Production and partial purification of invertase using *Cympopogan caecius* leaf powder as substrate

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Abstract The present study investigates the efficiency of *Aspergillus niger* to produce invertase, an industrially important enzyme by using powdered stem of *Cympopogan caecius* (Lemon grass) as sole substrate and sole carbon source for the microorganism. The molecular weight of invertase was estimated to be 66–70 kDa by sodium do decyl sulphate poly acrylamide gel electrophoresis (SDS PAGE). The production of the enzyme was studied at different pH scales ranging from pH 4.0 to 7.0 at a constant temperature of 30°C and 2% substrate concentration. The maximum production of invertase (specific activity –0.0516 $\mu\text{k}/\text{mg}$ protein) was obtained at pH 5.5 at 30°C temperature, and incubation for 48 h. The activity was found to be stable at pH 5.5 for 30 min. The enzyme was found to be stable in the temperature range of 20–55°C. The effect of divalent metal ions Cu^{2+} , Fe^{2+} , Co^{2+} on the activity of the enzyme invertase showed that these ions affected the activity by a certain factor. The study can be further industrially exploited in a country-like India where lemon grass is found in plenty and can be used as substrate for enzyme production. Moreover, the preparation of the substrate is also a simple process.

Keywords *Aspergillus niger* · *Cympopogan caecius* · Invertase · Specific activity · Enzyme characterization

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Introduction

Enzyme production is a rapidly growing component of biotechnology industry. Annual sales figures of this industry have touched the billion-dollar mark, with an increasing number of patents and research articles in this field. Fungal enzymes, in specific, have been a part of the dietary supplement industry for several decades. In this field these enzymes are primarily used for two applications – digestive and systemic. A wide range of microorganisms produces invertase and thus can utilize sucrose as a nutrient [1]. Commercially, invertase is biosynthesized by yeast strains, *Aureobasidium* spp, *Rhodotorula glutinis*, *Saccharomyces cerevisiae*, *Saccharomyces carlsbergensis*. *Aspergillus niger* is also thought to produce invertase [2]. The sugar industry is comparatively a minor user of enzymes but provides a few historically significant and instructive examples of enzyme technology [3]. The hydrolysis (inversion) of sucrose, completely or partially into glucose and fructose provides sweet syrups that are more stable than pure sucrose syrups. The most familiar “Golden Syrup” is produced by the acid hydrolysis of one of the less pure streams from the cane sugar refinery, but other types of syrups are produced by using yeast invertase.

Invertase, including acid invertase is an important industrial enzyme with applications in the production of non-crystallizable invert sugars and soft-centered chocolates. At sucrose concentrations lower than 10% (w/v); invertase hydrolyses sucrose to glucose and fructose. The age-old method of acid inversions was the main tool for inverting sugars and few actual users adopted this method for their requirement. This method is crude, leading to non-uniform product formation, in addition to the formation of hydroxyl

methyl furfural (HMF) and furfural, a highly neurotoxic product [4].

Traditionally, autolysing yeast cells on-site produced invertase. The autolysate was added to the syrup (70% w/v sucrose) to be inverted together with small amounts of xylene to prevent microbial growth and allowed to react at 50°C, pH 4.5 for 48–72 h followed by the removal of the enzyme and xylene. Lately, commercially produced invertase concentrates are being employed for the inversion process in all sugar industries. Invertase also finds application in the formulation of soft centered confectioneries, lactic acid production, fermentation of cane sugar molasses; calf feed production, ethanol production and the manufacture of invert sugar as a major feed for honey bees in apiculture farms [5].

The fungi *A. niger* produces a large array of extracellular enzymes and has been exploited industrially in the production of various enzymes. There are several reports that highlight the effective substrate usage of *Aspergillus* species. *A. niger*, *A. flavus*, *A. fumigatus*, *A. oryzae* and *A. parasiticus* show faster growth and intense invertase activity than yeast species [6]. A wide array of enzymes have been characterized from *A. niger* and are important in the biotechnology industry and genomes of three different strains of *A. niger* have been completely sequenced, thus could be exploited for further studies [7]. It is also an important model organism for important research areas like the eukaryotic protein secretion, effects of suppression or triggering of biomass degrading enzymes, and also, molecular mechanisms for the development of fermentation. This organism, in particular could be thus important in fermentation process development and media standardization [8]. *A. niger* belongs to non-pathogenic, ‘generally recognized as safe’ (GRAS) microbial organisms and is used for the production of enzymes (AMFEP, <http://www.amfep.org/list.html>). Enzymes from *A. niger* are made to over express for the purpose of food production [9]. Invertase from *A. niger* has been studied in detail and thus seems to be an appropriate choice for the study.

The aim of this paper is to report on the possibility of effective utilization of *Cynopogon caecius* as the sole substrate for *A. niger* for the production of invertase and to partially purify the enzyme. The study also assesses the thermo stability of the enzyme invertase and the effect of divalent metal ions (Cu^{2+} , Fe^{2+} , Co^{2+}) on the hydrolysis of sucrose. The findings can be further refined and applied industrially, such that *C. caecius* substrate could be used commercially to extract invertase that has a high industrial demand.

Materials and methods

Maintenance and preservation of the strain

The *A. niger* strain used for the present study was obtained from the Department of Biotechnology, Bharathiar University, Coimbatore. The culture was inoculated in potato dextrose agar and inoculated at 27°C for growth. A few spores were aseptically transferred to a cryo-vial containing glycerol to a final concentration of 30% and then stored at –20°C.

Preparation of the substrate

The substrate used for the production studies was the dried and powdered stem of lemon grass, *C. caecius*. The grass was collected from Kalapatti area in Coimbatore district. The leaves were removed and the stem was thoroughly dried and subjected to further blending so that it formed a fine powder. To 50 g of this powdered substrate, 1:8 parts of concentrated sulfuric acid:water is was added and allowed to stand for 7–12 h for digestion of lignin; followed by neutralization with 1N sodium hydroxide for 12 h at room temperature. It was then washed several times with distilled water until the pH became neutral. The wet substrate was then dried at 65–75°C and stored for further use.

Enzyme production and assay

The media for production of invertase was reconstituted in a series of flasks by taking 3% (w/v) dried powdered substrate supplemented with 0.5% peptone and the pH was adjusted to 4.0, 4.5, 5.0, 5.5, 6.0, 6.5 and 7.0, respectively with 0.1N hydrochloric acid and sterilized at 121°C, 15 lbs/inch² pressure for 15 min. A seed culture of *A. niger* was initiated and a 2% inoculum with 3.6×10^6 cells/ml was inoculated to all the flasks containing the substrate of different pH ranges and incubated at 30°C for 40–42 h. After incubation, the contents were filtered through a Whatman filter paper No. 1 and taken for assay. The reaction mixture was 5 ml of acetate buffer (0.4 M sucrose in 0.2 M acetic acid - acetate buffer) and 3 ml of the filtrate (enzyme solution). The enzyme activity was determined at 20°C, measuring the reducing sugars by Nelson-Somogyi method [10]. The total protein in the culture filtrate was accounted by Bradford’s method [11]. One microkat (μk) was defined as the amount of enzyme catalyzing the hydrolysis of 1 μM sucrose per second under the given conditions of reaction.

Enzyme purification

Invertase was purified from the crude culture filtrate, which was initially subjected to 40% precipitation with ammonium sulfate and pelleted at 10,000 rpm for 20 min at 4°C. The pellet obtained was dissolved in 1 ml of 0.2 M acetate buffer. The supernatant was further saturated to 80% concentration of ammonium sulfate and pelleted as before. The pellet was dissolved in 1 ml of the acetate buffer and the activity was estimated in the 40% and 80% fractions. The 80% fraction content that was precipitated showed higher activity and hence was applied to gel filtration column (Hi Gel 6B filtration) that was primarily equilibrated with acetate buffer. The fraction (1–20) was collected at different time intervals and the activity was estimated in all fractions. The fractions 8–12 had maximum activity and hence were pooled and dialyzed against distilled water overnight at 4°C. The molecular weight of the partially purified invertase was estimated by separating the sample on a 10% denaturing poly acrylamide gel electrophoresis (PAGE) with standard protein molecular weight marker (Qiagen). The gel was then subjected to silver staining [12].

Effect of temperature on enzyme activity

Invertase activity was examined at temperature scales of 20–55°C at pH 5.5. The enzyme preparation (1 ml) was added to the reaction mixture in acetate buffer and incubated at temperatures of 20–55°C and the activity of the enzyme was assayed as before by the Nelson-Somogyi method.

Effect of metal ions on enzyme activity

The divalent metal ions (Fe^{2+} , Co^{2+} and Cu^{2+}) were taken for the present study and were added to the reaction mixture and as chlorides at 10 mM concentrations at pH 5.5. The activity of the enzyme was assessed as before.

Results and discussion

Enzyme production

The production of enzyme invertase using the substrate was strongly dependent on the carbohydrate content of the substrate. The total carbohydrate content was found to be 1 mg/ml. The production of the enzyme invertase was studied at different pH scales (4.0–7.0) at a constant temperature (30°C) and an incubation period of 24 h. It was found that the production was maximal at a pH 5.5 (Fig. 1) and 30°C temperature (Table 1). Any change of pH beyond the optima shows drastic reduction in the activity of invertase, and it possible that the active site of the enzyme might get altered because of slight denaturation of the enzyme at pH above 5.5. Every enzyme has an optimum pH at which the rate of the reaction that it catalyzes will be maximized [13]. Slight deviation in the pH from the optimum leads to a decrease in the reaction rate. These might be due to changes in the ionization of the amino acid residues and disruption of the non-covalent interactions which maintain the 3D structure of the enzymes [14].

Table 1 Enzyme activity at different pH scales (30°C)

S. No.	pH	Total enzyme activity (μk)	Total protein (mg/ml)	Specific activity ($\mu\text{k}/\text{mg}$ protein)
1	4.0	0.0055	0.78	0.0070
2	4.5	0.0150	0.88	0.0170
3	5.0	0.0320	1.16	0.0275
4	5.5	0.0506	0.98	0.0516
5	6.0	0.0043	1.26	0.0034
6	6.5	0.0020	1.30	0.0015
7	7.0	0.0150	1.32	0.0011

Table 2 Purification scheme of invertase from *A. niger* at pH 5.5 and 30°C

Stage of purification	Total activity (μk)	Total protein (mg/ml)	Specific activity ($\mu\text{k}/\text{mg}$ protein)	Purification factor
Crude enzyme purification	0.0506	0.98	0.0516	1.00
40% ammonium sulfate precipitated	0.0409	0.78	0.0524	2.00
80% ammonium sulfate precipitated	0.0362	0.60	0.0603	3.00
Column eluted (Hi Gel 6B filtration)	0.0363	0.59	0.0615	3.00

Fig. 1 Effect of pH on the enzyme activity.

Fig. 2 Purification of invertase on Hi Gel 6B column. Figure showing the increase in the specific activity in different fractions collected at different time points.

The enzyme showed an activity of 0.0506 μk and a specific activity of 0.0516 $\mu\text{k}/\text{mg}$ protein. Production of the enzyme is highly dependent on the presence of available sucrose as a substrate. Concentration of sucrose above 10% is thought to repress the enzyme production [15]. The present study indicated that the enzyme production using lemon grass as substrate was a bit lower than expected. The possible reason could be the presence of lesser quantity of the inducible sugar in the substrate.

Partial purification of invertase

The purification scheme for invertase from *A. niger* is shown in Table 2. The specific activity of the final column (Hi Gel 6B filtration) eluted, fraction was found to be 0.0615 $\mu\text{k}/\text{mg}$ of protein, which represents a purification factor of 3.0 (Table 2 and Fig. 2). The molecular weight as determined by SDS PAGE that revealed a single band in the column purified preparation corresponding to a molecular weight of 66–70 kDa (Fig. 3). A similar result, for the molecular weight, was reported for the S-invertase produced by *Aspergillus nidulans* that produced a single large glycoprotein band of molecular weight 78 kDa [5].

Effect of temperature on the enzyme activity

The stability of invertase produced from *A. niger* using this cheaper substrate was determined from 20°C to 55°C. The invertase from *A. niger* was found to be stable till a temperature of 55°C without much loss in the activity (Table 3). This corresponds to a reduction in the activity by around 40% (Fig. 4). The stability also corresponds to the report where the invertase activity was optimal at 50°C and pH 5.5. Although, the stability of this from of invertase was much less when compared to the stability of the enzyme produced by *Rhodotorula glutinis*, which was stable at 60°C [4].

Fig. 3 SDS PAGE of partially purified invertase showing various stages of purification. Lane 7: Qiagen marker of range 30,000–200,000 DA, Lane 6 is the crude enzyme, Lane 5 corresponds to 40% ammonium sulfate precipitated fraction, Lane 4 is 60% ammonium sulfate fraction, Lane 3 shows bands for 80% ammonium sulfate fraction, Lane 2 has the dialysate and Lane 1 is the column eluted fraction with a single band on the SDS PAGE gel.

Effect of metal ions on the enzyme activity

Metal ions increased the activity of the enzyme by a factor of approximately 1.30–1.40 as compared to the control (Table 4). The effect of metal ions could be important in the use of raw materials with a high salt content as substrates for the production studies. It may be noted that, reports have shown that plant invertase activity was inhibited by copper ions and with other divalent ions like zinc, manganese and barium. However, addition of cobalt has been shown to reverse the inhibitions of the enzyme by chelators like ethylene diamine trichloro acetic acid (EDTA). It can be observed that the enzyme may be a metalloenzyme whose activity can be increased in the presence of specific metal ions like cobalt and ferrous [16].

The primary aim of this investigation was to decipher how effectively the substrate (*C. caecius* leaf powder) could be exploited for the production of an industrially

Table 3 Effect of temperature on enzyme activity

Temperature (°C)	Total activity (μk)	Total protein (mg/ml)	Specific activity ($\mu\text{k}/\text{mg}$ protein)	% stability
20	0.0363	0.59	0.0615	100
30	0.0363	0.58	0.0613	100
35	0.0342	0.62	0.0551	89.5
40	0.0332	0.63	0.0526	85.5
45	0.0332	0.63	0.0526	85.5
50	0.0316	0.69	0.0457	74.3
55	0.0310	0.80	0.0387	62.9

Fig. 4 Effect of temperature on enzyme stability.

Table 4 Effect of metal ions on enzyme activity

Metal ion	Total activity (μk)	Factor increase
Control	0.0615	—
Fe ²⁺	0.0826	1.34
Cu ²⁺	0.0865	1.40
Co ²⁺	0.0842	1.36

important enzyme like invertase by *A. niger*. The present study can thus be extended to the industrial scales. Being a cheaper substrate, it can be easily obtained and moreover, the processing of the substrate does not involve complex extractions. Thus the substrate can be widely used for the production of the enzyme; invertase and this substrate can also be used to produce other enzymes from the fungi.

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