TLC)

The sugars in the reaction mixtures were analyzed by thin-layer chromatography (TLC) on silica gel G-60 using chloroform/acetic acid/water (6:7:1 by volume) as a mobile phase system. After layer development and mobile phase evaporation under continuous warm air flow for 15 min, the spots on the chromatograms were visualized by spraying a mixture of sulfuric acid and ethanol (10:90) on the plate.

2.5.5. Optimum pH

The optimum pH for levanase production was studied over a pH range of 5.0–11 at 30 °C. The following buffer systems were used at 100 mM: sodium acetate (pH 5); phosphate (pH 6.0–7.5); Tris (pH 8.0–8.5); and glycine (pH 9.0–11). Inoculation was performed with a 10% (v/v) inoculum of each subculture and the enzyme production medium was incubated at 200 rpm for 36 h.

2.5.6. Optimum temperature

In order to determine the effect of incubation temperature on levanase production, the enzyme production medium was inoculated at 10% (v/v) (in Erlenmeyer flask of 250 ml), incubated at various temperatures (25, 30, 40, 55, 60 and 70 °C), pH 6.4 and at 200 rpm for 36 h. To measure the thermostability, 1.0 ml of crude enzyme was kept at various temperatures (30, 40, 50, 55, 60 and 70 °C) and pH 6.4 for different incubation time (up to 1 h). The enzyme thermostability was determined by measuring the residual activity after incubation time of the enzyme at 40 °C.

2.5.7. Reaction products from levan by the levanase

The degradation reaction was performed under the same conditions as those of the standard levanase assay conditions, except that the incubation time of up to 24 h was employed. At various time points, the reaction mixtures were heated in boiling water for 5 min to stop the enzyme reaction, and the sugars were analyzed by TLC.

2.6. Enzyme purification

2.6.1. Production step

One liter of culture was prepared, inoculated and shaken for overnight at 30 °C for 36 h. Then, the culture was centrifuged at 10,000 rpm for 20 min in order to remove cells. The resultant cell free supernatant from the culture was used as the starting material for enzyme purification.

2.6.2. Ammonium sulfate precipitation

100 ml of the extracellular enzyme was subjected to ammonium sulfate precipitation according to standard methods. Levanase enzyme precipitates between 35 and 80%. Both pellet and supernatant were assayed for levanase activity. Pellets from 35 to 80% precipitation were with high enzyme activity. The pellet was suspended in the minimum volume of phosphate buffer (20 mM, pH 6.4), and centrifuged again. The supernatant was dialyzed against 3 changes of phosphate buffer (20 mM, pH 6.4) at 4 °C for overnight to remove the ammonium salt. Dialysis was carried out using a cellulose dialysis membrane (220 mm in diameter) with a cut off point of 10 kDa.

2.6.3. Gel filtration chromatography

The lyophilized sample was dissolved in 1.0 ml of phosphate buffer (pH 6.5) and then loaded on a Sephacryl S200 column (2.5 cm × 150 cm) previously equilibrated with 20 mM phosphate buffer (pH 6.4). Proteins were eluted with the same buffer at a flow rate of 30 ml/h and fractions were collected as earlier described. The levanase activity and protein content of each fraction were determined. The active fractions were pooled, lyophilized, and stored at 4 °C until further use.