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Production and properties of invertase from a *Cladosporium cladosporioides* in SmF using pomegranate peel waste as substrate

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ABSTRACT

Objective: To use suitable fungal strain for production of invertase and to study the effect of external substances and metal ions that may enhances the production of invertase. **Methods:** The culture and nutrient requirements of *Cladosporium cladosporioides* (*C. cladosporioides*) for production of invertase in a medium at different pH, temperature, incubation period, carbon source and nitrogen source were quantified in present study. **Results:** The optimum pH, temperature and incubation period for enzyme production were 4, 30 ℃ and 4th day respectively. Among the carbon source pomegranate peel was recorded to be the best carbon source for enzyme production and yeast extract at 1% was ideal nitrogen source for the invertase production. The enzyme was purified by ammonium sulphate precipitation, dialysis and DEAE-Cellulose column chromatography. A trial for the purification of invertase resulted in an enzyme with specific activity of 197.50 Units/mL with 6.35 folds of purification. The purified invertase had a maximum activity at pH 6 and the K_m value for pomegranate was 0.26 mg/mL. Analysis of this enzyme for molecular mass was carried out by SDS-PAGE electrophoresis which revealed one band 61 kDa. **Conclusion:** From this study, it can be concluded that the pomegranate peel waste can be more effectively used as a substrate for the production of enzyme under optimized culture conditions.

1. Introduction

Invertase is used for the inversion of sucrose in the preparation of invert sugar and high fructose syrup (HFS). It is one of the most widely used enzymes in food industry where fructose is preferred than especially in the preparation of jams and candies because it is sweeter and does not crystallize easily D-glucose and D-fructose at concentrations lower than 10% sucrose, thus making these enzymes suitable for biotechnological applications[1]. It has wide range of commercial applications including the production of confectionery with liquid or soft centers, chocolates and fermentation of cane molasses into ethanol. It is also used in pharmaceutical industry as digestive aid tablets, powder milk for infants' foods, as calf feed preparation, assimilation of alcohol in fortified wines and in manufactured inverted sugars as food for honeybees[2].

Microbial invertase are usually produced either by free or immobilized cells. Immobilized cells have been used in a variety of applications such as biotransformation, biosensors, production of ethanol, degradation of phenol etc. At present, the immobilization technology is often studied for its potential to improve fermentation processes and bioremediation[3].

Biologically active enzymes may be extracted from any living organisms like plants, animals and micro organisms. Microbes are preferred to plants and animals as sources of enzymes because of less harmful materials than plant and animal tissues. The majority of enzymes used in industrial/ biotechnological applications are derived from particular fungi and bacteria[4]. The present study was started as a survey of new species to learn their capacity to produce

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invertase when grown in shaken cultures.

2. Materials and methods

2.1. Organism and inoculum preparation

Fungal strains were isolated from soil of sugarcane field Coimbatore, India by dilution plate method. Culture was screened for invertase enzyme production and fungal strain *C. cladosporioides* selected for the production of invertase was prepared from 4 d old slant culture.

2.2. Fermentation condition

2.2.1. Enzyme production under Submerged Fermentation (SmF)

The medium used for enzyme production under submerged fermentation comprised of (g/L): sucrose 20, yeast extract 10, ammonium sulphate 1.0, magnesium sulphate 0.75, potassium dihydrogen phosphate 3.5, pH 5.0. Cultivation was carried out in 250 mL Erlenmeyer flasks, each containing 50 mL of sterile medium. After inoculation (10^6 spores/mL) the flasks were incubated at 30 ℃ for seven days using the static and agitated (125 rpm) culture technique. At the end of fermentation, the supernatant was harvested by centrifugation at 10 000 rpm for 10 min (4 ℃) and was used as crude enzyme extract.

2.2.2.Enzyme production under Solid State Fermentation (SSF)

For solid substrate fermentation, polyurethane foam (PUF) with an average density of 17 mg/mL was cut into 0.5 cm cubes, washed and dried overnight in an oven at 75 ℃. It was then autoclaved at 121 ℃ for 10 min in an Erlenmeyer flask. Basal mineral medium with a composition of $NaNO_3$, 15.0; KH_2PO_4, 1.76; KCl, 0.76; $MnCl_2$, 0.001; $MgSO_4$, 0.76; $FeCl_2$, 0.001; $CuSO_2$, 0.001; $ZnCl_2$, 0.001 was supplemented with 100 g/L of sucrose autoclaved and inoculated with fungal strains. This was then added to the flask containing PUF, stirred manually before incubation at 30 ℃ for 32 h with an initial pH of 5.5. Extracts were obtained by gentle pressure of the PUF cubes. Filtrate obtained was assayed for its enzyme activity[5].

2.3. Enzyme assay

Invertase activity was determined using the method of Aranda *et al*[6] with slight modification by incubating 0.1 mL of enzyme solution with 0.9 mL of sucrose in 0.03 M acetate buffer (pH 5.0). To stop the reaction, 1 mL of dinitrosalicylic acid reagent was added and heated for 5 min at 100 ℃ in a boiling water bath. Finally the absorbance was read at 540 nm in spectrophotometer. One unit of invertase (IU) is defined as the amount of enzyme which liberates 1 μmol of glucose/minute/mL under the assay condition

2.4. Optimization of invertase

Optimization of medium composition is one of the essential steps to maintain a balance between the various medium components to minimize the amount of unutilized components at the end of fermentation[7]. The effects of various factors like incubation day, pH and temperature on the production of invertase were studied by incubating the flasks with varying pH (3-8) and temperature (20-30 ℃) for seven days and also the effect of various factors like inoculum size, carbon source (substituted substrates), nitrogen sources and its concentration was studied.

2.5. Purification of invertase

Crude extract was precipitated by 70% saturation with ammonium sulphate and then dialyzed against 100mM Tris HCl buffer (pH 7.5) for 24 h at 40 ℃. the filtrate was loaded onto a DEAE-cellulose chromatographic column (25 cm×2.6 cm) equilibrated with Tris-HCl buffer, 100 mM, pH 7.5. The enzyme was eluted with a linear salt concentration gradient (NaCl, 0-0.4 M) in the same buffer and 3.0 mL fractions were collected at a flow rate of 20 mL per hour. SDS-PAGE electrophoresis was carried out and molecular weight was determined. The protein content was estimated by Lowry method[8]. The purified enzyme is used for further studies.

2.6. Characterization of purified invertase of C. cladosporioides

The kinetic parameter of the purified protease enzyme was determined and the optimum pH 3.0-8.0 [The pH was adjusted using, the following buffers: 50 mM sodium citrate (pH 3.0-6.0) and 50 mM sodium phosphate (pH 7.0 & 8.0)], temperature (30-70 ℃) and substrate concentration on the activity of the enzyme was also assayed. Metal ions which included $MgCl_2$, $ZnCl_2$, NaCl, $CaCl_2$ and $CuCl_2$, at concentration of 0.05 M each, and inhibitors, namely aniline, diethylaniline and toluidine (0.01 M) were tested for their effects on enzyme activity.

3. Results

A comparative evaluation of fungal invertase enzyme production using submerged and solid-state fermentation was studied. For submerged fermentation the conical flask containing the fermentation medium (Czapek Dox medium) was inoculated with fungal spores (10^6 spores/mL) and incubated at 30 ℃ for seven days using agitated (125 rpm) culture technique. Solid state fermentation was carried out in basal mineral medium inoculated with the fungal spores (10^6 spores/mL) and incubated at 30 ℃. The enzyme was extracted and assayed.

The enzyme production was found to be 17.50 IU/mL in SmF and in SSF the production of invertase was found to be 15.28 IU/mL *C. cladosporioides*. From figure 1 it can be seen that SmF was found to support maximum invertase production than in SSF. Hence the following experiments were carried out using submerged fermentation.

Different agricultural byproducts such as pineapple peel, sweet lime, sugarcane bagasse, Mosambi peel, orange peel and pomegranate were tested for the production of enzyme (Figure 2). Of all the substrates tested, pomegranate was found to be the best substrates for the production of invertase.

Comparative evaluation of invertase enzyme production in submerged fermentation (SmF) and solid state fermentation (SSF) of *C. cladosporioides*.

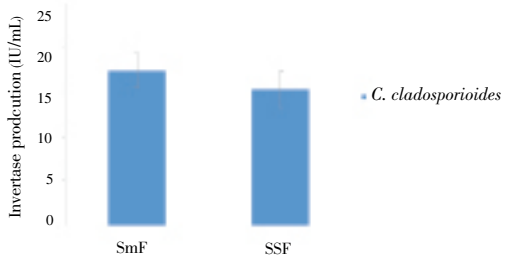


Figure 1

Values are Mean± SD of three samples. Means followed by a common superscript letter are not significantly different at 5% level by using DMRT analysis.

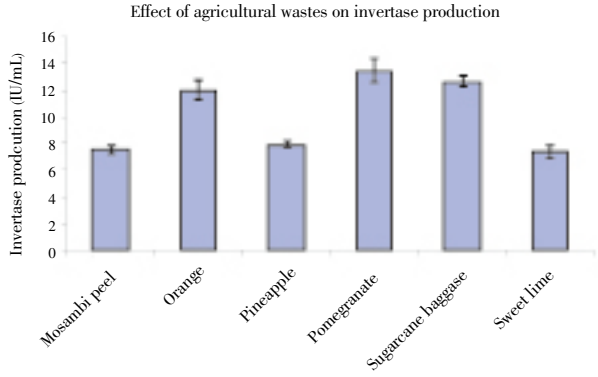


Figure 2

Values are Mean± SD of three samples. Means followed by a common superscript letter are not significantly different at 5% level by using DMRT analysis.

Invertase production by *C. cladosporioides* was studied in shaken flask culture technique by inoculating 10^6 spores/mL of fermentation medium (CD medium). The CD medium was inoculated with the fungal strain and incubated for various time intervals (1-7 d). The highest production was observed on fourth day (23.2 IU/mL) of incubation using *C. cladosporioides* (Figure 3).

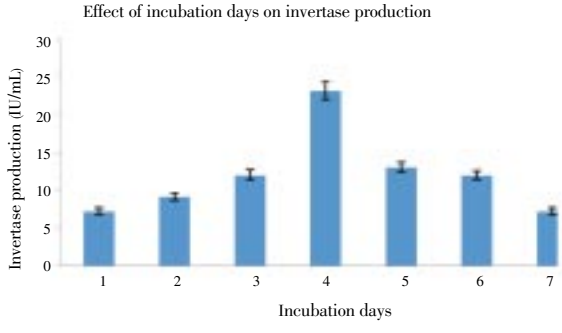


Figure 3

Values are Mean± SD of three samples. Means followed by a common superscript letter are not significantly different at 5% level by using DMRT analysis.

Production of invertase is largely dependent on optimum pH and temperature of the fermentation medium. Figure 4 shows the effect of pH on enzyme production by *C. cladosporioides*. Maximum production of invertase was obtained when initial pH of the fermentation medium was kept at 4.0 (18.5 IU/mL) and temperature at 30 ℃ (Figure 5) by *C. cladosporioides*.

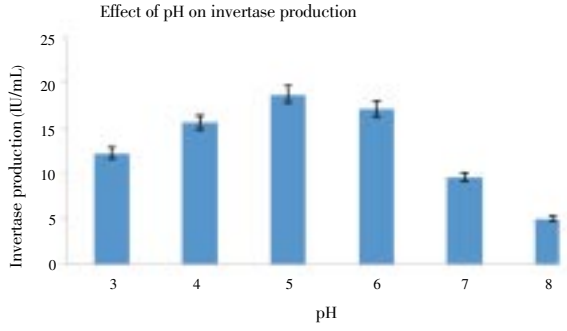


Figure 4

Values are Mean± SD of three samples. Means followed by a common superscript letter are not significantly different at 5% level by using DMRT analysis.

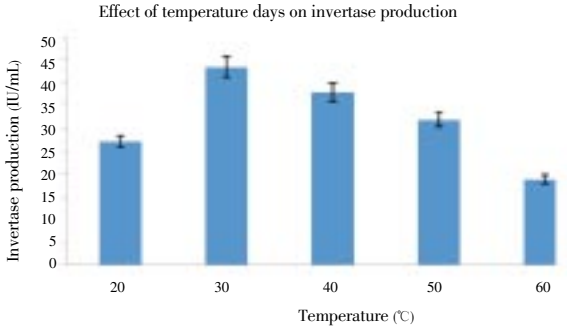


Figure 5

Values are Mean± SD of three samples. Means followed by a common superscript letter are not significantly different at 5% level by using DMRT analysis.

To evaluate the effect of inoculum size on invertase enzyme production, varied cell concentrations (1%-6%) were added to different flasks and then fermentation was carried out. In the case of *C. cladosporioides* it was evident from the Figure 6 that the maximum enzyme production occurred at 3% inoculum size for pomegranate peel as the substrate with a production of 31.0 IU/mL.

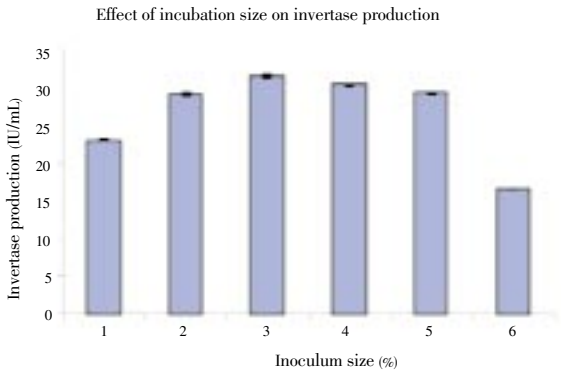


Figure 6

Values are Mean± SD of three samples. Means followed by a common superscript letter are not significantly different at 5% level by using DMRT analysis.

The effect of different nitrogen sources were tested by incorporating 1% different nitrogen sources like nutrient broth, peptone, urea and yeast extract into the fermentation medium. When the medium was incorporated with yeast extract better yield was observed for *C. cladosporioides* (Figure 7).

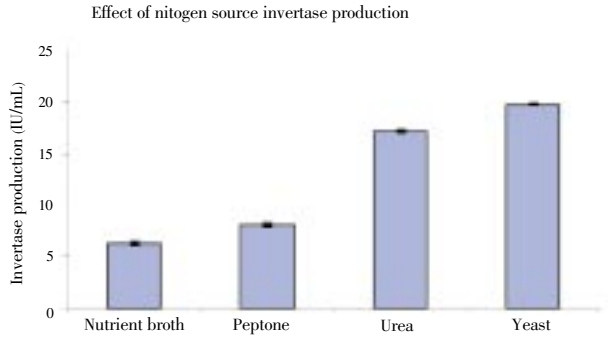


Figure 7

Values are Mean± SD of three samples. Means followed by a common superscript letter are not significantly different at 5% level by using DMRT analysis.

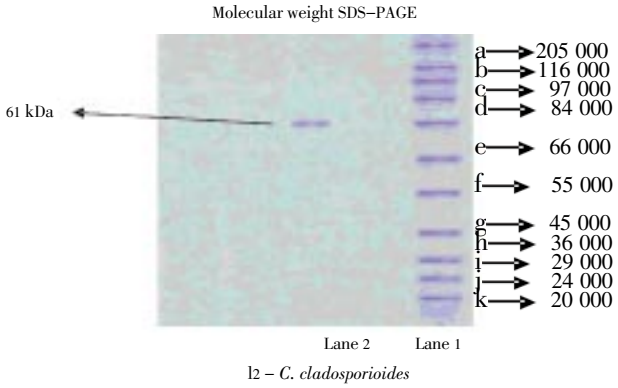
Table 1

Purification and recovery of invertase from *C. cladosporioides*.

Steps	Invertase activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification (fold)	Recovery (%)
Crude extract	9940.00	320.64	31.00	1.00	100.00
70% ammonium sulphate precipitation	6360.00	134.60	47.25	1.52	63.90
Dialysis	469.20	9.08	51.75	1.67	4.72
DEAE cellulose column chromatography	393.75	1.99	197.50	6.35	3.96

The crude enzyme of *C. cladosporioides* was precipitated at 70% saturation of ammonium sulphate with 1.52 fold purification and 63.9% recovery with a specific activity 47.25 U/mg. After dialysis, passage from DEAE cellulose column further purifies the enzyme to 6.35 fold with 3.96 % recovery (Table 1).

Molecular weight of the *C. cladosporioides* strains was determined by SDS-PAGE analysis which was found to be 61 kDa (Figure 8). The homogenate of the dialysate was checked by SDS-PAGE was performed as discussed by Laemmli[12].



l2 – *C. cladosporioides*

Figure 8

Molecular weight SDS-PAGE.

Temperature and pH are the most important factors, which markedly influence enzyme activity. The effect of pH on the enzyme activity indicates that the invertase is active at pH 6.0 (Figure 9). Maximum invertase activity was recorded at 50 ℃. Further increase in temperature resulted in decrease in the activity of invertase (Figure 10).

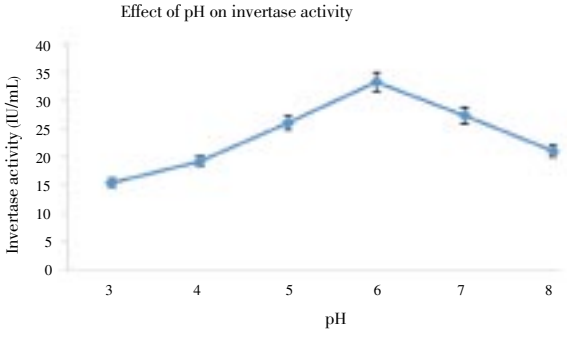


Figure 9

Values are Mean± SD of three samples. Means followed by a common superscript letter are not significantly different at 5% level by using DMRT analysis.

The kinetic parameters for purified extracellular invertase activity were determined using sucrose, in the concentration range of 0.2-1.0 mM. Reaction rate verses substrate concentration curve was plotted to determine whether the enzyme obeys Michaelis-Menten kinetics, and constants were determined from a Line Weaver-Burk plot. It can be

seen that the *C. cladosporioides* had lower V_{max} of 28.57 U/mg and K_m of 0.26 mg/ml (Figure 11).

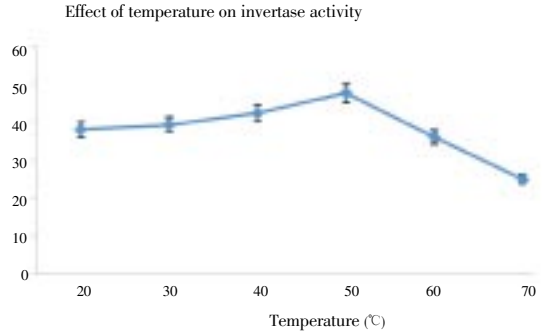
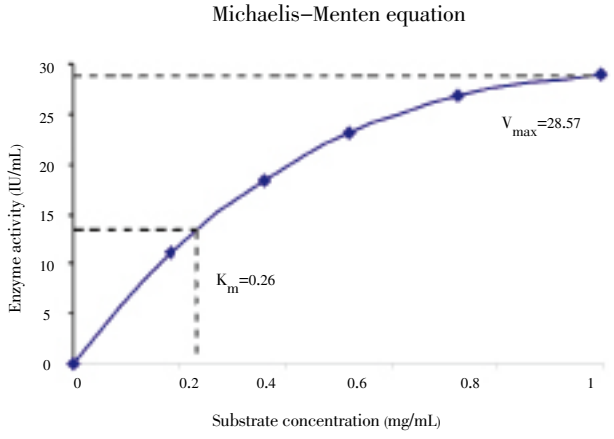
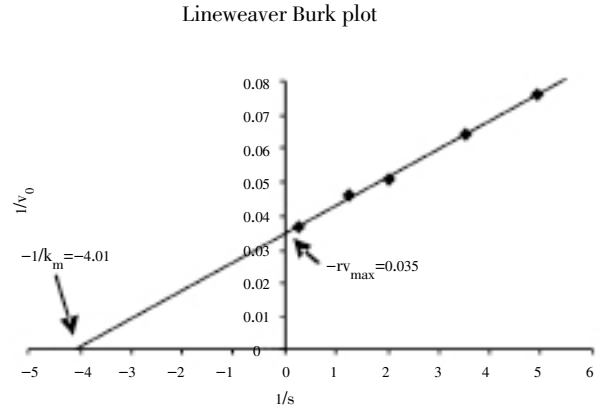


Figure 10

Values are Mean ± SD of three samples. Means followed by a common superscript letter are not significantly different at 5% level by using DMRT analysis.

Various metal ions were reported to be inhibitory while some others influenced the production of invertase. The effect of metal ions on activity of the enzyme invertase from *C. cladosporioides* was studied. The metal ions such as Zn^{2+}, Mg^{2+}, Ca^{2+}, Na^{+} and Cu^{2+} were tested. From the figure 12, it can be shown that the metal ions Na^{+} and Ca^{2+} supported the maximum enzyme activity whereas Mg^{2+}, Zn^{2+} and Cu^{2+} drastically inhibited the invertase activity particularly Zn^{2+} was found to be the potent inhibitor of invertase. Effect of enzyme inhibitors on invertase activity was tested by incubating the enzyme with various inhibitors, aniline, diethylaniline, toluidine at a concentration of 0.01 M (Figure 13).





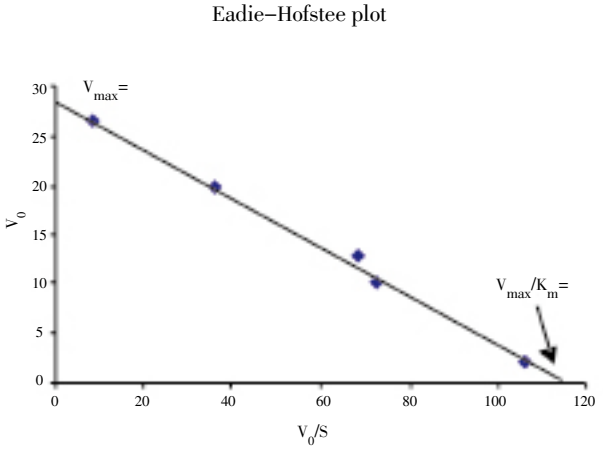


Figure 11

Enzyme kinetics of *C. cladosporioides*.

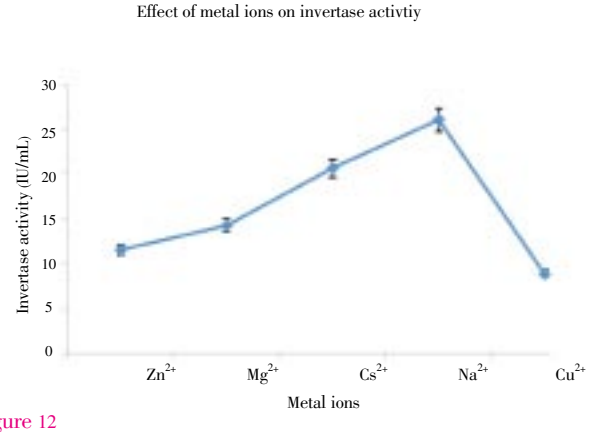


Figure 12

Values are Mean ± SD of three samples. Means followed by a common superscript letter are not significantly different at 5% level by using DMRT analysis.

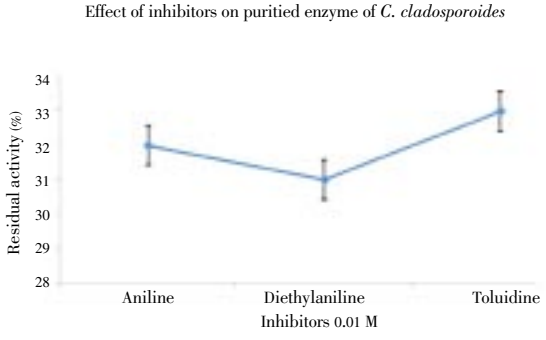


Figure 13

Values are Mean± SD of three samples. Means followed by a common superscript letter are not significantly different at 5% level by using DMRT analysis. *C. cladosporioides* shows maximum inhibitory effect with toluidine. It retained its activity of 33% at 0.01 M concentration and 32% with aniline and 31% with diethylaniline at 0.01 M.

4. Discussion

In the present study, invertase production was carried out in submerged (SmF) and solid state fermentation (SSF) for *C. cladosporioides*. The production was found

to be higher in SmF than in SSF for *C. cladosporioides*. Therefore, further study was conducted using SmF for optimization studies.

Our results were in accordance with the results reported by Baig *et al*[9] that the production of invertase from *Saccharomyces cerevisiae* was maximum when the strain was grown in submerged fermentation and when urea was provided as the inducer. The study on invertase production by *Penicillium chrysogenum* and some other fungi to determine the relative distribution of intra and extracellular invertase produced by them in SmF, and it was found that the proportion of the enzymes produced in SmF varied with organism and the period of fermentation[10].

The media optimization is an important aspect to be considered in the development of fermentation technology. However, there are only a few reports concerning the optimization of media composition especially for fungal strains in invertase production. The incubation period varies with enzyme productions. The present study was carried out to evaluate the effect of incubation period on invertase production by *C. cladosporioides* where in the maximum yield of the enzyme was obtained on the 4th day of incubation. Further increase in incubation period gave less invertase production. It might be due to decrease in the availability of nutrients in medium building capacity of yeast or depletion of sugar contents[11].

The result was supported by Rashad and Nooman[2] who observed that the invertase production was maximum on the 4th day of cultivation by *Saccharomyses Cerevisiae* NRRL Y-12632.

Guimaraes[12] reported that for Aspergillus ochraceus, invertase reached its highest level when supplemented with sugarcane bagasse at 96 hours i.e on 4th day at 40 ℃ under orbital agitation of 100 rpm. The maximum invertase production by *Aspergillus oryzae* on 4th day incubation at pH 5.5 was reported by Shankar and Mulimani[13].

Temperature is a critical parameter that has to be controlled and it varies from organism to organism and it determines the success of optimization system. Temperature strongly affects the production of invertase specifically or none specifically. The highest invertase production was found at 30 ℃. Similar behavior was reported for extracellular invertase production from *A. niger* and from *L. reuteri*[14].

Among physical parameters, pH of the growth medium plays an important role by inducing morphological changes in microbes and in enzyme secretion. The pH change observed during the growth of microbes also affects product stability in the medium[15]. Optimum pH for invertase was found to be in a range between 4.0 to 6.8 for the fungi species[16] and the present study recorded 4.0 as optimal, which agrees with earlier findings.

The extracted enzyme was subjected to partial purification viz., 70% ammonium sulphate, dialysis, DEAE column chromatography. It was observed that *C. cladosporioides* exhibited a single peak with 0-0.4 M NaCl. It is evident from our study that purification of the enzyme in the range of 7.85 folds in *C. cladosporioides*. Concurrently, the eluted sample was subjected to SDS PAGE and the molecular weight was determined (Figure 8). Our result was in consonance with the work of Guimaraes[12] who purified the enzyme to 7.1 fold with a recovery of 24%, by two chromatographic steps in DEAE-cellulose and sephacryl s-200, in *Aspergillus ochraceus*.

The purification of enzyme invertase from *Isotricha prostoma* in sepharose CL4B/octyl-sepharose CL4B chromatography gave a yield of 29% and a purification of 20.7-fold and DE52 DEAE-cellulose chromatography it was 26% with a purity of 115.4 fold[18]. Ishimoto and Nakamura[17] purified invertase from *Clostridium perfringens* AS40-75 by DEAE-cellulose chromatography, gel filtration through sephadex G-150 and hydroxylapatite chromatography and the maximum recovery was with ammonium sulphate ie 91% with specific activity of 1.42.

The molecular weight of invertase for many organisms was reported by many workers. In our present study, the molecular weight was determined by SDS-PAGE and was found to be 67 for *C. cladosporioides*. Ettalibi and Baratti[18] reported the molecular weight of invertase in *A. ficcum* to be 84 kDa. The effect of pH on the enzyme activity of invertase produced from *C. cladosporioides* exhibit stability over the pH range from 3.0 to 8.0 with different buffer (acetate and phosphate) and the maximum enzyme activity was observed at pH 6.0. Our results were in concordance with a number of authors, which reported a maximum activity of invertase at a pH 6.0 in *C. herbarum* by Ettalibi[19].

The effect of temperature on the enzyme activity was assayed on the temperature range between 20 ℃ to 65 ℃. The enzyme showed highest activity at 50 ℃ in *Saccharomyses cerevisiae* NRRL Y-12632[2]. The optimum temperature for invertase activity was 55 ℃ for *Saccharomycopis fibuligera*[20]. Similar observation was noticed by *A. niger*[22] *Cladosporium herbarum* 1, a psychrotropics fungi showed maximum activity at 55-60 ℃.

In the present study, the K_m values were 0.26 mg/mL and V_{max} were 28.57 IU/mg for *C. cladosporioides*. Similar results were obtained by Ishimoto[17] in *C. perfringens*. The K_m value for invertase in *S. fibuligera* was found to be 67 mM[20] and the substrate activity of about 32.8 mM for sucrose. Belcarz[21] reported that the K_m values of the S and F forms of invertase were found to be 41.54 mM in *Candida utilis* and was highly active against sucrose. Kinetic study of the K_m and V_{max} for sucrose was calculated as 0.227 M and 0.096 μ mol/min.

In the present study, the metal ions Na^+ and Ca^{2+} significantly increased the enzyme activity except a very few such as Zn^2, Mg^{2+} and Cu^{2+}. These results suggest that the concerned metal ions apparently protect the enzyme against thermal denaturation and play a vital role in maintaining the active confirmation of the enzyme at high temperatures.

Enrichment with 2 mM Co^{2+} and Mn^{2+} increased the enzyme activity by 13% and 15% respectively. Mn^{2+} ions stabilize the invertase within 12 h of incubation at 0 ℃. Cd^{2+} ions markedly inactivated the β-fructofuranosidase[22]. Invertase is strongly inhibited by heavy metal ions.

Amines are known to have inhibitory effects on invertase. In the present study, an inhibition of 60%-70% was observed in the presence of 0.01 M aniline,

diethylaniline, toluidine when added to the partially purified enzyme *C. cladosporioides*. Similar observation was reported by[17] in their study of purification properties of invertase from *Clostridium* species.

According to them, a slight inhibition of 50%-60% was observed in the presence of 0.01 M of aniline, diethylaniline, toluidine when sucrose concentration was 0.0125 M whereas at higher concentration of sucrose (0.125 M), inhibition was weak. Inhibitor study by p-hydroxymercuribenzoate (PCMB) on invertase production by *Clostridium pasteurianum* worked out by Laishley[7] showed that PCMB strongly inhibited the invertase activity and other sulfhydyl reagents had no effects.

Therefore it was concluded in our study that both nutritional and cultural conditions were required for optimum growth and production of invertase from *Cladosporium cladosporioides*. It can be concluded that the pomegranate peel waste can be more effectively used as a substrate for the production of enzyme under optimized culture conditions.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgement

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