Chapter 1: Isolation and identification of potent acidophilic amylase producing fungal strain from starchy waste

1.1. Introduction

The applications of different microbes in different industries like food, pharmaceutical etc. have been widely accepted. Particularly bacteria and fungi are the predominant producers of many extracellular compounds. Most of the extracellular enzymes are produced by microbes through fermentation. Commercial production of enzyme from fungi and bacteria is also cost effective.

In present days amylases have many applications in textile and paper industries (Pandey et al., 2000). Amylases are produced from different sources like animal, plant, microbes etc. (Kathiresan and Manivannan, 2006). Among the sources the most preferred source is microbial source because of its availability. The microbial extracellular enzymes can be released into the culture medium. Therefore, enzymes are easily found from culture medium. Soil contains different essential nutrients so it can be considered as the reservoir of microorganisms. Various factors like temperature, pH, pressure, air composition, minerals, carbon source, energy source, interaction between different microbes can affect the ecology and population dynamics of microorganisms in soil. Therefore the activities of microorganisms depend on soil. Microorganisms cannot take the macronutrients directly from the soil so they convert these macronutrients to monomeric substances by different extracellular enzymes secreted by them. After that the monomeric substances diffuse into the cell and generate energy. These monomeric substances act as catabolite inducer of enzyme synthesis.

There are several microorganisms which can produce amylase, was reported by Okolo et al. (2000), Mishra et al. (2005), Baks et al. (2008), Rasooli et al. (2008). Production of acidophilic amylase from *Bacillus cereus* MTCC 10205 was reported by Kumari et al. (2013) and from *Aspergillus niger* was reported by Gupta et al. (2008). Acidophilic amylases are mainly used in the glucose syrup producing industries.

Amylase from *Aspergillus* sp. had different important role in conversion of starch and starchy materials (Reddy and Yang, 2006). The raw starch degrading amylases have been reported from different fungal species (Forgarty and Kelly, 1979; Okolo, 2000). To study the activity of acidophilic amylase different fungi were isolated which were active in acidic condition.

1.2. Materials and Methods

1.2.1. Collection of sample

Soil samples were collected from Municipal Garbage, Midnapore bus stand; Vidyasagar University canteen; paddy field of Gopegarh; potato cultivation field of Dharma with predominant in starchy waste. For sampling, soil was collected from a depth of 5-8cm. The collected soil samples were mixed homogeneously and finally soil was taken in sterilized plastic bag and preserved at 4 ^{o}C at laboratory until use. Direct starchy waste like rotten potatoes in cultivation field, rice grain infected with fungi were also considered for sampling and isolation of microbes.

1.2.2. Dilution of soil sample

Each soil and starchy waste sample was suspended at a ratio of 1:10 (w:v) in sterilized distilled water in Erlenmeyer flask. The flasks were shaken thoroughly on a rotary shaker for 1 h. In each case the suspension was allowed to stand for some time to settle down heavy particles and serial dilution was performed in aseptic condition.

1.2.3. Media selection for isolation of acidophilic fungi

The primary screening was done by using Czapek dox agar with slightly modified composition as (in g/l): [$NaNO_3$, (3.0); $MgSO_4$, (0.5); KCl, (0.5); $FeSO_4$, (0.01); KH_2PO_4, (1.0)] and sucrose replaced with starch (1%) and pH was maintained at 3.0. For solidification 2% agar was added to media (pH- 3.0) and autoclaved. After that to avoid the unwanted bacterial growth 100 mg/l of Ampicillin was added. Then 0.1 ml of diluted sample (10^{-3}-10^{-9}) was poured on starch agar media (20 ml) containing Petriplates separately followed by spread plate technique. The Petriplates were incubated for 72h at 27

^{o}C. After incubation the plates were flooded with iodine solution and kept for 1 min for color changes.

1.2.4. Preparation of spore suspension

Various fungal isolates from the primary screening were subjected to formation of spore suspensions. The spore suspensions were prepared by addition of sterilized distilled water containing Tween 80 (2 drops/ 100 ml) to 72 h-old fungal isolate growing on slants of Czapek dox agar media. The final concentration of the spore suspensions were adjusted to about 2×10^2 spores / ml and preserved at 4 °C for secondary screening.

1.2.5. Preparation of potato peel

The potato peels were collected from Vidyasagar University canteen. Then the peels were washed with normal and warm water to remove dirt. The washed potato peels were then dried in sunlight. The dried peels were grinded in a mixture grinder into a particle size of around 0.8 mm and employed in the studies.

1.2.6. Secondary screening of the isolates

In secondary screening, those fungal isolates producing predominant clear zone were cultured in a medium containing salt solution (%, w/v): [$NaNO_3$, (3.0); $MgSO_4$, (0.5); KCl, (0.5); $FeSO_4$, (0.01); KH_2PO_4, (1.0)] with dry potato peel (0.5g), at pH 3.0. After inoculation with 1 ml of spore suspension, the fermentation was carried out at 27 ^{o}C for 72 h. The fungal strain was selected by highest α- amylase activity given by shortest fermentation period.

1.2.7. Assay for α- amylase activity

The amylolytic activity of isolate was determined by following method described by Miller, (1959). 0.5 ml of crude enzyme was incubated with 1 ml of soluble starch (1%, w/v) prepared in acetate buffer (0.2 M pH 3.0) at 37 °C for 60 min. The reaction was terminated by the addition of 1 ml of 3, 5- dinitrosalicylic acid (DNS) reagent. The released reducing sugars were estimated colorimetrically at 540 nm. One unit of amylase activity was defined as the amount of enzyme releasing 1 µg of reducing sugars (glucose equivalents) per minute under standard assay condition.

1.2.8. Identification of potent fungal strain

The potent fungal strain was identified based on macroscopic (morphological) and microscopic observation. Then the taxonomic study was performed by 18S r DNA gene sequencing. The amplified PCR product submitted to GenBank to search homologous sequences. Blast algorithm was used to search for homologous sequence in GenBank (for accession number). The 18S r DNA sequences were aligned using the Clustal – X program (Sivaramakrisnan et al, 2006) and the phylogenetic tree was generated by PHYLIP.

1.3. Results and discussion

1.3.1. Isolation and primary screening of α- amylase

In this study fifty amylase producing fungi were isolated from four different places for the primary screening (Table 1.1). During primary screening, on the basis of clear zone formation a total of twenty five (25) α- amylase producing fungal strains (RBP) were isolated (Table 1.2). Among them 4 strains were selected for secondary screening from each place. These four strains were named as RBP1, RBP5, RBP7, RBP15. According to clear zone formation RBP7 showed best result by 70 mm clear zone in 72h in starch containing Czapek Dox agar (Fig. 1.1, 1.2) (Table 1.3). Omemu et al. (2004) stated that *Aspergillus niger* AMO7 produce 7.0 mm zone of hydrolysis. Again, Malik et al. (2017) showed that amylase producing fungi FP2 and FBa3 produce 26 mm and 25 mm zone of hydrolysis respectively.

Table 1.1. Collection of soil sample from different sites of Midnapore

Site of collection	Number of sample collected	Character
Midnapore bus stand	RBP1-RBP10	Municipal garbage
Vidyasagar University	RBP11-RBP16	Canteen waste
Dharma	RBP17-RBP21	potato cultivation field
Gopegarh	RBP22-RBP25	paddy field

Table 1.2. Isolated fungal strains on basis of clear zone formation

Name of strains	Zone of clearance (mm)
RBP1	30
RBP2	25
RBP3	20
RBP4	15
RBP5	27
RBP6	19
RBP7	70
RBP8	22
RBP9	17
RBP10	12
RBP11	16
RBP12	10
RBP13	18
RBP14	24
RBP15	38
RBP16	21
RBP17	11
RBP18	14
RBP19	23
RBP20	19
RBP21	8
RBP22	11
RBP23	9
RBP24	10
RBP25	8

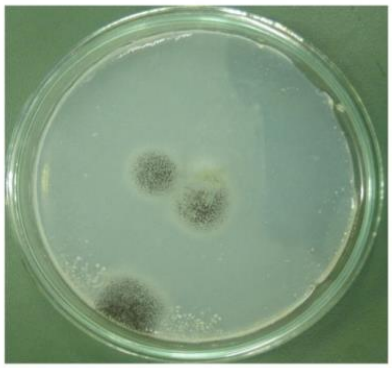

Fig. 1.1. Phenotypic appearance of the colony of RBP 7 on Czapek dox agar medium.

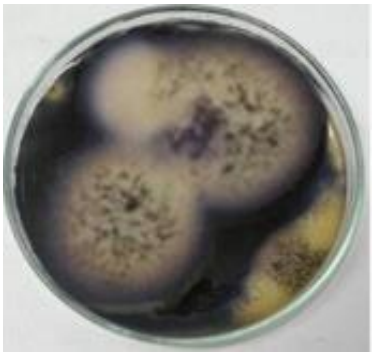

Fig.1.2. Zone of hydrolysis produced by RBP 7 on starch containing medium was visualized by iodine.

Table 1.3. Selection of fungal strain after primary screening

Sl. No.	Name of strains	Zone of clearance (mm)
1	RBP1	30
2	RBP5	27
3	RBP7	70
4	RBP15	38

1.3.2. Secondary screening of fungi

In secondary screening four primarily selected fungal strains RBP1, RBP5 and RBP7 from Midnapore Bus stand and RBP15 from Vidyasagar University canteen were examined for amylase production on starch containing liquid medium (Table 1.4).

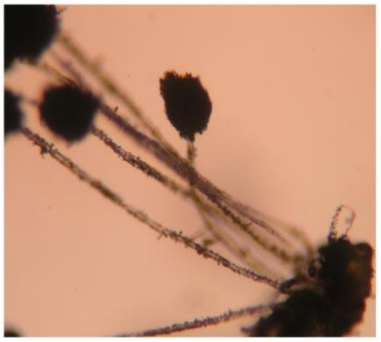

Fig.1.3. Lightmicroscopic picture of newly isolated RBP7

Among them the strain RBP7 was selected for its highest α-amylolytic activity. RBP7 and RBP15 showed highest amylolytic activity because waste material from canteen of Vidyasagar University and the garbage of Midnapore Central Bus stand contain residual food particles as well as starchy waste materials. This starchy products influence the growth of fungus. Naturally fungal hyphae has the ability to penetrate over the starchy raw materials to uptake nutrients. Sometimes, the hydrolytic products act as inducer for expression of amylolytic enzymes.

Whereas RBP1 and RBP5 showed lesser amylolytic activity because the soil sample of paddy field and potato field do not contain major amount of starchy waste and the result of the expression capacity of amylolytic gene from microorganisms including fungi will be low. Alternatively the soil of these two fields sometimes enriched with excess nitrogenous chemical fertilizers which may have detrimental effect on the production of amylase. Thus the expression level of isolates from starchy waste material (canteen of Vidyasagar University and Midnapore Central Bus stand) shown better results in comparison with potato field.

After the isolation of RBP7, it had been observed under light microscope (Fig. 1.3) and also under SEM (Fig. 1.4). In this study 135.7 U/ml amylase was produced by RBP7 in specified condition (at pH 3.0, temperature 27 ^{o}C, incubation period 72 h). Previous study showed that 0.37 U/ml amylase was produced by *Aspergillus niger* (Bedan et al., 2014). The fungal strain YPO3 and FP2 produce maximum 13.7 U/ml and 13.57 U/ml amylase respectively (Malik et al., 2017).

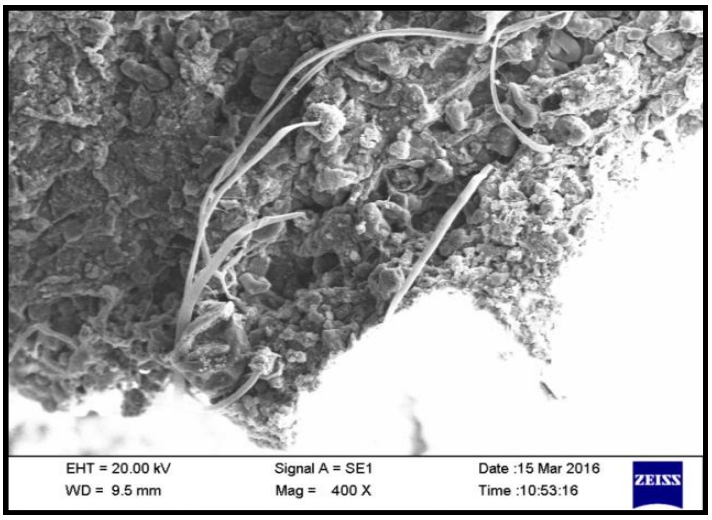

Fig.1.4. SEM image of the strain RBP7 showing the sporangium containing the conidia

Table 1.4. Production list of amylase by selected fungi in potato peel containing liquid medium

Sl. No.	Name of strains	Enzyme activity (U/ml)
1	RBP1	76.57
2	RBP5	97.35
3	RBP7	135.7
4	RBP15	81.55

1.3.3. Identification of the selected fungal strain

Microscopic observation of the isolated fungi showed morphology similar with *Aspergillus* sp. Phylogenetic analysis based on 18S rDNA sequences showed that the isolate has maximum similarities with *Aspergillus niger* species (Table 1.5). The sequence has been submitted to GenBank (accession number KX 100578.1) (Fig. 1.5). The identifying character of the strain RBP7 was also much similar with that of *Aspergillus niger* reported by Gupta et al., (2008). *Aspergillus niger* played a major role in the development of industrial processes. Furthermore, virtually all 25 species of *Aspergilus* secrete acidophilus amylase. The genus occupies a unique position in the industrial field by virtue of its large number of important species and the array of enzyme produced by them (Li et al., 2012).

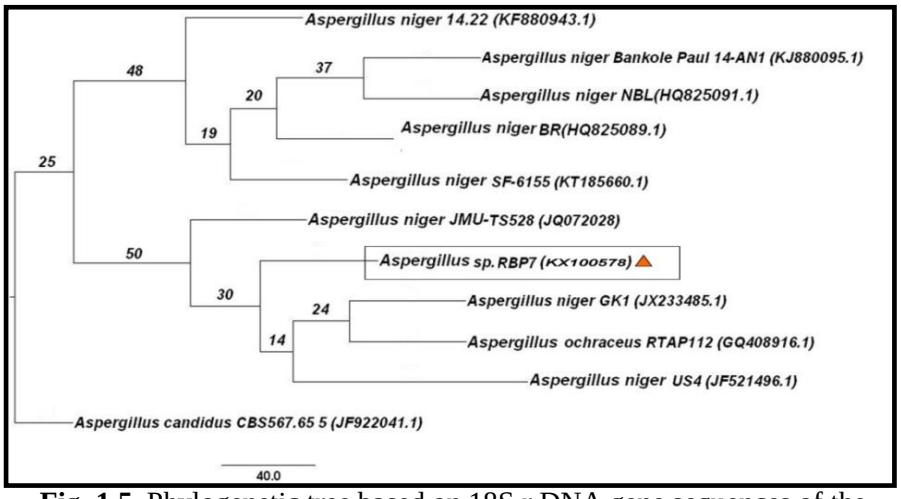

Fig. 1.5. Phylogenetic tree based on 18S r DNA gene sequences of the newly isolated strain *Aspergillus niger* RBP7

Table 1.5. Phenotypic and microscopic characteristics of potential fungal strain RPB7

Character	**RBP7**
Macroscopic Character	
Colony diameter (mm)	70±2.5
Colony color	dark black
Microscopic Character	
Conidia	dark black
Mycellium	Dull white
Conidia breadth (µm)	15.0-16.0
Conidiophore color	Hyaline yellowish or brown
Surface texture	Smooth
Conidiophore size(µm)	3.6-4.0
Conidiospore	Globose
Surface texture	Rough walled
Vesicle diameter (µm)	30.0-32.3
Philides (µm)	Biserinate
Length (µm)	7.0

The physicochemical parameters like optimum pH, temperature nutrient concentration of medium are the most important factors for enzyme production (Gupta et al., 2003). In primary selection process starch was used as carbon source. Ryan et al. (2006) stated that starch is easily accessible source of energy. Acidophilic amylase production by using fungal strain was reported by Prakasham et al. (2007), Pathak and Narula, (2013). The pH have immense effect on amylase production was studied by Ramachandran et al (2004).

2.4. Conclusion

The fungi *Aspergillus niger* RBP7 secrets significant amount of acidophilus amylase when grown in starch containing liquid medium. The genus *Aspergillus* had played a major role in the development of industrial process. The genus occupies a unique position in the industrial field by virtue of its large number of important species and the array of enzyme produced by them.