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International Journal of ChemTech Research
CODEN(USA): IJCRGG ISSN : 0974-4290
Vol. 3, No.3, pp 1514-1519, July-Sept 2011

Bioprospecting of Marine Yeast with special reference to Inulinase production

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Abstract: The marine yeast strain SY3 was isolated from gut region of *Lutjanus campechanus* which is found to secrete inulinase in YPD medium. The partial purification of the enzyme by dialysis, showed over of 62.7U/ml of inulinase activity produced within 48 hours of fermentation in shake flask. The potential strain was identified as *Cryptococcus* sp based on morphological and biochemical characteristics. The optimal medium for inulinase production was with 4.0% (w/v) inulin and 0.5% (w/v) yeast extract, while the optimal cultivation conditions for inulinase production were pH 4.0 and 37° C. Findings of the present study showed that marine yeast will be a potential source for inulinase production.
Key words: *Lutjanus campechanus,* inulinase activity, *Cryptococcus* sp.

INTRODUCTION

The oceans, which cover almost 70% of the earth's surface and over 90% of volume of its crust[1] contain a variety of species. Marine ecosystems are the host of different species ranging from microorganisms to the base of food web to large mammals[2]. Organisms from these habitats may hold keys to nutrient cycling, metal detoxification, and food-webs in extreme environments, as well as potentially provide unique bio molecules for industry and medicine[3]. First the marine yeasts were discovered from the Atlantic Oceans. Following this discovery yeast were isolated from different sources, viz., seawater deposits, seaweeds, fishes, marine mammals, and sea birds in all over the World. It has been found that marine yeast could produce bioactive substances including amylase, lipase, protease, phytase inulinase, vitamin C, amino acids, glutathione, glucan, killer toxin with potential application in mariculture, food, pharmaceutical, cosmetic, chemical industries and environmental protection[4].

Among the various products produced from marine yeasts, inulinase has received much attention as it can be widely applied in the production of fuel ethanol and high fructose syrup[5]. D-Fructose is a monosaccharide widely distributed in nature that shows sweetening power 70% higher than sucrose. It consists in a suitable table sugar, ingredient for some food formulation and in substrate for fermentative processes[6]. Inulin can be converted into fructose by chemical approaches. However, the chemical approach is associated with high investment and work load[7]. Fructose can also be produced from starch by

enzymatic method involving α-amylase, amyloglucosidase and glucose isomerase. The best procedure involves the use of microbial inulinase, which after one step enzymatic hydrolysis of inulin yields 95% pure fructose. The increasing potential of inulinase application prompts screening for newer inulinase producing microorganisms which can meet the conditions favorable to the industrial applications. Inulinases have also been used for the production of inulo-oligosaccharides – low caloric saccharides acting as growth factors for beneficial microorganisms in the intestinal flora[8].

Terrestrial yeasts which are reported to produce inulinase include *Candida* sp., *Sporotrichum* sp., *Pichia* sp., and *Kluyveromyces sp.* However a few studies including Gong *et al.,*[9] report on inulinase production by marine yeast *Pichia guilliermondii*, Goa *et al.,*[10] report on inulinase production of four marine yeasts *Pichia guilliermondii*, *Crptococcus aureus*, *Yarwonia lipolytica*, *Debaromyces hansenii*, Sheng *et al.,*[11] report on inulinase production by *Cryptococus aureus* and again Sheng *et al.,*[12] optimized the process parameters for high inulinase production by the marine yeast *Cryptococcus aureus* G7a were the existing reports on inulinase producing marine yeast. Hence, an attempt was made to exploit marine yeast for inulinase production.

MATERIALS AND METHOD

Isolation of Marine Yeast

Marine fish *Lutjanus campechanus* was collected from Mahabalipuram coastal area. The gut region was dissected and homogenized under aseptic conditions. In order to enrich the yeast population, 5 ml of homogenized sample was suspended in 5.0 ml of yeast extract peptone dextrose (YPD) broth [2.0% glucose, 2.0% polypeptone and 1.0% yeast extract supplemented with 0.05% chloramphenicol] and incubated in rotary shaker at 95 rpm for 5 days. Then the enriched suspension was serially diluted and plated on YPD agar by adopting standard spread plate method. Plating was done in duplicate and all the plates were incubated at 25ºC for 5 days. After incubation all the plates were observed and morphologically different yeast like colonies were selected, purified and subcultured on YPD slants[10].

Primary Screening for Inulinase Activity

All the yeast isolates were inoculated in to the medium containing 2 g/L of inulin, 10 g/L of yeast extract, 20 g/L of $MgSO_4.7H_2O$, 2 g/L of KCl, 10% of NaCl, 20 g/L of agar. Inulin was used as the sole source of carbon in this medium; thus, yeast growth after 48 hours of incubation at 37°C shows the presence of inulinase activity. Based on the results of primary screening, three potential yeast isolate was selected for further investigations.

Inulinase Production

One loopful of the selected yeast culture was transferred into 10 ml of yeast extract peptone dextrose medium (YPD) medium and incubated for 24 hours at28^0C. Five ml of yeast inoculum with a final concentration of 10^6 cells were transferred to 45 ml of production medium which contained inulin 4%, K_2HPO_4 0.3%, Yeast extract 0.5%, pH 5.0 prepared with sea water. The production flasks were incubated in a rotary shaker with 180 rpm at 28ºC for 72 hours[10].

Assay of Inulinase

The fermented broth was centrifuged at 3500 rpm for 10 minutes and the cell free supernatant obtained was collected and used as crude inulinase enzyme. To the 2ml 0.2% inulin, 2ml acetate buffer (pH 4.6) and 0.5 ml crude enzyme were added in the reaction tube and incubated at 50°C for 20 minutes. After incubation, the reaction tubes were kept in a boiling water bath for 10 minutes to stop the enzyme reaction and then cooled to room temperature. The reaction mixture was assayed for reducing sugar as fructose by DNS method by reading the absorbance at 575 nm. The calibration curve was prepared with fructose solutions of known concentration 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 µ mol of fructose under the assay conditions[13].

Partial Purification of Inulinase

The separation and dialysis of inulinase was carried out by adopting the protocol described by Green *et al.,*[14]. About 50 ml of crude enzyme produced from the yeast strain was taken and stirred with magnetic beads. To this 50 ml of 80% ammonium sulphate solution was added and slowly mixed for about one hour. The precipitate was allowed to form at 4ºC for 24 hours. Then the whole solution was centrifuged at 4,000 rpm for 10 minutes at 4ºC. The precipitate obtained after ammonium sulphate precipitation was dialysed using dialysis membrane against phosphate buffered saline (pH-7) for 24 hours. The buffer was changed occasionally. Then the dialysate was tested for inulinase activity by DNS method as described earlier.

Characterization and Identification of Potential Strain

Phenotypic characters such as micro morphology (Gram staining, capsule staining),

biochemical characteristics (sugar assimilation, urease test), and physiological characteristics (salt tolerance) of potential strain was studied by using standard procedures. Based on the results of phenotypic characteristics the potential isolate was identified at genus level.

Optimization of Inulinase Production

Effect of critical medium components on inulinase production was studied by adopting classical one factor at a time method. Factors which are studied include carbon source, nitrogen source, substrate concentration, temperature and pH[8].

Table 1: Assay for inulinase

Yeast strain	Inulinase activity (U/ml)
SY3	53.9
SY6	39.5
SY8	42.4

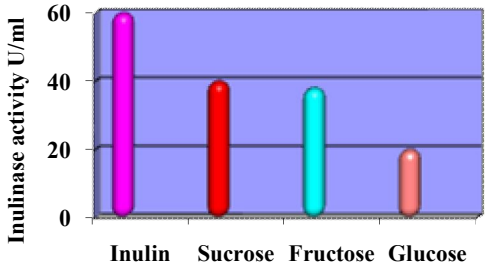

Fig.1: Effect of Carbon sources on inulinase production

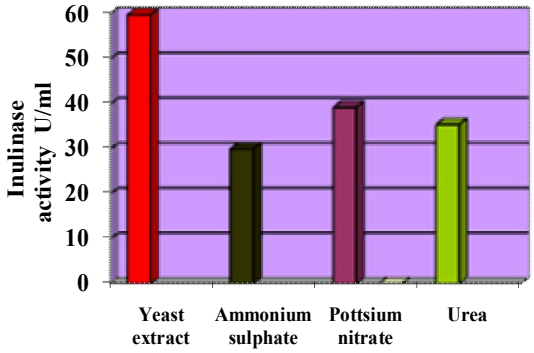

Fig. 2: Effect of Nitrogen sources on inulinase production

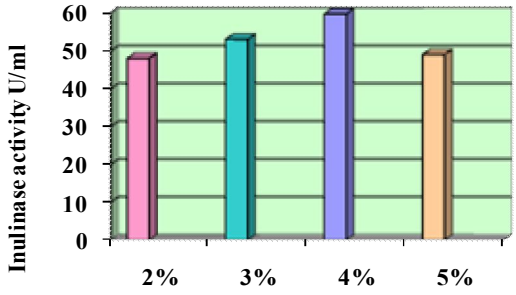

Fig. 3: Effect of Inulinase concentration on inulinase production

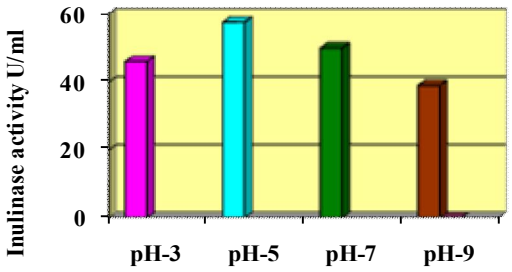

Fig. 4: Effect of pH on inulinase production

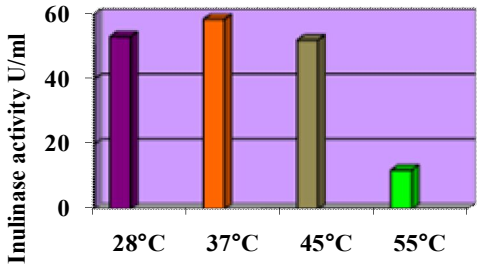

Fig. 5: Effect of Temperature on inulinase production

RESULT AND DISCUSSION

It is well known that different terrestrial yeasts were reported to produce a large amount of inulinase. The present study is attempted to study the inulinase producing potential of marine yeasts. Totally 25 yeast colonies were isolated from isolation agar. In the preliminary screening, three out of 25 isolates showed inulinase activity. The active isolates were subjected for inulinase production and assayed by DNS method (Table 1). Of this strain SY3 was selected that could secrete large amount of inulinase into the production medium and the quantity of the enzyme was found to be 53.9 U/ml.

Gao *et al.,*[10] used four strains of marine yeast for screening of inulinase production and reported the maximum production was with *Yarwonia lipolytica* that produced inulinase activity of 62.8 U/ml. The partial purification of the enzyme by ammonium sulphate precipitation and dialysis showed 62.7 U/ml of enzyme activity.

The colony of yeast strain SY3 on YPD agar was pink and butyrous, its vegetative cells are produced by budding. There were no pseudohyphae and ascospores, and the yeast produce capsule. The strain showed positive result in urease activity. The sugar assimilation test of the potent strain showed that it assimilates glucose, sucrose, lactose, xylose, maltose, cellobiose, fructose, L-arabinose, D-arabinose.

Gao *et al.,*[10] Sheng *et al.,*[11] reported *Cryptococcus aureus* with above similar characteristics for the production of inulinase. Sheng *et al.,*[12] reported that one of the marine yeast strain, which was identified to be *Cryptococcus aureus* G7a, was able to grow on a wide range of carbon sources and secrete a large amount of inulinase into the medium. The above characteristic of the marine yeast showed that it was *Cryptococcus* sp. The marine yeast SY3 isolated in this study showed good growth at 0-7.5% sodium chloride concentration. This confirmed its marine nature.

Effects of Carbon Source on Inulinase Production

There are numerous report on optimization of inulinase production from terrestrial yeasts[10] than the yeasts from marine origin. Indeed, as shown in graph 1, inulinase production by the marine yeast strain SY3 was also influenced greatly by different carbon sources (including inulin, fructose, glucose and sucrose) in the medium. It can be seen clearly from Figure 1 that inulin was the best carbon source for inulinase production with 60U/ml of enzyme activity, and sucrose was the second better source which influenced inulinase production by strain SY3. However, the lowest inulinase was produced in the medium containing glucose. Derycke *et al.,*[15] reported most of the mold and yeast strains produce inulinase efficiently in the presence of inulin. But the presence of simple sugars inhibits inulinase production.

Effect of Nitrogen Source

Different nitrogen sources such as yeast extract, ammonium sulphate, potassium nitrate, and urea were supplemented in production medium. Among this, yeast extract was found to be a good nitrogen source which increased inulinase production (58.9 U/ml) and potassium nitrate was found to be the second better nitrogen source (Figure 2). The lowest production was with ammonium sulphate. Inhibitory effect of ammonium salts on inulinase production by *Streptomyces* sp. and *K. fragilis* has also been reported earlier by Singh *et al.,*[16]

Effect of Inulin Concentration

Inulin concentration of 2%, 3%, 4%, 5% were evaluated in which maximum inulinase production (59.7%) was observed in the medium supplemented with 4% inulin substrate. Inulinase production was decreased when the concentration inulin increased further (Figure 3). In contrast, findings of a previous study reported that 2% inulin acted as suitable concentration for inulinase production from *Aspergillus ficuum*[17].

Effect of pH

Of the various pH range tested, maximum of 57.6 U/ml of inulinase was produced by the strain SY3 in the medium adjusted with pH 5. Inulinase production was decreased with increase in pH range (Graph 4). It is well known that yeast usually prefers acidic pH for their growth and hence the production of inulinase was best at pH 5. Sheng *et al.,*[12] reported the maximum production of inulinase of *Cryptococcus aureus* G7a was maximum at 5.5.

Effect of Temperature

Among the different temperature ranges tested, maximum of 58 U/ml of inulinase was produced at 37^0C (Graph 5). This may due to the fish gut have the temperature close to 37° C. Similar results were obtained by Shady *et al*.,[18] reported that A*spergillus tamarii* AR-IN9 showed significant inulinase production at the temperature of 30 to 40°C. Out of these conditions, the inulinase production decreased.

All these conditions are favorable to industrial application, avoiding microbial contamination and reducing the formation of undesirable products. The inulinase produced by the *Cryptococccus* sp SY3 isolated in this study will have wide applications in production of ultra high fructose syrups, high concentration of ethanol from inulin. Optimization studies using statistical methods like response surface methodology is in progress to prove its inulinase producing potential further.

ACKNOWLEDGEMENT

The authors sincerely thank Prof. K. R. Venkatesan, Principal, Sri Sankara Arts & Science College for his encouragement and also thank Management authorities for providing the research facilities. One of the author (Dr.RB) thank the Vice Chancellor and the Registrar of Periyar University, Salem, for their encouragement.

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