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SCREENING OF AQUATIC FUNGI FOR ANTIMICROBIAL AND AMYLASE EFFICACY ISOLATED FROM FISH CULTURING PONDS OF SOUTH-EAST COASTAL ANDHRA PRADESH, INDIA

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Keywords:

Penicillium SAPB-15, *Aspergillus* SAPB-20, Antimicrobial activity, Amylase production, Bioactive metabolites

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ABSTRACT: Objectives: The main intention of the current study was to isolate, antimicrobial assay and amylase screening of fungal strains SAPB-15 and SAPB-20 isolated from freshwater fish culturing ponds of Kaikaluru and Mudinepalli villages, South-East coast of Andhra Pradesh, India. **Methods:** The water samples were collected from different fish culturing aquatic ponds and transported to the laboratory. A serial dilution plate technique was employed for fungi isolation by using Czapek-Dox agar and Sabouraud dextrose agar media. Twenty fungal strains were isolated, all of them were screened for their antimicrobial and amylase production. The potential fungal strains were designated as SAPB-15 and SAPB-20. Partial characterization of the strains was carried out based on cultural, micro-morphological characteristics. **Results:** The potent fungal strains were provisionally identified as *Penicillium* species and *Aspergillus* species. The secondary metabolites produced by strains inhibited several test bacteria and fungi and the strains also exhibited a positive response to amylase production. **Conclusion:** Aquatic ponds are treasure houses for potential microbes. Among the twenty fungal strains, SAPB-15 and SAPB-20 were found to possess antimicrobial potential, and genuses of the strains were identified as *Penicillium* and *Aspergillus*.

INTRODUCTION: Rapid proliferation of new diseases by a variety of microbial pathogens is developing day by day. They have become most problematic to the cultivation of different aquatic organisms (fishes, prawns, oysterssters *etc*.). Therefore, there is an urgent need to screen novel antimicrobial agents that can play a crucial role in inhibiting pathogens; they were screened from unusual environments.

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An aquatic ecosystem includes freshwater habitats (rivers, lakes, ponds, streams, swamps, and wetlands) and marine water habitats (oceans, reefs, seabed, *etc*.) Freshwater Aquatic ponds are stagnant aquatic ecosystems flooded with nutritional resources usually seen in the southern coastal regions of Andhra Pradesh.

Moreover, the freshwater fish culturing ponds provide a unique ecological niche for the growth of diversified microorganisms (bacteria and fungi) that produce exclusive secondary metabolites of industrial importance. Many of which were causing diseases to the aquatic organisms especially fish [1]. Fungal diseases are the most serious cause of aquaculture losses, resulting in considerable economic losses in industries [2]. Many of the fungi

that affect fishes from surrounding water environment are considered opportunists [3], attacking the fishes when they are stressed or immune-compromised or when they have lost their mucus protection because of trauma or excessive handling or due to inadequate nutrition [4, 5]. Hence, it is necessary to identify fungal organisms associated with cultivated ponds in area [6]. Despite their ability to cause diseases, their importance as a beneficial role in different industries has gained more interest. Among the various microbes, Fungi were treated as an ideal source for producing a variety of natural compounds that can efficiently be exploited as bioactive properties. More specifically, fungi displayed antimicrobial, anticancer, anti-diabetic, anti-oxidant, antiviral, anti-inflammatory, anti-parasitic, and immune-modulatory properties [7, 8].

In the present study, Kaikaluru and Mudinepalli villages were selected because they are geographically situated near the Kolleru Lake, which was a naturally eutrophic lake situated between two major river basins of the Godavari and the Krishna, which functions as a natural flood balancing reservoir between the deltas of the two rivers. Kolleru lake wetland gained Ramsar convention of International importance in 2002. *Catla catla, Labeo rohitha, Cirhinus mrigala* and *Channa punctata* are these areas' major cultivated fresh water fishes.

In a screening of different Freshwater Aquatic ecosystems of Kaikaluru and Mudinepalli villages, Andhra Pradesh, the potent strain *Penicillium* (SAPB-15) and *Aspergillus* (SAPB-20) with broad-spectrum activity against Gram-positive and Gram-negative bacteria as well as fungi was found. An attempt has been made in the present study to identify the potent strains based on the cultural, micro-morphological, and antimicrobial approaches as well as enzyme production efficacy.

MATERIALS AND METHODS:

Sample Collection: Water samples were collected from different locations of freshwater fish culturing ponds of Kaikaluru and Mudinepalli villages, South-East coast of Andhra Pradesh, India. Water samples were collected randomly in sterile packed containers and transported carefully to the laboratory.

Isolation of Fresh Water Fungi: Serial dilution plate technique was used to isolate fungi by using collected water samples [9]. Sabouraud-dextrose agar (SDA) and Czapek-Dox agar (CDA) media amended with sodium chloride (2%) are used for fungi isolation. Streptomycin (30µg/ml) was added to retard the growth of bacteria. Aliquots of samples were prepared through the serial dilution plate technique. 0.1ml of each dilution (10^{-2} to 10^{-4}) of samples was spread over the SDA and CDA media. At 37°C, the plates were incubated and observed for the growth of fungi. After 5-10 days of incubation, the fungal colonies were picked up [10].

Maintenance of Fungal Cultures: The 20 fungal strains appeared, and they were purified through the streak plate method. The colonies were picked up, streaked over CD agar plates, and incubated at 37ºC. Further, the strains were maintained on CD agar slants and stored at 4ºC for further study.

Screening of Fungi for Antimicrobial Metabolites Production: All the isolated fungal strains were screened for antibacterial activity against *Staphylococcus aureus* (MTCC 3160), *Bacillus subtilis* (ATCC 6633), *Xanthomonas campestris* (MTCC 2286) and *Escherichia coli* (ATCC 9027) and antifungal activity against *Candida albicans* (MTCC 183) and *Alternaria*.

Identification of the Potent Fungal Strains: The potent fungal strains were identified based on colony characteristics (colony size, color, shape, appearance, and pigment production) and micromorphological (mycelium, conidiophores, and conidia) characteristics.

Cultural and Morphological Characteristics of the Strains SAPB-15 and SAPB-20: The potent fungal strains were cultured on different culture media such as SDA, CDA, NAM, YMD and PDA. After 5 days of incubation, colony characteristics were observed. The strains were initially identified by morphological characterization by plating the fungi on SDA and incubating them for 5 days. The growth and presence/ absence of pigmentation were noted. The fungi were identified by studying their cultural characteristics, spore formation, and mycelium arrangement. Slides were prepared by cover-slip culture technique using Lacto-phenol

Cotton Blue (LPCB) reagent under a compound microscope [11].

Growth Pattern of the Strains SAPB-15 and SAPB-20: The growth pattern of the two strains were determined by inoculating them into the 100 ml Sabouraud-dextrose broth with 2% NaCl. The culture broth was inoculated and incubated at 35±2° C on rotary shaker at 120 rpm. All the flasks were harvested at 3 days intervals up to 27 days and mycelium dry weight was calculated, determining the growth of the two strains. The culture filtrates obtained after separating the biomass were extracted with ethyl acetate and antimicrobial activity of the crude extract was determined by agar well diffusion method [12].

Metabolites Extraction and Antimicrobial Activity: The agar well diffusion method determined the antimicrobial activity of the two strains. The sterile culture suspension was prepared by suspending 5-day-old culture in saline, was used to inoculate CD broth (seed medium) and incubated at 35°C for 7 days on a rotator shaker at 120 rpm. The 10% seed culture was transferred to CD broth (fermentation medium). The fermentation was carried out at 35±2°C for 27 days under agitation at 120 rpm. The antimicrobial metabolites were recovered from the above filtrate by extraction with ethyl acetate. The ethyl acetate was added to the filtrate (1:1) and vigorously shaken. The solvent extract was evaporated to dryness in a water bath, and thus obtained residue was used to determine its antimicrobial assay. Ethyl acetate alone was used as a negative control. About different concentrations (25, 50, 75 and 100µl) of two cultures of crude extracts (SAPB-15 and SAPB-20) and negative control were also poured into separate wells. The zone of inhibition (mm) was measured after the plates were incubated at 37°C for 48 h [13].

Screening of Fungal Strains for Amylase Activity: Amylase is one of the most important enzymes with great significance in food, pharmaceutical, and fine chemical industries and contributes 25% of the total enzyme market [14]. The production medium, such as starch agar medium (1% soluble starch) was employed to detect the amylase production efficiency of both strains. The medium contains (g/L) Starch, 10; peptone, 10; yeast extract, 20; KH_2PO_4, 0.05; $MnCl_2.4H_2O$, 0.015; $MgSO_4.7H_2O$, 0.25; $CaCl_2.2H_2O$, 0.05; $FeSO_4.7H_2O$, 0.01 and Agar, 30. The pH was adjusted to 6.5, and medium was sterilized in an autoclave for 15 min at 121ºC. The strains SAPB-15 and SAPB-20 were inoculated over the starch agar medium plates and incubated for 48-72h. After incubation, plates were flooded with 1% Gram's iodine solution. Starch hydrolysis by formation of clear zone around the culture colonies against the blue back-ground was taken as positive.

Enzyme Production and Enzyme Assay: The Starch culture broth was prepared and used for amylase enzyme assay. The strains SAPB-15 and SAPB-20 were inoculated into 250mL Erlenmeyer flasks containing 50mL of production broth (Starch culture broth). The flasks were incubated at 30°C in a rotary shaker at 150 rpm for 10 days. After the incubation was completed, the fungi were harvested by filtration, and the filtrate was used as the crude enzyme preparation.

Amylase activity was estimated using Miller, 1959 with minor variations [15]. The reaction mixture was prepared by mixing 1 ml of supernatant with 1 ml of solubilized starch solution and incubated for 10 min at 60ºC. 2 ml of dinitrosalicylic acid (DNS) reagent was added to stop the reaction. The mixture was cooled for 10-15 minutes in a water bath and then centrifuged 2000 rpm for 5 minutes. The enzyme quantity was measured by using Vis-Spectrophotometer at 540 nm.

RESULTS AND DISCUSSION:

Isolation and Screening of Fungi from Water Samples: A total of twenty fungal strains were isolated from the water samples of Kaikaluru and Mudinepalli fish culturing ponds and designated as SAPB-1 and SAPB-20. They were subjected to screening against selected test bacteria and fungi. Interestingly, the isolates SAPB-15 and SAPB-20 exhibited potent activity against the trailed pathogens. The strains SAPB-15 and SAPB-20 were subcultured on CDA incorporated with 2% NaCl and preserved for further studies **Fig.1A, B.**

Cultural and Morphological Characteristics of the Strains SAPB-15 and SAPB-20: Cultural characteristics of the strains SAPB-15 and SAPB-20 were studied on five different media *viz*. SA, CDA, NAM, YMD and PDA **Table 1** and **Table 2**.

The strain SAPB-15 exhibited excellent growth and produced dark brown pigment on SA, CDA and PDA media. There was no pigmentation on NAM, and YMD.

The SAPB-15 colonies are white to dark brown with a fluffy texture, reaching 25 mm diameter after 5 days of incubation at 35°C **Fig. 2A**. The strain *Penicillium* SAPB-15, exhibited typical filamentous hyphae with asexual spore such as conidia.

The hyphae are colorless, slender, tubular, branched and septate hyphae. The hyphae are formed from several threads of mycelium, which can get intertwined into a hyphal network [16].

The strain SAPB-20 exhibited good growth and produced yellow pigment on CDA, SA and PDA media. There was no pigmentation on NAM, and YMD. The SAPB-20 colonies are white to dark yellow-green with fluffy texture, reaching 20-30 mm diameter after 5 days of incubation **Fig. 1B**.

The strain *Aspergillus* SAPB-20 on Czapek-Dox agar, colonies are granular, flat, and white at first but quickly become light greenish yellow. Conidial heads are typically radiated, later splitting to form bi-seriate and few conidial heads with phialides borne directly on the uni-seriate vesicle. Conidiophores are coarsely roughened, and conidia are globose to sub-globose [17] **Fig. 2B.**

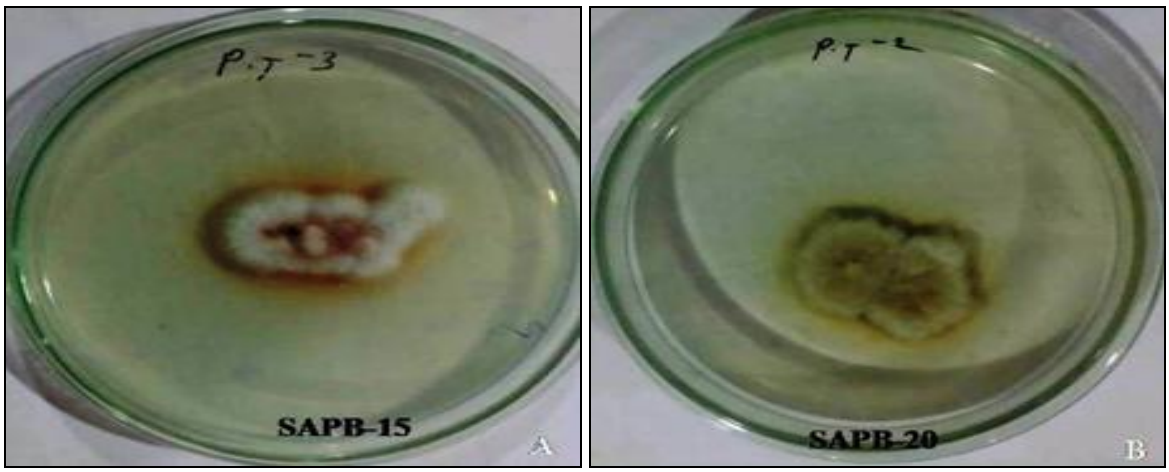

FIG. 1A-B: THE STRAINS SAPB-15 AND SAPB-20 GROWN ON CZAPEK-DOX AGAR PLATES

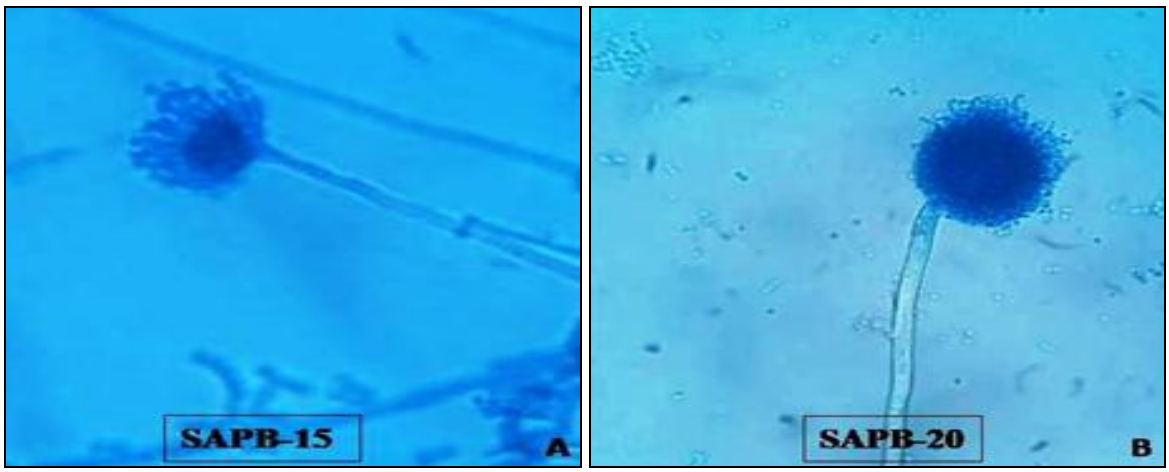

FIG. 2A-B: COMPOUND MICROSCOPE MICROGRAPHS OF THE STRAINS SAPB-15 AND SAPB-20 (40X)

TABLE 1: CULTURAL AND MORPHOLOGICAL FEATURES OF SAPB-15

S. no.	Media	Colony colour	Colony size (mm)	Texture; Growth	Pigmentation(+/-)
1	C.D.A	Pale pink with white periphery	25	Woolly; Excellent	Pale pinkish brown
2	S.A	Pale pink with white periphery	20	Woolly; Excellent	Pale pinkish brown
3	N.A.M	-	-	-	-
4	Y.M.D	Pale pink with white periphery	4	Woolly; Poor	-
5	P.D.A	Pale pink with white periphery	17	Woolly; Good	Pale pinkish brown

TABLE 2: CULTURAL AND MORPHOLOGICAL FEATURES OF SAPB-20

S. no.	Media	Colony colour	Colony size (mm)	Texture; Growth	Pigmentation(+/-)
1	C.D.A	Black colony with white periphery	20	Woolly; Excellent	light greenish-yellow
2	S.A	Black colony with white periphery	18	Woolly; Excellent	light greenish-yellow
3	N.A.M	-	-	-	-
4	Y.M.D	Black colony with white periphery	4	Woolly; Poor	-
5	P.D.A	Black colony with white periphery	12	Woolly; Good	light greenish-yellow

(CDA- Czapek-Dox agar, SA- Sabouraud-Dextrose agar, NAM- Nutrient agar, YMD- Yeast extract malt extract dextrose agar, PDA- Potato dextrose agar).

Growth Pattern and Antimicrobial Profile of SAPB-15 and SAPB-20: The growth curve and antimicrobial profile of SAPB-15 and SAPB-20 were studied at three-day intervals up to twenty-seven days in batch culture. Both strains entered into the log phase on the sixth day, which extended up to 18th day. The strain *Penicillium* SAPB-15 entered into stationary phase is extended from twenty-one to twenty-four days of incubation and finally entered into decline phase **Fig. 3A**. The bioactive metabolites obtained from twenty-one day-old culture of SAPB-15 showed high antimicrobial potential against the trailed pathogens (*Xanthomonas campestris* (22±0.2mm) and *Candida albicans* (20±0.7 mm) **Table 3**. Whereas the strain SAPB-20 entered into stationary phase is extended from fifteen to eighteen days of incubation and finally entered into decline phase **Fig. 3B**. The metabolites obtained from eighteen day-old culture of SAPB-20 showed high sensitivity against the *Escherichia coli* (21±0.2 mm) **Table 3.**

Production of antimicrobial metabolites by 20-day-old culture extracts of *Ascotricha sinuosa* VJCH-18 showed high antimicrobial activity against *E. coli*, *P. vulgaris* and *C. albicans* [20]. Rani *et al*., (2017) reported that the secondary metabolites of isolate NS1 produced the maximum diameter of growth inhibition zone against the *E. coli* with 20 mm clear zone [21]. The antimicrobial assay of both strains SAPB-15 and SAPB-20 cultured on CD broth was tabulated in **Table 3**. The antimicrobial assay was performed in triplicates and Values are the means of three replicates ± SD, statistically analyzed and found to be significant at 5% level.

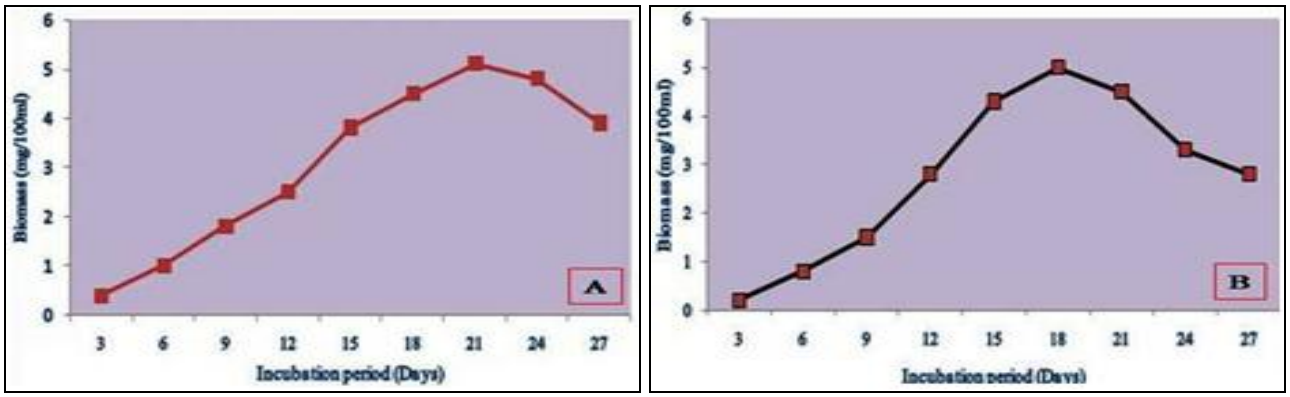

FIG. 3: A-B: GROWTH PATTERN OF *PENICILLIUM* SAPB-15 AND *ASPERGILLUS* SAPB-20

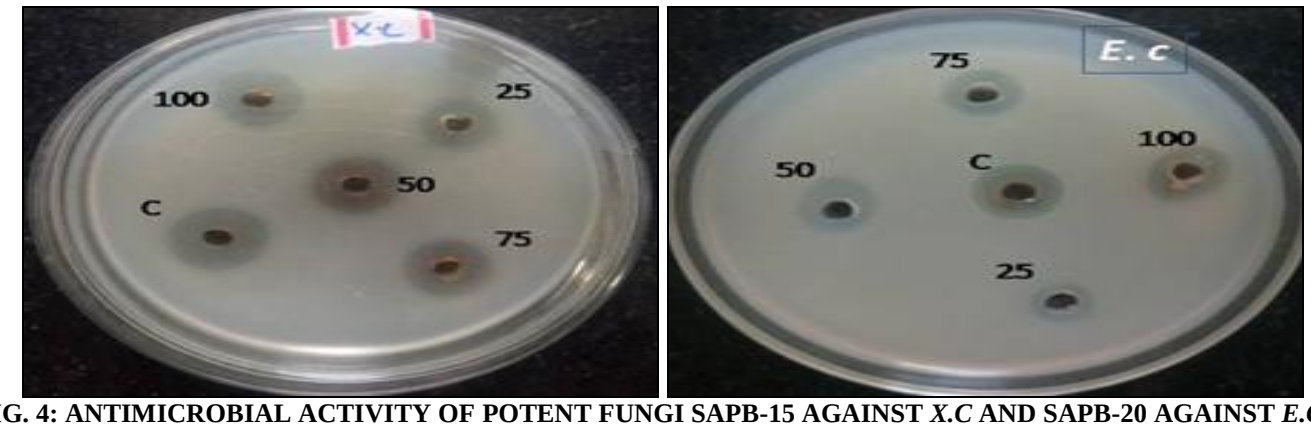

FIG. 4: ANTIMICROBIAL ACTIVITY OF POTENT FUNGI SAPB-15 AGAINST *X.C* AND SAPB-20 AGAINST *E.C*

TABLE 3: ANTIMICROBIAL ACTIVITY OF POTENT FUNGI SAPB-15 AND SAPB-20 AGAINST THE TEST ORGANISMS

Test Strains	Zone of inhibition (mm)	
Bacteria	**SAPB-15**	**SAPB-20**
Escherichia coli	18±0.9	21±0.2
Xanthomonas campestris	22±0.2	14±0.3
Bacillus subtilis	14±0.5	18±0.9
Staphylococcus aureus	10±0.1	16±0.11
Fungi		
Candida albicans	20±0.7	18±0.15
Alternaria	17±0.2	12±0.4

*The results are analyzed statistically and found to be significant at 5% level.

Quantification of Amylase Activity: Amylases are a group of hydrolyses that can specifically cleave the O-glycosidic bonds in starch. Two important groups of amylases are glucoamylase and α-amylase. Glucoamylase (exo- 1, 4-α-D-glucan glucano-hydrolase) hydrolyzes single glucose units from the non-reducing ends of amylose and amylopectin [18]. The α-amylases (endo-1, 4-α-D-glucan glucohydrolase) are extracellular enzymes that randomly cleave the 1, 4-α-Dglucosidic linkages between adjacent glucose units inside the linear amylose chain [19]. Both strains exhibited a positive response to the appearance of a clear zone around the culture colonies after the plates were flooded with Gram's iodine solution (1%). The extracellular amylase activity was determined by dinitrosalicylic acid (DNS) method. The crude fractions of enzyme absorption were measured at 540nm; the measurement provides the validation of amylase production. After seven days of the incubation period, the strain SAPB-15 produced 60.3 (U/ml^{-1}) as the maximum enzyme production. Whereas the same strain produced 8.5 (U/ml^{-1}) as the lowest amylase production after two days of incubation (**Fig. 6A**. The strain SAPB-20 produced the 88.5 (U/ml^{-1}) as maximum amylase production after the six days of the incubation period. Similarly, 10.1 (U/ml^{-1}) was recorded as the minimum production of amylase for the two days of incubation period **Fig. 6B**. Balkan and Ertan, (2005) reported [22], α-Amylase production by *P. chrysogenum* grown in culture broth reached its maximum production at 6–8 days, at 30°C, with a level of 155 (U/ml^{-1}). Similarly, Metin *et al.*, (2010) noticed 180 (U/ml^{-}) as the highest amylase production [23].

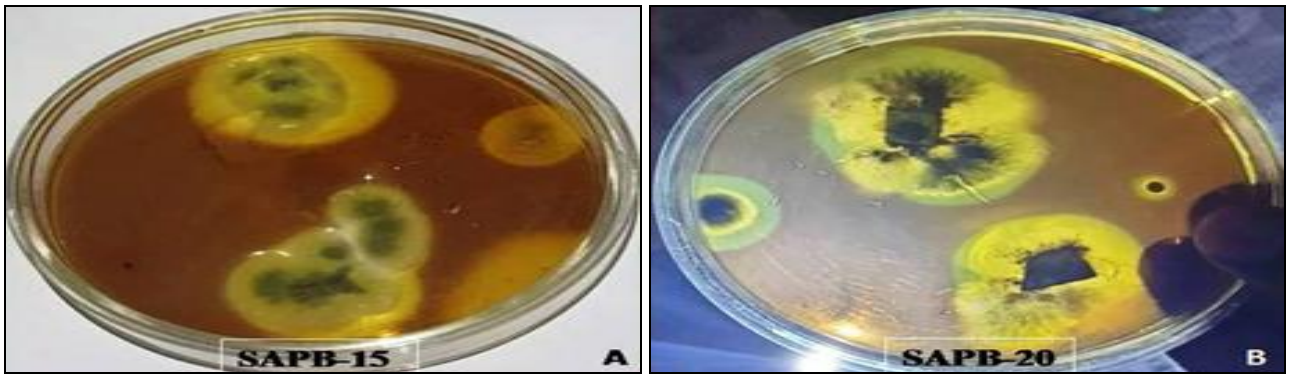

FIG. 5A-B: AMYLASE SCREENING OF THE STRAINS SAPB-15 AND SAPB-20

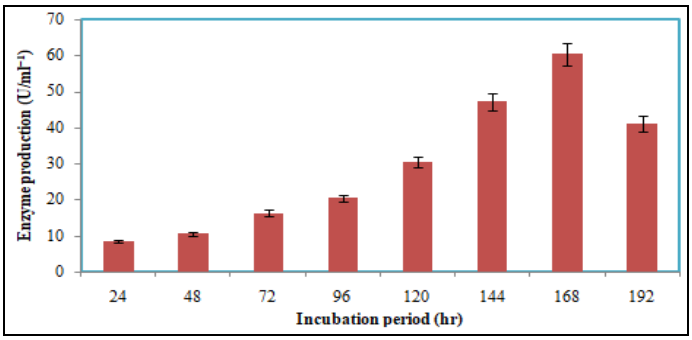

FIG. 6A: EFFECT OF INCUBATION PERIOD ON AMYLASE PRODUCTION BY *PENICILLIUM* SAPB-15. (*Values are the means of three replicates ± SD, statistically analyzed and found to be significant at 5% level).

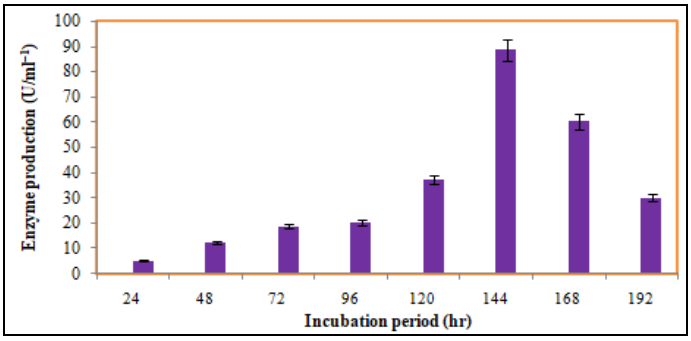

FIG. 6B: EFFECT OF INCUBATION PERIOD ON AMYLASE PRODUCTION BY *ASPERGILLUS* SAPB-20. (*Values are the means of three replicates ± SD, statistically analyzed and found to be significant at 5% level)

CONCLUSION: The present study suggests that bioactive fungal strains isolated from freshwater fish culturing ponds of the South-East coast of Andhra Pradesh can produce many bioactive metabolites necessary for industrial applications.

Among the isolated fungi, the strains SAPB-15 and SAPB-20 were potent against trailed pathogens with significant amylase production. Hence, further studies on molecular characterization and optimization parameters of *Penicillium* SAPB-15 and *Aspergillus* SAPB-20 for improved metabolite production are in progress.

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CONFLICTS OF INTEREST: The authors declare no conflict of interest.

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