Orange Peel as Novel Substrate for Enhanced Invertase Production by A. niger in Solid State Fermentation

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Effective invertase enzyme production was achieved with orange peel as carbon source compared to all other tested also residues. Among different nitrogen sources, yeast extract supported maximum enzyme production. Various fermentation parameters (pH of the medium, incubation temperature, time, volume in addition to carbon and nitrogen sources) also influenced the rate of invertase production. Maximum enzyme production of 55 units was observed in the medium of pH 4 containing 2% of orange peel having particle size of 3-1.5 mm containing 1% of sucrose and 1% yeast extract in 96 hours of incubation.

Keywords: Orange peel, Invertase, Solid state fermentation and Aspergillus niger.

Abstract

Invertase [β-fructofuranosidases (EC.3.2.1.26)] is an enzyme that catalyses the hydrolysis of sucrose (table sugar). The resulting mixture of fructose and glucose is called inverted sugar syrup. Invertase, cleave the O-C (fructose) bond. It is namely used in the food and beverage industry to produce candies, chocolates, lactic acid and glycerol, etc (Aehlew, 2004). Among microorganisms Saccharomyces cerevisae commonly called bakers yeast in the primary strain used for the production of invertase commercially. The common microorganism used for the study is Aspergillus niger and Candida utilis (Icrwin et al., 2001 and Schuster et al., 2002).

The objective of present study is to utilize the agro-industrial residue which is primarily composed of complex polysaccharides that strengthens microbial growth for the production of industrial important enzymes. The solid state fermentation process of enzyme production have potential advantages i.e. simplicity in operation high productivity, less favourable for contamination (Singhania et al., 2009).
Materials and Methods

The microorganism *Aspergillus niger* was isolated from the soil of sugarcane field of Bidar, (India) by serial dilution method. The cultures of these were obtained from the plate inoculated with diluted sample of $10^{-8}$. The fungal strain is propagated on potato-dextrose agar medium (PDA) at 30º C and maintained at 4º C.

Fermentation conditions / culture medium

The medium used for the production of enzyme under solid state fermentation has constituents (gm/l) of 25gm sucrose, 10gm yeast extract, 1gm ammonium sulphate, $[(NH_4)_2SO_4]$, 0.1gm calcium chloride ($CaCl_2.2H_2O$), and Potassium dihydrogen phosphate ($KH_2PO_4$). The pH of the medium adjusted to 5.

Processing of the substrate

The fruit peel waste (orange, pomegranate, sapota peel and pineapple) were collected from the market and juice centre washed, sliced and shade dried and grinded stored in polythene bag at room temperature. They were autoclaved at 15 lbs for 20 minutes before use (Uma et al., 2010).

SSF: Solid- state fermentation

The powdered substrate 40 gm (orange / pomegranate / sapota / pineapple) was taken in 250ml Erkenmeyer flask and moistened with culture medium/ solid state medium in the ratio of 2:1 (w/v). The substrate is mixed thoroughly and autoclaved for 20 minutes at 121ºC 15 lbs and cooled to room temperature. The sterilised medium was inoculated with $10^6$ spores/ml inoculums. After thorough mixing the contents flasks were incubated in a incubator at 35ºC for 36hrs intervals. All the sets were prepared in duplicate. At the end of fermentation 50 ml of distilled water was added to the fermentation substrate and kept on rotatory shaker at10000 rpm for 30 minutes and the supernatant used as crude enzyme for assay.

Enzyme assay

The estimation of reducing sugar was done by dinitrosalicylic acid (DNS) method. 0.1 ml enzyme solution was incubated with 0.9ml sucrose in 0.03M in acetic buffer (pH 5). To stop the reaction 1 ml of dinitrosalicylic acid (DNS) reagent was added and heated for 3 minutes in a boiling water bath. The solution was cooled to room temperature. Finally the absorbance was read at 540nm using spectrophotometer (Miller, 1959). One unit of invertase (1U) is defined as the amount of enzyme which liberates one mole of glucose/minute/ml under the assay conditions.

The optimization of the medium on the production of invertase was done by studying the effects of various factors like Inoculum size: 4ml inoculum size, Incubation time: 96 hours, Carbon sources: sucrose 1%, Nitrogen sources: Yeast extract 1%, pH: 5 and Temperature: 30ºC (4 days old culture of 4ml inoculum size was taken for the study of parameters).

Optimization study

The optimization of parameters like incubation time, incubation temperature, inoculums size, initial pH and the nutritional sources like different substrates, addition of carbon sources, nitrogen sources are known to influence the enzyme production. These parameters were optimized by the conventional methods of optimizing one independent parameter at a time while fixing other values (Miller 1959). The parameter optimized in one experiment was maintained in subsequent experiments (Shafiq et al., 2003 and 2004).
Results and Discussion

Effect of incubation period

To estimate the optimum incubation period for invertase enzyme production, fermentation flasks were incubated for different time duration from 1 to 6 days. After every 24 hours, the exhausts were evaluated for invertase activity. Maximal filters value of enzyme production were reached between 72 and 96 hours. Further increase in incubation period resulted in a decrease in invertase production (Fig 1; Tables 1 and 2). This might be due to reduction in the availability of nutrients in the medium and accumulation of toxic products of metabolism (Shafiq et al., 2003).

Effect of incubation temperature

Temperature plays an important role for the production of the invertase by A. niger. The effect of temperature on invertase production was studied by incubating the culture media (production media) at various temperatures such as 25, 30, 35, 40ºC. The strain has shown maximum enzyme production at a temperature of 30ºC (Fig 2 and Table 3) and the same results were observed by Shafiq et al., 2004. Hence it was found favorable for A. niger however, the enzyme activity was not significant because of denaturation of active sites of enzyme at higher temperatures.

Effect of inoculum volume

Different volume of inoculums such as 1, 2, 3, 4 and 5ml were tested for their ability to induce invertase production in the production medium. The maximum invertase activity was observed at the 4ml (45 IU/ml) of inoculum level. The inoculum size was further increases the production of enzyme gradually decreased due to the fact that at high level of inoculum size. Fungi grow fast by consuming the essential nutrients at the initial stages and rapid accumulation of byproducts into the fermentation medium observed reference 4. the reason the low production of enzyme at the inoculum size below than optimal was due to the slow growth of the organism and extended time period to utilize nutrients properly (Schuster et al., 2002).

Effect of pH

The effect of optimum pH for invertase production by A. niger was determined by adjusting the pH values of 3, 4, 5, 6, 7 and then inoculated with 4ml inoculum prepared from 4 days old culture and incubated at 30ºC for 4 days.

The strain has shown maximum invertase production at the medium pH 5 (Fig. 3 and Table 2) the results of others (Vitolo et al., 1995) are evidenced with this result. This shows that enzyme is not stable towards alkaline conditions so the sucrose inversion efficiency is also affected indirectly (Balasunbaram and Pandit, 2001).

Effect of carbon sources

Different carbon sources such as glucose, fructose, lactose, sucrose and raffinose at 1% concentration were added to the medium for the invertase production. The pH of the medium was adjusted to 5 and 4ml inoculums of 4 day old culture at 30ºC for 4 days.

Among all the carbon sources tested sucrose gave the best result Vitolo and Yassuda 1991 and Rubio and Navarro 2006). These results were also supported by the findings of Cairns et al., (1995), who reported that invertase production in some other fungi were induced by sucrose, glucose and fructose are not involved in the induction synthesis of invertase in A. niger (Rubio and Navarro 2006).
Effect of nitrogen sources

The effect of different nitrogen sources were tested by adding 1% different nitrogen sources like peptone, urea, yeast extract to the production medium (pH 5) containing sucrose as the carbon sucrose with 4ml culture inoculum of 4 days old culture (Kamble and Borate 2012). The flasks were incubated at 30°C for 96 hours.

Table 1. Effect of carbon source on invertase production by *A. niger*

| Conditions of carbon sources | Enzyme activity (IU/ML) |
|------------------------------|-------------------------|
| Fructose                     | 44                      |
| Glucose                      | 38                      |
| Lactose                      | 26                      |
| Sucrose                      | 50                      |
| Maltose                      | 25                      |

Table 2. Effect of nitrogen source on invertase

| Conditions of nitrogen sources | Enzyme activity (IU/ML) |
|--------------------------------|-------------------------|
| Peptone                        | 42                      |
| Urea                           | 35                      |
| Yeast extract                  | 52                      |
| Malt                           | 40                      |
| Casein                         | 40                      |

Table 3. Optimized conditions for invertase production by *A. niger*

| Optimized parameter          | Optimized conditions |
|------------------------------|----------------------|
| Incubation time              | 96 hours             |
| Incubation temperature       | 30°C                 |
| Inoculums volume             | 4 ml                 |
| Initial pH                   | 5                    |
| Carbon source                | Sucrose              |
| Nitrogen source              | Yeast extract        |

Fig. 1. Effect of incubation time on invertase production using *A. niger*
**Fig. 2** Effect of incubation temperature on invertase production using *A. niger*

![Graph showing enzyme activity at different temperatures](Image)

**Fig. 3** Effect of inoculum volume on the invertase production using *A. niger*

![Graph showing enzyme activity at different inoculum volumes](Image)

**Fig. 4** Effect of pH on the invertase production using *A. niger.*

![Graph showing enzyme activity at different pH levels](Image)
The maximum invertase production was shown using yeast extract as nitrogen source (Fig. 4 and Table 2). Similar results that yeast extract was the best nitrogen source for invertase from a cladosporium cladosprioides in SmF (Uma et al., 2012). Whereas some reported that the peptone + yeast extract was significant in invertase production by
Saccharomyces cerevisiae (Kamble and Borate 2012) (Fig. 5–7).

**Effect of substrates on enzyme activity**

Different agricultural byproducts such as orange peel pomegranate peel sapota peel, pineapple peel and lemon peel were tested for production of invertase enzyme. The maximum invertase production was recorded using orange peel (55 IU/ml) supplemented medium. In our investigation 5 agricultural residues such as peels of orange, Pomegranate, Pineapple, Lemon and Sapota have been used as substrate maximum invertase production (IU/ML) was recorded with orange peel similar results as orange peel as the best substrate for the maximum production of invertase was observed using Saccharomyces cerevisiae (Pandey et al., 2001, Alegre et al., 2009, and Shankar et al., 2013) and also using A. niger (Asha et al., 2016). Some investigated as the best agro residue as carbon source using A. niger Vijaykumar et al., 2016).

In conclusion, the investigation suggests that the orange peel could be an alternative and promising substrate for the production of invertase by A. niger.

The solid state fermentation (SSF) is considered as most eco-friendly process. In addition, this work will act as first time information to researchers who want to explore the possibilities of converting waste to wealth and value addition. Since orange peel utilized within process are readily accessible agricultural (horticultural) waste with little or no cost and also contain an appreciable amount of invertase. These agricultural wastes are regarded as low cost substrate using A. niger. This work will not only lead to the reduction in the production cost of invertase but also help to decrease the pollution load resulting from these agricultural wastes

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