JOURNAL OF PURE AND APPLIED MICROBIOLOGY, August 2014. Vol. **8**(4), p. 3147-3153

Quantitative Estimation of α-amylase of *Bacillus spp*. Isolated from Water and Sediment of Bhitarkanika Mangrove Ecosystem

Priti Binita Lakra, Sukantibala Mohapatra and Pratima Ray

Orissa University of Agriculture and Technology, Center for Post Graduate Studies, Department of Microbiology, Bhubaneswar - 751003, Orissa, India.

(Received: 11 January 2014; accepted: 18 March 2014)

Over millions of years, mangroves have evolved both morphologically and physiologically to adapt to swampy and saline environments. Similar adaptive characteristics in the form and function may occur with the associated micro flora in such environments. The present study was carried out to isolate and identify microbes in general and *Bacillus spp.* in particular from soil and sediment sample of Bhitarkanika mangrove ecosystem of Orissa coast to explore the extracellular enzymatic potential of the isolates. Generally *Bacillus spp.* are gram positive rods and resistant to high temperature. Soil and sediment samples were collected and was exposed to a temperature of 65°C for one hour in a water bath to remove undesirable soil bacteria and to isolate the heat resistant *Bacillus* spp. by dilution plate technique using *Bacillus* Hi-Chrome Agar. After Gram staining, spore staining and biochemical studies following Bergey's Manual of Systematic Bacteriology (Pelczar,Chan & Krieg), it was observed that the collected soil sample has a rich flora of *Bacillus spp.*. After biochemical test, the isolates were found to be *Bacillus cereus, Bacillus subtilis, Bacillus sphaericus, Bacillus polymyxa, Bacillus macquariensis, Bacillus smithii, Bacillus licheniformis, and Bacillus pasteurii.* All the isolates were screened for production of alpha-amylase and were found positive .Among all, *B.smithii* was the highest amylase producer i.e. 18.07 unit/ml at 55°C. After 4th day of incubation, the qualitative and quantitative assay of the enzyme was done following 3, 5-Dinitro salicylic acid method (Miller, 1959). Again the enzyme production was optimized at different pH, 'C' sources, and 'N' sources.

Key words: *Bacillus*, Amylase, Biochemical identification, Qualitative and Quantitative assay.

Amylases have been reported to occur in microorganisms, although they are also found in plants and animals. Two major classes of amylases have been identified in microorganisms, namely α-amylase and glucoamylase. Alpha-Amylases (endo-1,4-a-D-glucan glucohydrolase) are extracellular enzymes that randomly cleave the 1,4-a-D glucosidic linkages between adjacent glucose units in the linear amylose chain. Glucoamylase (exo-1, 4-a-D-glucan glucanohydrolase), hydrolyze single glucose units from the non reducing ends of amylose and amylopectin in a stepwise manner. Among various extracellular enzymes, alpha-amylase ranks first in terms of commercial exploitation. Spectrum of applications of a-amylase has widened in many sectors such as clinical, medicinal and analytical chemistry. Besides their use in starch saccharification, they also find applications in baking, brewing, detergent, textile, paper and distilling industry. Amylase catalyzes the hydrolysis of starch. It has also application in pharmaceutical, food and detergent industries. Biotechnological importance of these enzymes has been realized by beverage industries for the purpose of saccharification. The use of enzyme in automatic dish washing detergents is also

* To whom all correspondence should be addressed.
Mob.: +91-7894742376;
E-mail: preetibinita@gmail.com

becoming popular. Amylase is also used in high maltose syrup (HMS) in pharmaceutical industries. The objective was to isolate *Bacillus* species from the soil and sediment of mangrove forest ecosystem and qualitative and quantitative estimation of α-amylase production by the isolated *Bacillus* spp. in search of a good amylase producer. Mangroves, over millions of years, have evolved both morphologically and physiologically to adapt swampy and saline environments. Similar adaptive characteristics in the form and function may occur with the associated micro flora in such environments. Some reports are available on the occurrence of free living and symbiotic microorganisms in such saline habitats. However information on micro flora of Bhitarkanika mangrove ecosystem is scanty. Keeping this in view, the present study was carried out for evaluation of extracellular activities of the microbial diversity in the Bhitarkanika mangrove ecosystem of Orissa coast.

MATERIALS AND METHOD

Collection of samples

Location

Soil and sediment samples were collected from different locations including outer estuaries, creeks at lower elevation, creeks at higher elevation and transitional areas.

Season

Soil and sediment samples were collected during winter by random sampling.

Source

Various types of soil and sediment present in Bhitarkanika mangrove ecosystem served as source material.

Isolation

Undesirable bacteria were removed by heating the samples at 65°C in a water bath for one hour to isolate the heat resistant *Bacillus* species by dilution plate technique using *Bacillus* Hi-Chrome Agar. The *Bacillus* species were identified from selected colonies following Bergey's Manual of Systematic Bacteriology (1981).

Microscopic examination

Gram staining

A drop of sterilized distilled water was taken on the middle of the clear slide. Then a loop full of bacterial suspension (young culture) was transferred to the sterilized drop of water, mixed properly, and a very thin film was prepared on the slide by spreading uniformly. The film was fixed by passing it over the gentle flame for two to three times. The slide was flooded with crystal violate solution and allowed to stand for 30 second and then washed thoroughly with gentle stream of tap water. The slide was then immersed in iodine solution for one minute and washed thoroughly with 95% alcohol for 10 second. Excess alcohol was drained off and washed thoroughly with gentle stream of tap water. The slide was then flooded with safranin for one minute, washed with tap water, blotted dry, and examined under microscope under 100x objective.

Spore staining

The spores were stained with malachite green stain. The bacterial smear was flooded with the stain, steamed (avoid boiling) over a flame for 10 minutes, washed under tap water, counter stained with safranin and observed under 100X objective. The spores took green stain under oil immersion objective.

Biochemical test

Mannitol motility

The formation of mannitol was indicated by the acid production which changed the color of the butt from red to yellow. Diffused growth along the stabbed line was considered positive test for motility while growth only on the stabbed line indicated a negative test for motility.

Catalase Test

One drop of 30% hydrogen peroxide was placed on a slide. One loop full of fresh bacterial culture was taken by a sterile needle and placed on the drop of hydrogen peroxide previously taken on the slide. Bubble production indicates positive result.

Urease test

Urease splits urea to ammonia and carbon dioxide, and was detected by inoculating the bacterial cultures into tubes containing urea broth having filter sterilized urea, and incubated for 72 hrs. Purplish pink coloration of the medium indicates a positive reaction.

Indole production test

One loop full of fresh bacterial culture (24 hour old) was inoculated in tryptone broth and incubated at 37°C for one to three days. After incubation, Kovac's or Salkowski's reagent was

added (1:1) volume and shaken vigorously for one minute. A pink ring at the interface of two solutions indicates positive result.

Nitrate reduction test

Nitrate reduction test was carried out in nitrate broth. The freshly prepared cultures were incubated in sterile nitrate broth containing tubes and incubated at 37°C for 24 hours. After incubation sulphanillic acid and α-napthyl amine mixture (1:1) was added. The appearance of deep pink color showed that bacterial isolates reduced nitrate to nitrite.

Voges Proskauer test

Voges Proskauer test was carried out in one ml of fresh bacterial culture grown in MRVP broth. After incubation, a mixed solution of α-napthol and potassium hydroxide was added. Development of crimson red color indicating presence of acetyl methyl carbinol confirmed positive for Voges Proskauer test.

Hydrolysis of starch

Hydrolysis of starch was carried out by taking 1% soluble starch on NA plates and spot inoculating the bacterial culture over that plate. After incubation for 24 hour at 30°C, the plates were flooded with iodine solution for five minutes. Hydrolysis of starch was indicated by a clear zone around the growth and unhydrolysed starch gave a blue color.

Quantitative assay of amylase

Preparation of inoculums

Each bacterial isolate was transferred from stock culture to 100 ml nutrient broth. The broths were then centrifuged at 10,000 rpm for 10 min. After centrifuging the pellets were re-suspended in 10 ml of sterile water and absorbance was recorded at 660 nm to have a suspension size 4.5×10,000 cells/ml.

Assay of amylase (By 3, 5-Dinitro salicylic acid (DNSA) method of Miller, 1959)

In DNSA assay method, the media composed of 0.5% soluble starch, 0.3% Di-potassium hydrogen phosphate and 0.1% $MgSO_4.7H_2O$ was used. A volume of 100ml of the medium was put in a conical flask and inoculated with 1ml of bacterial suspension. After 96 hours of incubation, the broth was centrifuged at 8,000 rpm for 10 min. In 2 ml of supernatant, 100 µl of 1% starch was incubated with 1 ml of phosphate buffer (pH 6.5). The mixture was incubated for 20 minutes before stopping the reaction by adding 0.5 µl DNSA reagent and cooling in a water bath for 10 minutes. A volume of 2.5 ml of distilled water was added, the absorbance was read at 540 nm using UV visible spectrophotometer against glucose as standard curve. One unit of enzyme activity is defined as the amount of enzyme which release 1µmole of glucose equivalent per minute under the assay condition (1 U/ml/min).

Effect of different carbon sources on amylase activity

To study the effect of different carbon sources (glucose, maltose, sucrose, and soluble starch), the isolates were inoculated in to assay medium with pH 7.3 at 37°C for four days in a water bath shaker to obtain a uniform growth with different carbon sources .

Effect of different nitrogen sources on amylase activity

To study the effect of nitrogen on amylase production, the study was carried out at different nitrogen sources like yeast extract, casein, urea and peptone. The isolates were inoculated in to assay medium containing different nitrogen sources with pH 7.3 at 37°C in water bath shaker for four days to obtain a uniform growth.

Determination of the effect of pH on amylase activity

The enzyme assay was carried out in media having different pH i.e. (4.5 to 8.5) by DNSA method.

Determination of the effect of different incubation temperature on amylase activity

The enzyme assay was carried out in media having different temperature i.e. (25, 30, 35, and 40°C) by DNSA method.

RESULTS AND DISCUSSIONS

Ten Bacillus strains were isolated, five from soil and five from the sediment. The pure cultures were subcultured on nutrient agar, gram stained and processed for spore staining in malachite green. All the ten isolates were identified biochemically following Bergey's Manual of Systematic Bacteriology. The results of the biochemical test and starch hydrolysis for amylase activity were presented in table -1, 2 respectively. The ten isolates were identified as *Bacillus sphaericus* (2 no.), *Bacillus polymyxa*, *Bacillus*

macquariensis, *Bacillus smithii* (2 no.), *Bacillus licheniformis*, *Bacillus cereus*, *Bacillus pasteuri* and *Bacillus subtillis* (Table 3).

Standard curve of Glucose

The result obtained from the test conducted to find out the standard curve for glucose is given in Fig. 1. The best result of the OD value of glucose when plotted against the concentration of glucose gives a straight line indicating the standard curve for the test. This standard curve was used to find out the unit of amylase activity throughout the study.

Enzyme assay

As per the objective of the study, the quantitative assay of amylase of the *Bacillus* species isolated from the soil and sediment sample

Table 1. Results of Biochemical tests

Isolates	Citrate test	Urease test	MR test	VP test	Indole production test	Catalase test	Nitrate reduction test	Mannitol, motility test
W1	+	-	+	+	-	+	-	+,-
W5	-	-	-	+	-	+	+	+,-
W8	-	-	+	-	-	+	+	+,-
W11	+	-	-	+	-	+	-	-,+
W12	+	-	-	-	-	+	+	+,+
S11	-	-	-	+	-	+	-	+,+
S13	-	+	+	-	-	+	+	-,+
S14	-	+	-	-	-	+	-	-,-
S19	-	-	-	+	-	+	+	+,-
S24	+	+	+	-	-	-	+	+,-

W stands for water S stands for Soil

Table 2. Starch hydrolysis test

Organism	Starch hydrolysis
W1	+
W5	+
W8	+
W11	+
W12	+
S11	+
S13	+
S14	+
S19	+
S24	+

Table 3. Identification of Isolates

Organism	Name of the isolates
W1	*Bacillus sphaericus*
W5	*Bacillus polymyxa*
W8	*Bacillus macquariensis*
W11	*Bacillus sphaericus*
W12	*Bacillus smithii*
S11	*Bacillus smithii*
S13	*Bacillus licheniformis*
S14	*Bacillus subtillis*
S19	*Bacillus cereus*
S24	*Bacillus pasteuri*

Table 4. Optimization of amylase production (Unit per ml) on different incubation period

Organism	Day1	Day 2	Day 3	Day 4	Day 5
W_5	1.68	7.5	13.36	13.36	1.73
W_8	8.98	7.28	12.07	12.07	12.41
S_{11}	1.6	8.48	18.07	18.07	2.74
S_{13}	6.93	7.10	11.39	11.39	2.05
S_{14}	8.48	1.31	18.07	18.07	2.74
S_{19}	8.48	9.07	11.39	10.86	1.28
S_{24}	9.07	7.34	10.87	14.39	17.38

collected from Bhitarkanika mangrove forest, was obtained by 3, 5- Dinitro salicylic acid method (Miller ,1959) and were put in to a tabular form after matching with the standard curve derived for glucose. The OD values obtained from the sample when put in to the standard curve indicate the quantity of glucose in mg/ml released in 20 minutes of incubation time. From the value of glucose, the amylase unit was calculated following the definition given in the material and methods. The results obtained from these bacterial isolates with respect to their amylolytic unit are given in the table 4-8.

The incubation period considered in the present experiment was with the reported range of other workers (Riaz *et al*, 2009; H.Anto *et a*l, 2006; Gangadharan *et al*, 2006). The incubation time is governed by characteristics of the culture and also based on growth rate and enzyme production. Fig-1 shows a gradual increase in enzyme production through 24, 48 and maximum at 72hours i.e. 18.7 unit/ml by *Bacillus smithii* and *Bacillus subtilis*. The enzyme yield showed a gradual decrease on further extention of fermentation after 4th day of incubation.. The decrease in enzyme yield after the optimum level may be because of denaturation or decomposition of á-amylase due to interaction with other component in media. After 4th day of incubation *Bacillus pasteuri* and *Bacillus*

Table 5. Optimization of pH for Amylase production (Unit per ml)

Organism	pH 3.5	pH 4.5	pH 5.5	pH 6.5	pH 7.5	pH 8.5
W_5	8.1	9.0	9.16	12.1	11.2	10.8
W_8	9.8	12.4	11.2	13.2	12.8	11.9
S_{11}	8.7	11.6	12.4	11.2	11.9	9.6
S_{13}	9.9	12.8	12.0	9.5	13.4	10.2
S_{14}	10.4	13.1	15.6	10.5	14.6	10.5
S_{19}	11.2	9.8	10.5	10.6	10.8	9.3
S_{24}	9.8	13.2	9.50	9.2	13.8	13.1

Table 6. Optimization of different carbon sources for Amylase production (Unit per ml)

Organism	Glucose	Sucrose	Dextrose	Starch
W_5	1.45	10.70	8.65	16.8
W_8	9.33	18.07	12.07	8.9
S_{11}	12.24	10.10	14.81	1.6
S_{13}	9.42	14.64	9.07	6.9
S_{14}	9.16	17.81	1.22	8.4
S_{19}	12.33	12.07	15.76	8.4
S_{24}	9.07	8.9	11.20	9.07

Table 7. Optimization of different nitrogen sources for amylase production (Unit per ml)

Organism	Urea	Casein	Beef extract	Peptone
W_5	13.27	14.64	6.02	16.87
W_8	11.22	11.56	11.47	8.98
S_{11}	11.82	12.50	10.70	5.3
S_{13}	10.62	11.64	9.50	6.9
S_{14}	1.02	11.56	8.48	8.4
S_{19}	1.02	11.90	9.59	8.4
S_{24}	11.64	11.56	11.64	9.07

Table 8. Optimization of incubation temperature for amylase production (Unit per ml)

Organism	25°C	30°C	35°C	40°C
W_5	3.4	3.6	6.7	4.3
W_8	2.6	3.9	7.1	4.1
S_{11}	2.4	4.2	7.2	3.1
S_{13}	3.1	4.8	6.5	3.5
S_{14}	2.8	3.2	7.3	3.9
S_{19}	2.6	2.9	6.4	4.1
S_{24}	3.2	3.1	6.9	4.0

maquariensis produce larger quantities of enzymes instead of 72 hours at which all other strains produce maximum amount of enzyme. This contradicts the report of previous authors. Further standardization is necessary for each strain to consider the optimum incubation period.

Among the physicochemical parameters, the pH of the growth medium plays an important role by inducing the morphological changes in enzyme secretion. The production of á-amylase is very sensitive to initial pH of the fermentation medium (Haq *et al*, (1995) reported that a pH range of 7.5-8.0 to be the best for the production of á-amylase by *Bacillus subtilis*, which corroborates the present finding. Both the used strains produce optimum amount of amylase at neutral pH. *Bacillus smithii* produce 15.67 unit/ml and *Bacillus subtilis* 14.6 respectively. Variation in pH resulted from the substrate consumption and metabolite production affects the enzyme activity.

Different carbon sources affect differently the production of α-amylase and the most commonly used carbon source is starch (Bajpai and Bajpai, 1989). High enzyme titers were

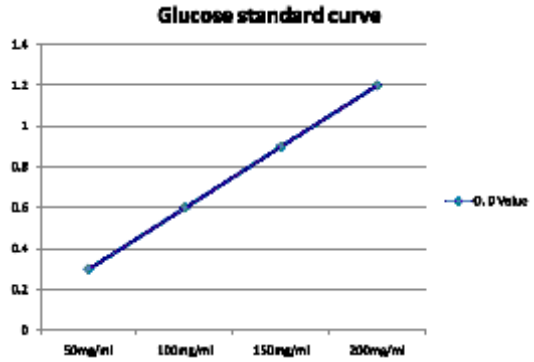

Fig. 1. Glucose standard curve

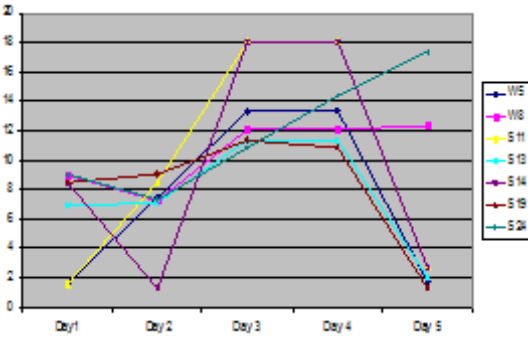

Fig. 2. Optimization of amylase production (Unit per ml) on different incubation period

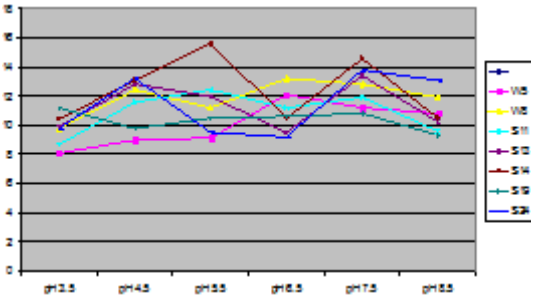

Fig. 3. Optimization of different pH for Amylase production (Unit per ml)

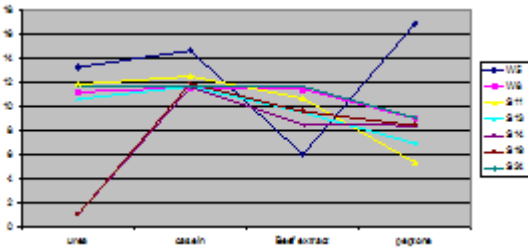

Fig. 4. Optimization of different carbon sources for Amylase production (Unit per ml)

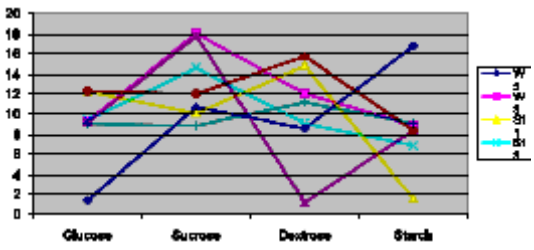

Fig. 5. Optimization of different nitrogen sources for Amylase production (Unit per ml)

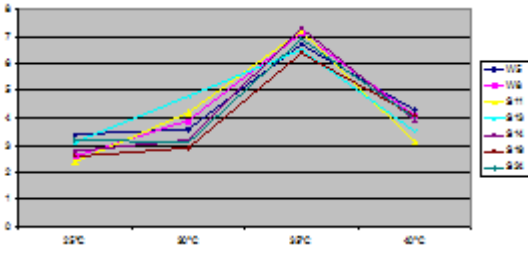

Fig. 6. Optimization of incubation temperature for amylase production (Unit per ml)

obtained in sucrose supplemented fermentation media than the other carbon sources. In the present study amylase production was more on sucrose and less on starch as compared to other carbon sources at 55°C. From the Table -8, it was observed that the *Bacillus maquariensis* produced more enzymes (i.e.) 18.07 unit/ml in medium containing sucrose as carbon source. Again *Bacillus smithii* produces 17.81 unit/ml of enzyme in the presence of sucrose. The present result cannot be correlated with the findings of other workers because no literature is available in the favor of above two organisms. In contrast carbon sources such as glucose, dextrose and starch did not enhance the amylase production in the above two organisms. *B.lichenifermis* produced 14.64 unit/ml of enzyme when sucrose is the carbon source and for *B.subtilis* , the enzyme activity was 17.81 unit/ml.

Added nitrogen sources have been reported to have an effect on the production of various enzymes including α-amylase (Pedersen and Neilsen, 2000). Earlier report showed that various nitrogen sources favor growth and enzyme production. Lower level of nitrogen is inadequate for the enzyme production and excess nitrogen is equally detrimental causing enzyme inhibition (Dharani and Aiyer, 2004). Presence of beef extract was found to be the best studied nitrogen source for amylase production.

The supernatant amylolytic activity was assayed at different temperatures ranging from 25ºC to 45ºC. Fig. 4 showed the effect of different temperature on the production of á-amylase by *Bacillus spp*. Maximum production of amylase was obtained at 35ºC which was supported by Vidyalaxmi *et.al.* (2009). Increase in incubation temperature further decreased the production of enzyme. The production of enzyme was greatly inhibited at 40ºC, which may be due to less growth of bacteria at that temperature.

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