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ANTIMICROBIAL POWER OF ENDOPHYTES FROM MEDICINAL PLANTS AND ENZYME PRODUCTION

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Abstract

Evaluation of some endophytes have been carried for their possible antimicrobial activity from various parts of medicinal plants belonging to Shreeyash Institute of Pharmaceutical Education and Research Botanical garden Aurangabad Maharashtra (India).A total of eight bacterial endophytes and nine fungal endophytes were isolated from the aerial and underground parts of selected medicinal plants. Isolated endophytic fungal species possess antifungal and antibacterial activity respectively. Endophytic microorganisms are recognize as a potential source of novel chemical molecules that might be useful in the treatment of various diseases. Bioactive compounds of endophytic fungi exhibit antimicrobial, antioxidant, anticancerous, antiviral and insecticidal properties. This research paper focused on the isolation, production, screening and separation of exploitable bioactive compounds from the plants Tulsi, Alovera, curry leaves, gulvel and turmeric leaves and stems. The obtain culture of endophytic fungi were tested for antimicrobial activity against commonly found both Gram-positive and Gram-negative microorganisms i.e. Staphylococcus aureus, Escherichia coli.. An inhibitory zone around the colony of endophytic fungi was evident of good antimicrobial activity of the fungi. From the isolated endophytic fungal species i.e. aspergillus oryzae extracted the enzyme amylase.

Keywords :- (endophytes, antimicrobial activity, antioxidant, anticancerous , antiviral, antibacterial, antifungal , aspergillus oryzae)

Introduction :-

The usage of medicinal plants have been rapidly improved due to their medicinal property. Endophytic fungi are the microorganisms that are present in living tissues of various plants, establishing mutual relationship without causing any symptom of diseases.

• In the current study, endophytes isolated from medicinal plants were assessed for their antimicrobial activity. various medicinal plants use for the microbial activities are belonging from various categories that are

1 aloe vera

2 ocimum tenuiflurom

3 tinospora cardifolia

4 Murraya koenigii

All are the the medicinal plants shows antimicrobial activity .

Endophytes may enhance host's growth, nutrient acquisition and improve the plants ability to tolerate Abiotic stresses by enhancing plant resistance to insects, pathogen and herbivores. The wide presence of an endophytes in plant tissue creates an effective barrier preventing an attack of the pathogens to the host plant. It has bees observed that metabolites produced by endophytes inhibit the growth of pathogens.

Endophytic fungi are of biotechnological interest due to their potential use as source of genetic vector, metabolites and biological control agents such as antimicrobial, antiviral anticancer agent. The increased use of various antibiotics leads to the bacterial resistance currently there are increasing problems worldwide and multi resistant .

endophytes of medicinal plant provide countless drug for the numeral diseases aware of multi resistance pathogenic organisms and and their producing capacity of antimicrobial compound by endophytes it is an indispensable the search of antibiotic substance with new mechanism of action less therapeutic toxicity Endophytic fungi live inside the part for atleast one part of their life without causing any symptoms to the host. Along with the identification of ENDOPHYTES the enzyme production of amylase is also going to be perform and the species which we are going to use for enzyme production is aspergillus oryzae .

Fungal amylases have been widely used for the industrial production due to advantages such as cost effectiveness, consistency, less time and space required for production and ease of process modification and optimization.

Amylase is the extracellular enzyme present which catalyzes hydrolysis of glycosidic bond in starch and fungal amylase in mainly use for the commercial applications likein food processing industries.and in antisalting industry.(baking

industry) for the production of enzyme both methods can be used that are SSF n SMF solid state fermentation and submurged fermentation.

These amylases have a high efficacy saccharification of starch when compared to bacteria alpha samylases. A. Orzae has an efficient system for secretion of protein and is extensively used to produce industrial enzyme.

the enzyme production is also n important part of work the sensitive action at the time of enzyme production are many of one among them is tempture, Effect of Temperature: Enzymes are most sensitive to heat in dilute solution and in the absence of substrate. ... The conversion of starch by a-Amylase increases in rate with rising temperature to a maximum of about 80oC. Heating above this temperature begins to destroy the activity.

Material and methods:

1. Collection of plant sample: Fresh leaves, stem, collected from selected medicinal plant tulsi, gulvel, curry leaves, aloe were collected from the Shreeyash Institute of Pharmaceutical Education and Research Botanical garden Aurangabad Maharashtra (India).

2. Isolation of Bacterial Endophytes:The isolation technique was followed from the standard protocol. The fresh plant leaves were subjected to surface sterilization procedure. The leaves were cut in to small pieces (0.5-1.0cm) and

washed in running tap water and rinsed in 70% ethanol for 30 second finally wash in sterile water and pat dried. Then it is placed on nutrient agar and incubated 37°c for 7 days. After an incubation period the plates were observed for the growth of endophytic bacteria and fungi.

3. Microorganism: Aspergillus orzae species was isolated from medicinal plant and maintain on nutrient agar slant and subcultured .

4. Screening for amylase producing fungi :The isolated strain was streaked into starch agar plate and incubated at room temperature for 72 hours. After incubation 1% of iodine solution was layered on the agar plates and zone of clearance was observed for screening the fungi.

5. Extraction of amylase from the fermentation medium : After incubation the fermentation medium was harvested by centrifugation at 5000 rpm for

20 min at 4°c. They Supernatant was collected and subjected to estimate the amylase activity.

6. Effect of temperature: To study the effect of temperature on amylase production the submerged fermentation was carried out at different temperature. (25°C,30°C, 35°C ,40°Cand 45°C).

7. Effect of pH: The fermentation medium was prepared by varying the pH values (5.0, 6.0,7.0,and 8.0) for the production of amylase.

8. Submerged fermentation of Amylase : Submerged fermentation was carried out in the Ehlenmeyer flasks by taking 100 ml of amylase production medium (Bernfed, 1951); containing Peptone (6.0g/L), MgSO4,(0.5g/L), KCl (0.5g/L), Starch (1g/L). In addition to this certain vegetables waste were used as a submerged fermentation medium.

It was modified with certain carbon source, nitrogen source and heavy metals. Carbon

sources (each 2g/L) included are glucose, fructose, mannitol, mannose, starch, sucrose, lactose and dextrose. Nitrogen sources included are NH4Cl, NaNO3, NH4NO3, KNO3, Peptone, Urea and Yeast extract (2g/L) as nitrogen source. Certain heavy (each 1mm/L) metals like Ca2+, Cu2+, Mg2+, Fe2+, Hg2+, Zn2+ Mn2+, K2+ and P2+ were also

included in the production medium. The medium was maintained at a pH range of 3, 6 and 9, at 30°C on a shaker with 120rpm for 6 to 18 days.

9. Enzyme assays : Five different enzyme assays, plus weight loss tests were conducted to investigate the abilities of the endophytes to produce wood degrading and other enzymes. Enzyme production can be separated into intracellular and extracellular production. The cellulose test only measured extracellular cellulose production, while the other tests measured both intracellular and extracellular enzyme production.Extracellular enzyme production ratio = the ratio of clear zone diameter to that of colony diameter.The extracellular enzymatic reactions of the following tests were,

classified into 4 types:

1. Strong reaction: the extracellular enzyme ratio was higher than or equal to 2.

2.Medium reaction: the extracellular enzyme ratio was less than 2 but more than 1.

3.Weak reaction: the extracellular enzyme ratio was equal to or less than 1.

4. No reaction: there is no reaction at all.

For intracellular enzymes (those endophytes with extracellular enzyme ratio equal to or less than 1), the diameter of the clear zone was used as a measurement of the amount of enzyme production . Amylase Starch agar was prepared and autoclaved. The test fungi were inoculated onto the agar plates and incubated for ten days. The plates were flooded with a dilute iodine solution (Lugol's iodine). After flooding with iodine, the starch stains blue-black and the zone of degradation around the colonies is either stained brown or remains colourless (Peterson and Bridge, 1994). Starch solution was prepared by dissolving 10 g soluble starch in 50 ml distilled water. This was stirred to give an even paste and then heated at 70-80ºC for 1-2 minutes before adding to medium. Cellulose azure agar was used to test for the presence of it .

Result:-

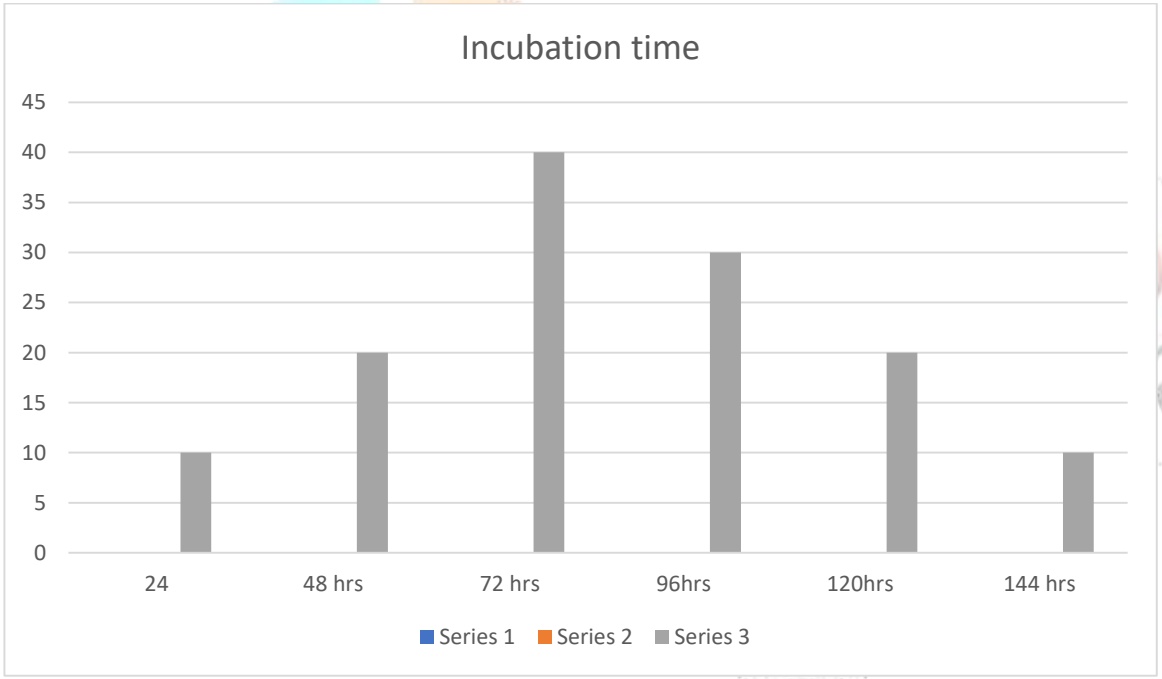
Selection of strain- Among nine different strains of fungi only two strain gave good amylolytic activity which was identified as Aspergillus oryzae, Aspergillus niger. They show good amylase activity.

Optimization for amylase production

The optimization of culture conditions were studied based on the stepwise modifications of the governing parameters such as, incubation time, inoculum size, inoculum age, pH, temperature, and effect of nitrogen sources.

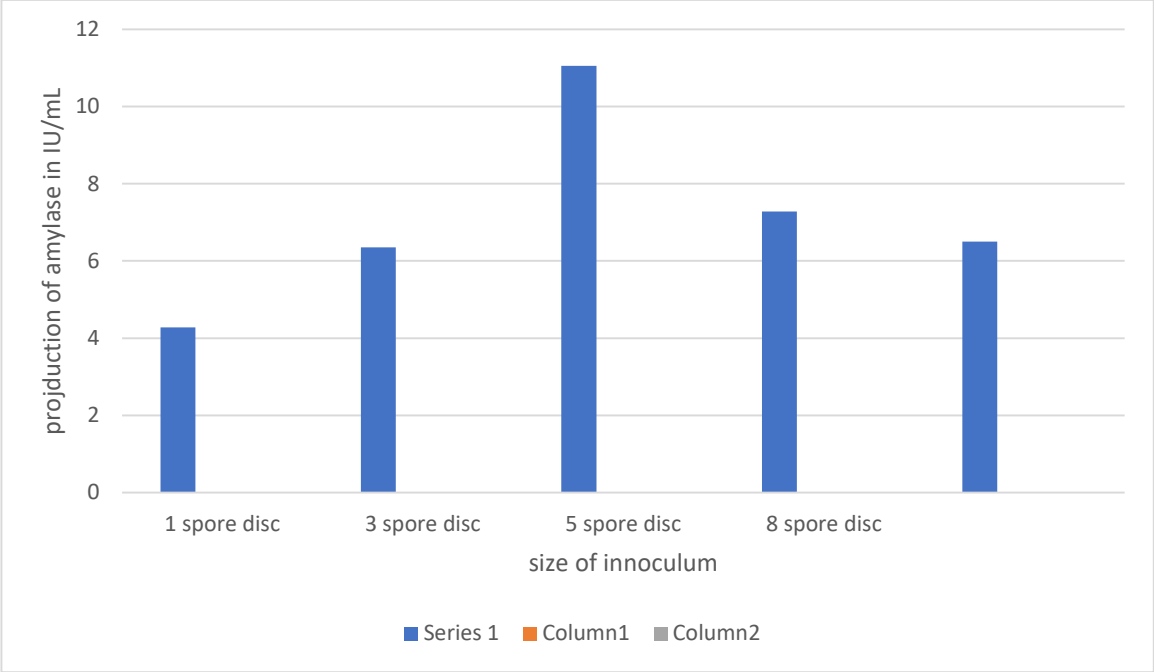
Incubation time

Time course play a very crucial role in fungal metabolic activity and growth. The incubation time necessary for optimal biosynthesis varies between different enzymes produced from one substrate (Smiths et al., 1996). Then flasks were incubated at different time duration: 24 to 144 hrs and amylase production determined at 24 hrs intervals. Optimum fermentation period was found at 72 hrs for A. oryzae in submerged state fermentation . Further increase in incubation time will decrease the enzyme production. It might be due to deficiency of nutrient, accumulation of toxic substances (Chamber etal., 1999, Shafique et al., 2009). The α-amylase production was increased with the increase in the incubation period at initial and found to be maximal after 72 hrs of inoculation. The results indicated that enzyme was secreted early in active growth phase and reached maximum towards the end of exponential growth phase.



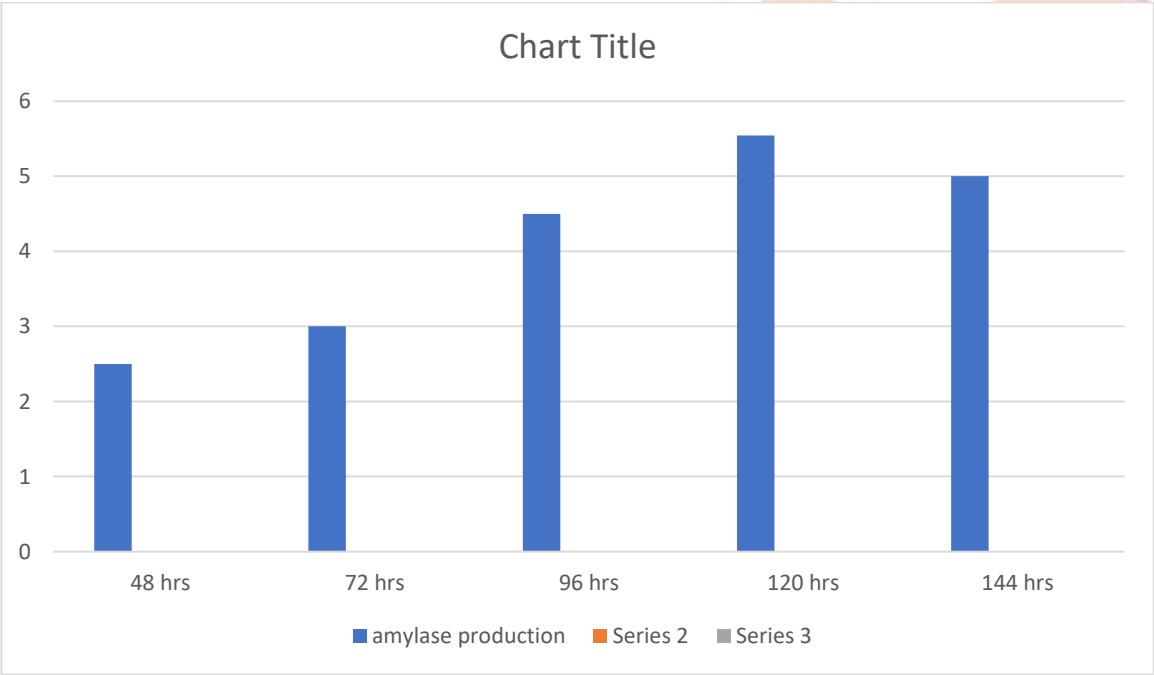
Inoculum size

It is one of the most important parameter which affects the enzyme activity by affecting substrate utilization rate.To determine the optimum inoculum size for enzyme biosynthesis, it was examined with addition of different inoculum size from 1, 3, 5, 8 and 10 spore discs of 8 mm size of A. oryzae, in to culture medium. Maximum amylase production was 11.05 IU/mL found when inoculum size was 5 discs . Further increase or decrease in inoculum size decrease in amylase production. Initial microbial load also affects the growth and primary metabolite production. The smaller inoculum size may extend the lag phase of fungal growth (Sharma et al., 1996). An increase in inoculum size generally improves the growth and growth related activities of the fungal culture up to certain level, but then there could be a reduction in microbial activity due to nutrient limitation. This requires a longer time to grow to yield optimum number to utilize the substrate and to form desired product.



Inoculum age

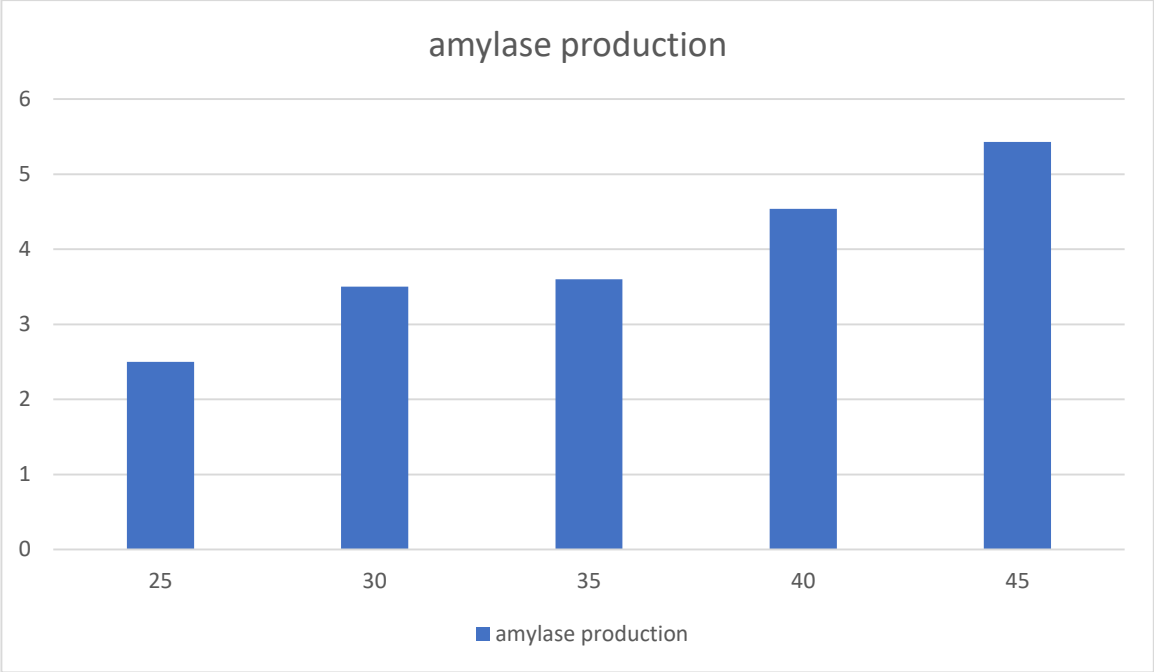
Different age of Aspergillus oryzae from 48 to 216 hrs was selected for production of enzyme. Result indicates a maximum amylase production (5.543 IU/mL) observed at age of 120 hrs of A. oryzae. Inoculum age plays a very critical role in fungal metabolic activity and growth. The inoculum age necessary for optimal biosynthesis varied between different enzymes produced from one substrate (Smiths et al., 1996). This type of observation due to, the substances are initially more susceptible, making a rapid rise in biosynthesis of enzymes. But with the prolongation of cultural time, the susceptible portions are completely hydrolyzed by microorganism, which inhibits the enzyme secretion pathways (Haq et al., 2006).



Temperature

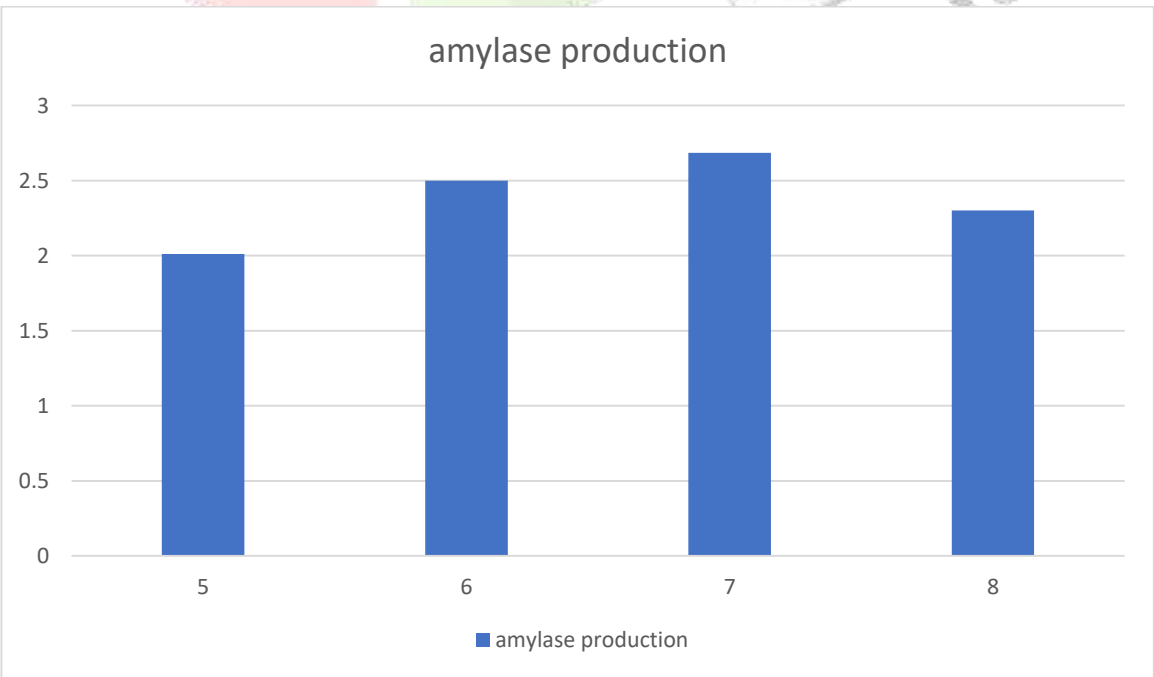
The effect of varying incubation temperature, 15ºC, 25ºC,35ºC and 45ºC checked on the production of α-amylase using A. oryzae. Result presented in shows that maximum amylase production was observed at 45ºC. Probably the most important factor among all the physical variables affecting the performance is the incubation temperature, because both cell growth and the production of enzymes and other metabolites are usually sensitive to temperature (Krishna C., 2005). α-amylase production by fungi is related to the growth which sequentially depends upon the incubation temperature (Muhammad et al., 2012). Hence, the optimum temperature depends on whether the

culture is mesophilic or thermophilic (Sivaramakrishnan et al.2006). The decrease in enzyme activity was observed at higher temperature because of change in membrane composition and cause protein catabolism as well as inhibition of fungal growth. Also the deleterious effect of high and low temperature on spore germination, cell growth, product formation, sporulation and consequently on the overall productivity of the fermentation process is reported by Moreaux (1980). The result also indicated that the enzyme production corresponded closely the growth of the fungus. Similar types of observation were recorded by Kheng and Omar (2005); Sudgen et al. (1994).



pH

The hydrogen ion concentration has a marked effect on enzyme production. The effect of different pH 5, 6, 7 and 8 on amylase production checked using A. oryzae is Amylase production was maximum 2.685 IU/mL at pH 7. At lower or higher pH, may be the affect stability of extracellular enzyme values and causes the rapid denaturation (Kalra and Sandhu, 1986). Further increase in the initial pH resulted decreased in the enzyme production. Initial pH of the medium is known to affect the synthesis and secretion of α-amylase (Muhammad et al., 2012). α-amylase production by microbial strains strongly depends on the extracellular pH as it influences many enzymatic reactions as well as the transport of various components across the cell membrane (Ellaiah et al., 2002).



Nitrogen source

The effect of various organic and inorganic nitrogen sources viz. peptone, urea, ammonium sulfate, yeast extract, sodium nitrate and potassium nitrate on the production of amylase was checked. The effect of nitrogen source on production of α-amylase using A. oryzae in SmF. In case of organic nitrogen sources yeast extract was found better for amylase production (5.248 IU/mL). The production of primary metabolites by organisms is highly influenced by their growth which depends on the nutrients provided. Similar type of observation found by Muhammad et al., (2012) when using A. niger . Many workers Oshoma et al., (2010); Valaparla, (2010); Anto et al., (2006); Pederson and Neilson (2000), reported that yeast extract as an organic nitrogen source produces maximum amylase production.

Total α- amylase (IU) Total Protein (mg) Specific activity Purification fold

Crude 18.6 23.69 0.785 1

30% 1574.7 7.80 201.8 84.66

70% 346.32 39.46 8.776 18.62

100% ND* ND ND ND

*ND- Not detected

Production and partial purification of amylase

Total amylase activity was found 1574.7 IU. The extracellular enzyme was partially purified to homogeneity from the culture filtrate. A summary of purification achievement is presented . The overall 84.66 fold purification of α-amylase was achieved with specific activity of 201.8 . Kinetic properties of amylase Kinetic properties of the partially purified α-amylase viz., substrate concentration, pH and temperature were determined.

Substrate concentration

Maximum α-amylase activity was found 46.56 IU/mL for

partial purified amylase with 1.5% starch as the substrate Beyond 1.5% starch concentration, there was no increase in α-amylase activity. The α-amylase activity from this organism was greatly influenced by the concentration of the substrate. With fixed enzyme concentration, an increase in the concentration of substrate results in increase in enzyme activity until a saturation point is reached. This is probably because at high substrate concentration, ineffective complexes are formed between enzyme and substrate. Also, since the substrate molecules are too many around the enzyme molecules, they may be bound to regions on the enzyme, which are not the active site or alternatively, may crowd the active site (Dixon and Webb, 1971). The Km and Vmax values of α-amylase determined through Lineweaver–Burk plot for hydrolysis of soluble starch at 50°C, pH 4.8 and reaction time 15 min, were 1.4 mg ml-1 and 37.037 U ml-1, respectively. The Km values of 1.9, 3.5 and 10 mg ml-1 for starch have been reported for glucoamylases from F. solani (Bhatti et al., 2007); Acremonium sp. (Marlida et al., 2000); A. niger (Selvakumar et al., 1996).

pH

Most fungi are able to grow in a wide pH range. Studies on the influence of pH on α-amylase activity revealed pH 7.0 as optimum . The enzyme activity is markedly affected by pH. This is because substrate binding and catalysis are often dependent on charge distribution on both, substrate and enzyme molecules (Shah and Madamwar, 2005).

Temperature

The effect on enzyme activity was studied in the in them range of 25°C to 45°C. The optimum α-amylase activity was found at 45ºC i.e. 5.563 IU/mL. At 30ºC, 40ºC remarkably lesser enzyme activity is observed as seen in . At higher temperature amylase activity was decreased because of enzyme denaturation (Bakare et al., 2005).

CONCULISON

Total 9 fungal cultures were isolated from medical plants which was collected. From the primary screening it was found that all the isolates have ability to inhibit the growth of micro-organisms and to produce amylase enzyme and showed clear zone of solubilization on starchy organic waste(leaves woods etc.) agar plate. Among the 9 isolates

Aspergillus oryzae as exhibiting good amylolytic enzymes production. After parametric optimization maximum amylolytic activity was observed, when pH of mineral salt medium was 7.0, incubation temperature of 45°C, after incubation of 120 hrs by using 50 mL of starchy organic waste as sole carbon source with 5 discs of A. oryzae. After studying the kinetic properties of α-amylase, its maximum activity was found at pH 7, temperature of 45°C, with 1.5% of substrate concentration. The Vmax and Km value were 37.037 IU/mL and 1.4 mg/mL respectively for α-endocellulase. From these results, we can say that starchy organic waste can use for large scale amylase production as cheap carbon source, so reduce production cost of amylase and can also solve pollution problem.

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