Production, Characterization Of Proteases By Solid State Fermentation Using Sugarcane Bagasse By Warcupiella Spinulosa.

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Abstract: Solid state fermentation is well appropriate for the production of various enzymatic complexes. Microbial proteases are the most important industrial enzymes with significant applications in food, leather and pharmacy industries. In the present study the endophytic fungal strain was isolated from mangrove forest situated in Pichavaram, TamilNadu, India. The aim of this study was to confirm the ability of endophytic fungi in production of industrially important extracellular enzymes. The production of protease by Warcupiella spinulosa was studied under Solid State Fermentation, with substrates like cotton seed, sugarcane bagasse, rice straw, rice bran, wheat bran, and mixing of all the above-mentioned substrate with feathers. The results displayed that the optimum conditions for maximum protease production were found to be Sugarcane bagasse (10,616 U/ml) as the substrate, 7th day of Incubation(10,833 U/ml) , Inoculum size 3%(13,216 U/ml) and Beef extract(1%) (13,216 U/ml) as Nitrogen source, the Metal Ions Fe_2SO_4(0.1%) (14,083 U/ml) supported the high enzyme activity, at pH 7 and Temperature RT. The production of protease was carried out at various selected substrates like Sugarcanebagasse,3%, Beefextract (1%), Fe_2SO_4(0.1%) ,pH(7),Temperature(RT) ,Day-7 and the results show that best for maximum neutral protease production (20,366 U/ml). The study was proved that Warcupiella spinulosa was able to produce a very high level of protease under Solid State Fermentation using inexpensive Sugarcane bagasse.

Index Terms: Endophytic Fungi, Protease, Solid state fermentation, Sugarcane bagasse

1 INTRODUCTION

The specific regions where mangrove plants grow are called Mangrove ecosystem. Mangroves are fast decreasing in the number of species with increasing freedom [1] (Bandaranayke, 1998). Fungi of Mangroves are called Manglicolous fungi. They are important for nutrient cycling and they can yield all the required enzymes to degrade lignin, cellulose and other plant components (Fell JW et al., 1984; Findlay et al., 1986; BremerGB ,1995) [2,3,4]. Enzymes are mainly produced by bacteria and fungi, they are also found in plants, animals and microorganism (Sidney Lester, 1972) [5]. Endophytic fungi have extensive variety of proteolytic enzymes than bacteria. The filamentous fungi have a potential to grow under varying environmental conditions such as pH, temperature, wide variety of substrates as nutrients (Haq et al.,2006) [6]. Several species of fungal strains including Aspergillus flavus, Aspergillus melleu, Aspergillus niger, Chrysosporium keratnophilum, Fusarium graminarum, Penicillium griseofulvin, Scedosporium apiosermum are reported to produce protease (Ellaiah et al., 2002) [7]. Substrate also plays an important role in determining the growth of micro-organisms. Substrate is chosen such a way that it should provide physical support as well as nutrients to the growing culture (Kim SS,2001) [8]. Solid state fermentation was one of the important research parts.

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Natural raw materials such as cassava, barley, wheat bran, rice bran, sugarcane bagasse, cassava bagasse, various oil cake like coconut oil cake, palm kernel cake, soybean cake, ground nut oil cake, etc. fruit pulps e.g. apple pomace and corn cobs, saw dust, seeds e.g. tamarind, jack fruit and coffee husk, coffee pulp, tea waste are used as nutrient source for enzyme production (Hesseltine CW, 1972)[9]. Protease production was high in Solid state fermentation than that of submerged fermentation **(**Hesseltine 1972; Ghildyal et al., 1985) [9,10]. Solid state fermentation has many advantages including superior volumetric productivity, cost effective, less effluent release, reduced pollution, high tilters, aeration process is easy, use of simpler machinery, use of inexpensive substrates, simpler downstream processing and lower energy requirements when compared with submerged fermentation (Paranthaman et al., 2009) [11]. The total hydrolysis of proteins catalyzed by microbial proteases are considered as degradative enzymes (Raju et al.,1994; Haq et al., 2006) [12,6]. Microbial proteases are predominantly extracellular. Protease constitutes a large and complex group of enzymes that plays an important nutritional and regulatory role in nature (Singh et al., 2009) [13]. Proteases are necessary for living organisms, they are prevalent and found in a wide diversity of sources. Protease is the most important industrial enzyme of interest accounting for about 60% of the total enzyme market in the world and approximately 40% of the total worldwide enzyme sale (Godfrey et al., 1996; Chouyyok et al., 2005) [14,15]. They are mostly used in detergents (Barindra et al.,2006) [16], food industries, leather, meat processing, cheese making, silver recovery from photographic film, production of digestive and certain medical treatments of inflammation and virulent wounds (Rao et al.,1998; Paranthaman et al., 2009) [17,11]. The thermal stability of the enzymes may be due to the presence of some metal ions or adaptability to carry out their biological at higher temperature (Haq et al., 2006) [6]. Fungal proteases are having more advantages than bacterial protease as they can utilize low cost material, high protease can be produced, which are easier to be recovered from fermentation broth. (Prakasam et al.,2006; Viswanatha et al., 2010) [18,19]. The present study was aimed

to optimize the protease production by Warcupiella spinulosa under SSF by using a suitable substrate and by adjusting suitable substrate, Nitrogen sources, Metal ions and the influence of physical parameters such as Incubation time, Inoculum size, pH and Temperature.

2 MATERIALS AND METHODS

2.1 Chemicals

All chemicals used for the study were of AR grade, it was procured from Merck Ltd., Hi Media Laboratories Pvt Ltd.

2.2 Collection of plant samples

Samples were collected from mangrove forest situated in Pichavaram, TamilNadu, India. Healthy leaves of Suaedo monoica were collected in sterile bags and transported to the laboratory aseptically.

2.3 Surface- sterilization and isolation of endophytic fungi

Isolation of endophytic fungi from S.monoica was carried out using the protocol of (Sunitha et al.,2013) [20]. These samples were cut into small pieces of 1 cm^2 size, washed under running tap water for 10 minutes. It was sterilized in series with 70% ethanol for 1 minute,1.0% sodium hypochlorite for 10 min and further cleaned by passing through two sets of sterile distilled water. The sterilized bits were carefully laid on the Potato Dextrose Agar (PDA) medium plates and incubated for 5 days at 25± 2˚C. For further studies the fungal cultures were sub cultured, and pure culture was maintained in PDA slants.

2.4 Morphological analysis

Fungal isolates were identified by studying morphology of the fungal culture on upper and lower surface of the culture plate.

2.5 Microscopical analysis

Isolated endophytic fungi were stained by lactophenol cotton blue staining procedure and visualized under microscope at 40X magnification. The fungi were identified on the basis of mycelia and spore characteristics.

2.6 Molecular Taxonomy of the potent isolates

2.6.1 DNA Extraction

1. Lysis/homogenization: Cells grown in monolayer should be lysed by suspending 1-3 colonies aseptically and mixed with 450 µl of “B Cube” lysis buffer in a 2 ml micro centrifuge tube and lyse the cells by repeated pipetting.
2. Add 4 µl of RNAse A and 250 µl of “B Cube” neutralization buffer.
3. Vortex the content and incubate the tubes for 30 minutes at 65˚C in water bath. To minimize shearing the DNA molecules, mix DNA solutions by inversion.
4. Centrifuge the tubes for 20 minutes at 14,000 rpm at 10 ˚C.
5. Following centrifugation, transfer the resulting viscous supernatant into a fresh 2 ml micro centrifuge tube without disturbing the pellet.
6. Add 600 µl of “B Cube” binding buffer to the content and mix thoroughly by pipetting and incubate the content at room temperature for 5 minutes.
7. Transfer 600 µl of the contents to a spin column placed in 2 ml collection tube.
8. Centrifuge for 2 minutes at 14,000 rpm and discard flow-through.
9. Reassemble the spin column and the collection tube then transfer the remaining 600 µl of the lysate.
10. Centrifuge for 2 minutes at 14,000 rpm and discard flow-through.
11. Add 500 µL “B Cube” washing buffer I to the spin column. Centrifuge at 14,000 rpm for 2 mins and discard flow-through
12. Reassemble the spin column and add 500 µl “B Cube” washing buffer II and Centrifuge at 14,000 rpm for 2 mins and discard flow-through
13. Transfer the spin column to a sterile 1.5-ml microcentrifuge tube
14. Add 100 µl of “B Cube” Elution buffer at the middle of spin column. Care should be taken to avoid touch with the filter.
15. Incubate the tubes for 5 minutes at room temperature and Centrifuge at 6000 rpm for 1 min.
16. Repeat the above-mentioned step 14 and 15 for complete elution. The buffer in the microcentrifuge tube contains the DNA.
17. DNA concentrations were measured by running aliquots on 1% agarose gel.
18. The DNA samples were stored at -20°C until further use.

2.6.2 PCR Protocol

Polymerase Chain Reaction (PCR) is a process that uses primers to amplify specific cloned or genomic DNA sequences with the help of a very unique enzyme. PCR uses the enzyme DNA polymerase that directs the synthesis of DNA from deoxynucleotide substrates on a single stranded DNA template. DNA polymerase adds nucleotides to the 3`end of a custom-designed oligonucleotide when it is annealed to a longer template DNA. Thus, if a synthetic oligonucleotide is annealed to a single-stranded template that contains a region complementary to the oligonucleotide, DNA polymerase can use the oligonucleotide as a primer and elongate its 3`end to generate an extended region of double stranded DNA.

2.6.3 Composition of the Taq Master Mix

- Taq DNA polymerase is supplied in 2X Taq buffer
- 0.4mM dNTPs,
- 3.2mM MgCl2 and
- 0.02% bromophenol blue.

2.6.4 Primer details

Primer Name	Sequence Details	Number of Base
LR7	5' TAC TAC CAC CAA GAT CT 3'	17
LROR	5' ACC CGC TGA ACT TAA GC 3'	17

Add 5 µL of isolated DNA in 25 µL of PCR reaction solution (1.5 µL of Forward Primer and Reverse Primer, 5 µL of deionized water, and 12 µL of Taq Master Mix). Perform PCR using the following thermal cycling conditions.

1. Denaturation

The DNA template is heated to 94°C. This breaks the weak hydrogen bonds that hold DNA strands together in a helix,

allowing the strands to separate creating single stranded DNA.

2. Annealing
The mixture is cooled to anywhere from 51℃. This allows the primers to bind (anneal) to their complementary sequence in the template DNA.

3. Extension
The reaction is then heated to 72° C , the optimal temperature for DNA polymerase to act. DNA polymerase extends the primers, adding nucleotides onto the primer in a sequential manner, using the target DNA as a template.

2.6.5 PCR conditions

STAGES	TEMP in ˚C	TIME	
Initial Denaturation	94˚C	3 min	
Denaturation	94˚C	45 sec	30 cycles
Annealing	51˚C	30 sec	
Extension	72˚C	2min	
Final extension	72˚C	10 min	
Hold	4˚C	∞	

2.6.6 Purification of PCR Production
Removed unincorporated PCR primers and dNTPs from PCR products by using Montage PCR Clean up kit (Millipore). The PCR product was sequenced using the primers. Sequencing reactions were performed using a ABI PRISM® BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq® DNA polymerase (FS enzyme) (Applied Biosystems).

2.6.7 Sequencing protocol
Single-pass sequencing was performed on each template using below 16s rRNA universal primers. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were resuspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems).

2.6.8 Bioinformatics protocol

1. The sequence was blast using NCBI blast similarity search tool. The phylogeny analysis of query sequence with the closely related sequence of blast results was performed followed by multiple sequence alignment.
2. The program MUSCLE 3.7 was used for multiple alignments of sequences (Edgar, 2004) [21]. The resulting aligned sequences were cured using the program Gblocks 0.91b. This Gblocks eliminates poorly aligned positions and divergent regions (removes alignment noise) (Talavera and Castresana 2007) [22]. Finally, the program PhyML 3.0 aLRT was used for phylogeny analysis and HKY85 as Substitution model.
3. PhyML was shown to be at least as accurate as other existing phylogeny programs using simulated data, while being one order of magnitude faster. PhyML was shown to be at least as accurate as other existing phylogeny programs using simulated data, while being one order of magnitude faster. The program Tree Dyn 198.3 was used for tree rendering. (Dereeper et al., 2008) [23].

2.7 Isolation of micro organism
Fungal strains were isolated from mangrove leaves. The isolated fungal cultures were screened for protease enzyme production. The organisms were identified using lactophenol cotton blue mounting method (Konemann et al.,1997) [24]. The isolated culture Warcupiella spinulosa was purified by routine sub culturing and stored at 4˚C for further analyses.

2.8 Solid state fermentation condition
Solid state fermentation was carried out in 250ml conical flask contains 10 grams of each substrate with 10 ml of Salt solution(g/L) [Composition, KNO3 2.0, MgSO4.7H2O 0.5, K2HPO4 1.0, ZnSO4.7H2O 0.437, FeSO4.7H2O 1.116, MnSO4.7H2O 0.203], pH 7.0 and the flasks were plugged tightly with cotton wool and autoclaved at 121˚C for 30 minutes. After sterilization, the flasks were inoculated with 1mL of fungal spore solution (10^6spores/mL) and incubated at 30˚C for seven days in an incubator shaker at 125 rpm (Chinnasamy muthulakshmi et al., 2011). [25]

2.9 Extraction of Crude Enzyme
At the end of the fermentation, cultures were extracted by adding 100mL of distilled water to each flask and shaken for 2 hours, filtered through cheese cloth. Cell free supernatant was obtained by centrifuging the extract at 10,000 rpm for 20 minutes, followed by filtering through Whatman filter paper. The amount of filtrate that contained the crude enzyme was measured and used for the protease assay (Chinnasamy muthulakshmi et al., 2011). [25].

2.10 Screening of Substrate
Different agricultural byproducts such as cotton seed, sugarcane bagasse, rice straw, rice bran, wheat bran, wheat bran +chicken feather powder(5:1), sugarcane bagasse + chicken feather powder (5:1), Rice straw+ chicken feather powder (5:1), Cotton seed + chicken feather powder (5:1), Rice bran + chicken feather powder (5:1), Mix of soya flour: wheat flour: wheat bran in the ratio of 3:4:3 and Mix of wheat bran+ sugarcane bagasse +rice straw and chicken feather in the ratio of 4:3:3:2 were evaluated for the production of protease in the present work. These substrates purchased from the local market, were grounded in dry blender until the size of the particles reached the desired size and stored at room temperature for further use. The best source (sugar bagasse with salt solution) was used for further study.

2.11 Effect of incubation days on protease production
The fermentation medium- (sugarcane bagasse with salt solution) was inoculated with the fungal strain and incubated for various time intervals 1-8 days. Enzyme production was analyzed at the end of every 24 hours. (Chinnasamy muthulakshmi et al., 2011). [25]

2.12 Effect of inoculum size on protease production
Size of inoculum is a major biological factor in the production of the enzyme. The fermentation medium -(sugarcane bagasse with salt solution) was inoculated with various

percentage of the fungal strain 1%, 2%,3%,4%and 5%. Highest enzyme production was analyzed (Chinnasamy muthulakshmi et al., 2011). [25]

2.13 Effect of various nitrogen sources on protease production

Effect of nitrogen sources on protease production was tested using various sources like Peptone, Yeast extract, NaNO3, and Beef extract at a concentration of 1% were added to the fermentation media. The flasks (sugarcane bagasse with salt solution) were inoculated with 1ml of fungal spore solution (10^6spores/ml) and incubated at 30˚C for seven days in an incubator shaker at 125 rpm. At the end of the incubation period, protease production in cell free supernatant was determined (Chinnasamy muthulakshmi et al., 2011). [25]

2.14 Effects of metal ion concertation on protease activity

The effect of metal ions on production of protease activity was tested using $MgSO_4$, $CuSO_4$, Fe_2SO_4, CaCO3 and $ZnSO_4$ at 0.1% concentration. The flasks (sugarcane bagasse with salt solution) were inoculated with 1ml of fungal spore solution (106spores/ml) and incubated at 30˚C for seven days in an incubator shaker at 125 rpm. At the end of the incubation period, protease production was determined (Chinnasamy muthulakshmi et al., 2011). [25]

2.15 Effect of pH

The effect of pH for protease production was determined by culturing the fungi in the production media with different pH. The experiment was carried out individually at various pH 1,3,5,7,9,11 and 13. The pH was adjusted using the following buffers; 50 mM Sodium citrate (pH 1.0 - 6.0) and 50 mM Sodium phosphate (pH 7.0 - 13.0) (Shyam Sunder Alariya et al., 2013). [26]

2.16 Effect of Temperature

Temperature is an important role for the production of protease. The effect of temperature optima of the protease production was studied by the incubating the reaction mixture at various temperature -20˚,RT, 40˚, 60˚, 80˚, 100˚C (Shyam Sunder Alariya et al., 2013). [26].

2.17 Solid state fermentation combined conditions

Solid state fermentation was carried out in 250ml conical flask. The fermentation medium contained 10 grams of sugarcane bagasse with 10 ml of Salt solution(g/L) [Composition, KNO3 2.0, MgSO4.7H2O 0.5, K2HPO4 1.0, ZnSO4.7H2O 0.437, FeSO4.7H2O 1.116, MnSO4.7H2O 0.203], pH 7.0 and Nitrogen source - Beef extract (1%), Metal ion - Fe_2SO_4 (0.1%) were added to the fermentation media, the flasks were plugged tightly with cotton wool and autoclaved at 121˚C for 30 minutes. After sterilization, the flasks were inoculated with 3% of fungal spore solution and incubated at 30˚C for seven days in an incubator shaker at 125 rpm (Chinnasamy muthulakshmi et al., 2011). [25]

2.18 PROTEASE

Acid, Neutral and Alkaline protease

2.18.1 Assay of Acid protease

The acid protease activity in crude enzyme extract was analyzed according to the modified method of (Srividya shivakumar, 2012) [27], using BSA as substrate, the reaction mixture containing 0.5ml of enzyme solution, 0.5ml of 1%(w/v) BSA in 0.2M Phosphate buffer (pH5) was incubated at 30°C for 10 minutes .The reaction was stopped by adding 1ml of 10% Trichloroacetic acid containing 0.22M acetic acid and 0.33M sodium acetate. The reaction mixture was allowed to stand for 30 minutes at 30°C and then was filtered. Take 2 ml of the filtrate and add 5ml of 0.55M sodium carbonate, followed by the addition of 1ml (3 times diluted) phenol reagent. The blue colour was measured at 660nm by using a schimadzu uv1800 spectrophotometer. One unit of activity was defined as the amount of enzyme producing a change of absorbency equivalent to 1µg of tyrosine per min, under the above conditions.

2.18.2 Assay of Neutral protease

The Neutral protease activity in crude enzyme extract was analyzed according to the modified method of (Srividya shivakumar, 2012) [27], using Casein as substrate, the reaction mixture containing 0.2ml of enzyme solution and 0.5ml of 1%(w/v) Casein in 50mM Phosphate buffer was incubated at 40°C for 20 minutes. Except where specified, enzyme reaction was carried out at pH7.0. The enzyme reaction was stopped by adding 1ml of 10% Trichloroacetic acid. The reaction mixture was allowed to stand for 15 minutes at room temperature. After centrifugation (10,000 rpm 5 minutes), 2.5ml of 0.4M sodium carbonate was added, followed by the addition of 1ml of 3 times diluted phenol reagent and incubated at room temperature in dark 30 minutes. The blue colour was measured at 660nm by using a schimadzu uv1800 spectrophotometer. One unit of activity was defined as the amount of enzyme producing a change of absorbency equivalent to 1µg of tyrosine per min, under the above conditions.

2.18.3 Assay of Alkaline protease

The alkaline protease activity in crude enzyme extract was analyzed according to the modified method of (Srividya shivakumar, 2012) [27], using casein as substrate, the reaction mixture contained 1ml of enzyme solution and 1ml of 0.5%(w/v) casein in 0.2M Tris-HCl buffer, incubated at 75°C for 1 hour. Except where specified, enzyme reaction was carried out at pH7.4. The enzyme reaction was stopped by adding 2ml of 10% Trichloroacetic acid. Reaction mixture was allowed to vortex mixer after centrifuged at 3000rpm for 10 minutes, Supernatant was read at 440nm by using a schimadzu uv1800 spectrophotometer. One unit of activity was defined as the amount of enzyme producing a change of absorbency equivalent to 1µg of tyrosine per min, under the above conditions.

3. RESULTS AND DISCUSSION

3.1 Sample Collection, Isolation of endophytic fungi

Endophytic fungus in mangrove plant leaves of Suaedo monoica was isolated to evaluate their capacity to produce extracellular enzyme. Each isolate was sub cultured in PDA Medium and stored at 4˚C for further studies. The shape, structure and type of colony were analyzed on different types of medium and further the lactophenol staining was performed. This strain was subjected to molecular identification. (Table 1)

3.2 Moprhology Of Collected Plant

Suaeda monoica: It is a Shrub 1.2 -5 m high, leaves alternately crowded, linear-oblong or spathulate, flat or sub terete, obtuse or rounded at tip, narrowed at base, black on drying.

3.3 Identification

Six strains were isolated from Suaedo monoica. Of the six strains, the best was chosen based on the protease production. Based on the morphological and molecular analysis the strain was identified as Warcupiella spinulosa (Fig 1, Table1,). The nucleotide sequence was subsequently deposited in Gen bank with accession number MH587019.

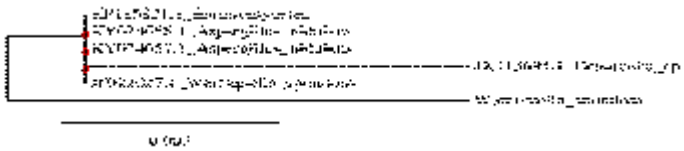

Fig 1: *Phylotree structure of Warcupiella spinulosa*

Table 1. *Molecular identification*

Isolate code	Identity	Colony morphology on PDA Medim	Host tissue	28s rRNA(bp)[a]	NCBI number	Gen bank accession no.
BWC F13	Warcupiella spinulosa	Brown powdery.	leaves	985 bp	EJNMH4UW015	MH587019

3.4 Effect of various substrates on protease production

Table 2. *Effect of various substrates on protease production*

S. No	Various substrates	Acid protease (U/ml)	Neutral protease (U/ml)	Alkaline protease (U/ml)
1	Cotton seed	1066	1083	8.888
2	Sugarcane bagasse	5866	10,183	20
3	Rice straw	5546	6500	133.33
4	Rice bran	2880	10616	46.66
5	Wheat bran	4800	6500	48.88
6	Cottonseed+feather powder (5:1)	3733	1083	35.55
7	Sugarcane bagasse+feather powder (5:1)	1386	2600	22.22
8	Rice straw+feather powder (5:1)	3306	9100	111.11
9	Rice bran+feather powder (5:1)	1920	4333	28.88
10	Wheatbran+feather powder (5:1)	4906.66	8233	48.88
11	Mix of soya flour: wheat flour: wheat bran in the ratio of 3:4:3	2666	5416	46.66
12	Mix of wheat bran+ sugarcane bagasse +rice straw and feather in the ratio of 4:3:3:2	3093	6500	44.44

The solid substrate provides nutrients to the organisms in addition to anchorage. So, the selection of a best solid substrate is a critical factor that involves the screening of substrates for microbial growth and product formation. Different substrates Cotton seed, Sugarcane bagasse, Rice straw, Rice bran, Wheat bran, Wheat bran +chicken feather powder(5:1), Sugarcane bagasse + chicken feather powder (5:1), Rice straw+ chicken feather powder (5:1), Cotton seed + chicken feather powder (5:1), Rice bran + chicken feather powder (5:1), Mix of soya flour: wheat flour: wheat bran in the ratio of 3:4:3 and Mix of wheat bran+ sugarcane bagasse +rice straw and chicken feather in the ratio of 4:3:3:2 were evaluated for the production of protease in the present work. Of all the substrates tested, Sugarcane bagasse (10,616 U/ml) was found to be the best substrate to produce Neutral protease (Table 2). Among acid protease, neutral protease and alkaline protease, Neutral protease level is high in various substrates. Alkaline protease production level is low in maximum substrates except in mixing of Rice straw and chicken feather powder (5:1). Heneri et al., 1988 [28] used different protein substrates for the production of proteolytic enzyme by Aspergillus niger and soybean meal was best substrate under solid state fermentation, in our studies we used mix of soya flour: wheat flour: wheat bran in the ratio of 3:4:3 but protease production was very low. Srividya shivakumar 2012 [27] reported that wheat flour produced highest acid protease production in Aspergillus sp but sugarcane bagasse produced high Neutral production in our studies. Hag et al., 2004[29] reported that Soybean meal was a best substrate for the production of protease from Penicillium griseoroseum under solid state fermentation, but our results showed that low production of protease. Chakraborty et al., 2000; Chinnasamy Muthulakshmi et al., 2011 [30,25] reported the high production of protease from Aspergillus niger using wheat bran as a substrate in our studies wheat bran produce less protease production than sugarcane bagasse at under solid-state fermentation. Hamid mukhtar and Ikram -UI- Haq 2009,[31] reported that different varieties of substrate like wheat bran, sunflower meal, soybean meal, cottonseed meal and rapeseed meal were evaluated for the biosynthesis of protease production by Aspergillus niger and sunflower meal produced maximum enzyme activity (5.2U/g) because it may have adequate supply of proteins , carbohydrates and minerals needed to the organism for growth synthesis of protease at 30˚C for 72hrs. Raghunath et al., 2012 [32] reported that rice bran showed the lovastatin production up to the highest levels [0.98±0.01 mg/g] of protease production but our results showed protease production level is low in wheat bran than sugarcane bagasse as the substrate. Benluvankar et al., 2015[33] reported that ground nut oil cake was the best substrate for protease production (2728.3U/g) by Penicillium sp. Dhaliwal et al.,2018,[34] using comparative study of wheat bran and rice bran as substrates, Aspergillus niger produced that the

maximum level of protease production, among them wheat bran produced higher activity protease as 1.785U/ml.

3.5 Effect of incubation days on protease production

Table. 3. *Effect of incubation days on protease production*

S.No	Incubation Days	Acid protease (U/ml)	Neutral protease (U/ml)	Alkaline protease (U/ml)
1	Day -1	4266	6283	8.888
2	Day -2	4480	6500	11.11
3	Day -3	5013	7150	11.11
4	Day -4	5120	7366	11.11
5	Day -5	5226	7800	22.22
6	Day -6	5333	8666	28.88
7	Day -7	5890	10,833	33.33
8	Day -8	5333	9100	28.88

The protease production was studied by conducting the fermentation using Warcupiella spinulosa for different time intervals (1,2,3,4,5,6,7 and 8 days). The enzyme production was gradually increased with the passage of time and highest Neutral protease activity (10,833 U/ml)(Table 3) was obtained on 7th day of incubation. It was also observed that prolonged incubation decreased the enzyme activity. However, the growth of the microorganism was not significantly affected. The present study was carried out for the production proteases by Warcupiella spinulosa was studied at 7th day. These results were supported by Johnvesly et al., 2002; Chinnasamy Muthulakshmi et al., 2011 [35,25] who reported that high protease enzyme production, occurred during 7th day of incubation by using A.flavus using Wheat Bran as a substrate. Similarly, in Aspergillus flavus, maximum protease production was showed between the 5th day and 7th day of incubation and declined thereafter Malathi and Chakraborthy,1991;Santhi (2014)[36,37] reported that the maximum protease production by Aspergillus niger,punica granatum peel waste as the substrate on the 7th day incubation and decreased further and these results were supported by our present study for the production proteases by Warcupiella spinulosa on the 7th day incubation and regularly it was increased from first day to 7th day but Srividya shivakumar 2012 [27] explained the protease production in Aspergillus sp was observed at 120hrs of incubation further incubation reduced the production. Karuna and Ayyana 1993 [38] reported that maximum protease activity with Aspergillus oryzae was obtained after 72 hrs of incubation. Paranthaman et al.,2009[11] using different varieties of rice broken, among all tested varieties of rice broken, Aspergillus niger produced maximum yield of protease during 72h. The incubation period was directly related to production of enzymes and further increase in incubation time resulted in decreased protease production and this can be created to the reduced availability of nutrients, competition and the production of toxic metabolites (Romero et al., 1998) [39]. Tremacoldi et al., 2004 [40] recorded Aspergillus clavatus was showed the highest acid proteolytic activity (80 U/ml) at the 3rd day of incubation. Benluvankar et al., 2015[33] reported that the maximum protease production (2899.1U/g ds) in Penicillium sp was observed on the 6th day of incubation and ground nut oil cake was the best substrate. Dhaliwal et al.,2018, [31] using comparative study of wheat bran and rice bran as substrates, Aspergillus niger produced that the maximum level of protease production at 120hrs.

3.6 Effect of inoculum size on protease production

Size of inoculum is a major biological factor in the production of the enzyme. At lower inoculum levels the production was very low. The decrease level seen with large inoculum size could be due to the shortage of the nutrients available for the large biomass and faster growth of the culture (Hesseltine et al., 1972) [9]. During the present study the maximum Neutral protease synthesis was identified with (13,216 U/ml) 3% inoculum size (Table 4) and these results were supported by Kalisz 1988; Haq et al., 2004 and Chinnasamy Muthulakshmi et al., 2011 [41,42,25]. The inoculum size is essential for optimum growth and production of enzyme by the microorganisms. Hamid mukhtar and Ikram -UI- Haq 2009,[31]reported that the enzyme production was found to be maximum the organism of Aspergillus niger for the production of protease for 72hrs and the inoculum ranged from 5% to 20%(0.5ml to 2ml) and it was produced maximum amount of enzyme (8.6U/g) at 10%(1ml)of spore inoculum at 30˚C(7.2 U/g) at pH 4.5(7.0 U/g) and sunflower meal as a substrate under solid state fermentation. At higher concentration there was a decrease in protease synthesis, it might be due to clumping of cells which could have reduced sugar and oxygen uptake rate and also enzyme release (Chinnasamy Muthulakshmi et al., 2011) [23]. Benluvankar et al., 2015[33]reported that maximum protease production by Penicillium sp increased with an increase in inoculum size up to a level(2g/kg) 5% on the 6th day of incubation and ground nut oil cake was the best substrate and was observed at pH 9.when the amount of mycelium increased , it rapidly consumed majority of the substrate for growth hence decreasing enzyme production(Carlile et al.,2001;Sathya et al 2009)[43,44].

Table .4. *Effect of inoculum size on protease production*

S.No	Inoculum size	Acid protease (U/ml)	Neutral protease (U/ml)	Alkaline protease (U/ml)
1	1%	5866	8450	8.888
2	2%	6080	9966	13.33
3	3%	7040	13,216	33.33
4	4%	6826	10,833	20.00
5	5%	5333	10,833	11.11

3.7 Effect of various nitrogen sources on protease production

In the present study the various nitrogen sources like Peptone, Yeast extract, NaNO3, and Beef extract were screened for synthesis of protease production. In that Beef extract was found to be the most suitable Nitrogen sources (19,926 U/ml) for acid protease production and (13,216 U/ml) in neutral protease production (Table 5). Some nitrogenous salts have a tendency to decrease the pH of the culture medium and had the opposing effect on enzyme production although they supported the growth of the organism (Wang et al., 1996) [45]. Benluvankar et al., 2015[33] reported that the Yeast extract was found to be the best nitrogen sources for maximum protease production (2899.1U/g ds) by Penicillium sp on the 6th day of incubation and ground nut oil cake was the best substrate. Phadatare et al., 1993 [46] report that the enrichment of protease production by the use of Yeast extract by Conidiobolus coronatus and Aspergillus terreus. Haq et al .,2003, [47] reported that peptone as a best nitrogen source for the production of protease by Aspergillus niger and

Rhizopus oligosporous. Our results are related to Hamid mukhtar and Ikram -UI- Haq 2009,[31]reported that the enzyme production was found to be maximum the organism of Aspergillus niger for the production of protease were used different nitrogen sources like urea, nutrient broth, meat extract, peptone , beef extract and Beef extract was found to be the best nitrogen sources giving maximum enzyme production 8.5U/g. Srividya shivakumar 2012) [27] reported that the Potassium nitrate showed the maximum protease production in Aspergillus sp.

Table. 5. *Effect of various Nitrogen sources on protease production*

S.No	Nitrogen sources	Acid protease (U/ml)	Neutral protease (U/ml)	Alkaline protease (U/ml)
1	Peptone	7466	7150	55.55
2	Yeast extract	6400	7583	86.66
3	NaNO3	7893	7800	86.66
4	Beef extract	19,926	13,216	46.66

3.8 Effects of metal ion concentration on protease activity

The effects of various metal ions ($MgSO_4$, $CuSO_4$, Fe_2SO_4, CaCO3 and $ZnSO_4$) on protease activity is shown in (Table 6). It can be shown that the metal ions $MgSO_4$ (15,573 U/ml)and CaCO3 (13,440 U/ml)supported the high enzyme activity in Acid protease production, and Fe_2SO_4 (14,083 U/ml) in Neutral protease production Whereas $CuSO_4$ (5333 U/ml) intensively low acid protease activity than Fe_2SO_4 (10,666 U/ml) and $ZnSO_4$ (9600 U/ml). The enzyme activity of acid protease from Monuscus sp was not affected by metal ions like Hg2+, Ca2+, Mg 2+, Mn2+, Zn2+ and Co2+(Yasuda et al.,1984) [48]. Nehra et al., (2004) [49] reports that Mg2+ was found to be an activator of the alkaline protease enzyme produced by Aspergillus sp, suggesting these metal ions had a capability to protect enzyme against denaturation that supported by our results in Acid protease production. Chinnasamy Muthulakshmi et al., 2011 [25] reported in Aspergillus flavus shown that the metal ions Zn 2+ and Cu2+ supported the maximum enzyme activity whereas Na2+ and Ca2+ inhibited the protease activity particularly Mg2+was found to be the inhibitor of protease. Srividya shivakumar 2012) [27] reported that the Aspergillus niger showed the maximum acid protease production in Ca2+ and Mg2+ ions that supported to Warcupiella spinulosa, while additions of Fe3+, Cu2+, and Zn2+ showed a decrease activity in acid protease production.

Table. 6. *Effects of metal ion concentration on protease activity*

S.No	Metalion concentration	Acid protease (U/ml)	Neutral protease (U/ml)	Alkaline protease (U/ml)
1	$MgSO_4$	15,573	10,833	31.11
2	$CuSO_4$	5333	6500	20
3	Fe_2SO_4,	10,666	14,083	22.22
4	CaCO3	13,440	10,833	33.33
5	$ZnSO_4$	9600	12,566	40

3.9 Effects of on pH protease activity

protease production by microbial strains strongly depends on the extracellular pH because culture pH strongly influences many enzymatic processes and transport of various materials through cell membrane, which in turn support the cell growth and product production (Elliah et al.,2002) [7]. The characterization of protease activity of Warcupiella spinulosa used in the study of the enzyme activity showed(fig 2) a gradual increase in activity and reached a peak at pH 7. It was related to Rhizopus enzyme (Fukumoto et al.,1967) [50]: Rhizopus oryzae at pH optimum of 5.5 (Sushil et al., 2005; Hamid mukhtar and Ikram -UI- Haq 2009) [51,31] pH 4.5(7.0 U/g) of protease production. The enzyme of Monascus sp was active in acid regions with maximum protease production at about pH 3.0 (Yasuda et al., 1984) [48] During ph 3.0 .Aspergillus flavus, Aspergillus candidus showed at pH 4.0 (Dworschack et al 1952; Nasuno and Onara, 1972)[52,53]. High pH reduces contamination risk during fermentation processes of Xylanase production by Bacillus sp under solid state fermentation (Gessesse and Mamo,1990) [54]. pH 8.0 for Bacillus cereus (Kim et al., 2001) [8], The Bacillus sps showed the protease activity for the optimum pH 7.0 and pH 7. Thermophilic Bacilus sps (Nascimento et al., 2004) [55]to other proteases Aspergillus enzyme pH 2.5~3.08 (Ichishima et al., 2004) [56] and B.cereus BGI(Ghorbel-Frikha et al ., 2005) [57]for,Bacillus sp HS08(Huang et al ., 2006) [58] and Bacillus sp, (Jung et al ., 2007)[59], Bacillus subtilus (Yandri et al ., 2008)[60] Aspergillus flavus showed maximal protease production at pH 4(Chinnasamy Muthulakshmi et al., 2011)[25]. Paranthaman et al.,2009[11] reported that Aspergillus niger produced maximum yield of protease production at pH 7.0 in all varieties of rice broken. Teufel and Gotz,1993 [61] reported that a neutral metalloprotease from Staphylococcus epidermidis showed optimal pH in the range 5.0-7.0. Benluvankar et al., 2015[33] reported that the Yeast extract was found to be the best nitrogen sources for maximum protease production (2899.1U/g ds) by Penicillium sp on the 6th day of incubation and ground nut oil cake was the best substrate and was observed at pH 9. Dhaliwal et al.,2018, [34] using comparative study of wheat bran and rice bran as substrates, Aspergillus niger produced that the maximum level of protease production at pH6.

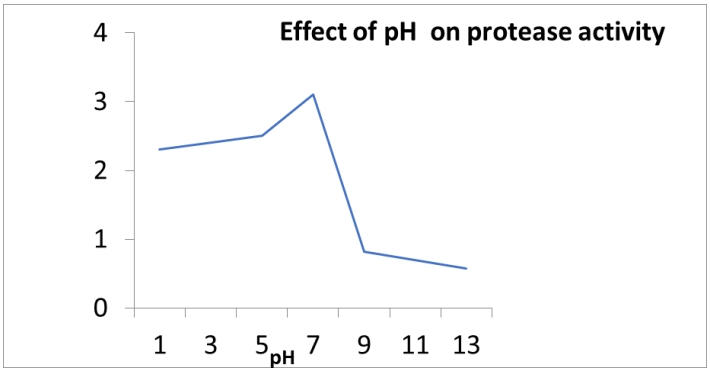

Fig .2. *Effects of on pH protease activity*

3.10 Effects of Temperature on protease activity

Fermentation was carried out at various temperatures like -20˚, RT, 40˚, 60˚, 80˚, 100˚ C to study its effect of protease activity. There was sudden increase in enzyme production by Warcupiella spinulosa from -20˚C to RT (fig 3). Then the enzyme production was slightly decreased up 40˚ to 100˚. Hence the optimum incubation temperature for the production of protease was found at RT. Our results are supported with, Dhaliwal et al.,2018, [34] reports and using comparative study

of wheat bran and rice bran as substrates, Aspergillus niger produced that the maximum level of protease production at room temperature28±2˚ C. Our results are related with Tramacoldi et al., 2004 [40] 80 (U/ml) at 30˚ C(Aspergillus clavatus); (Hamid mukhtar and Ikram -UI- Haq 2009[31] 30˚ C(7.2 U/g) showed the highest acid proteolytic activity. The different species of Penicillium including P.citrinum, P.perpurogerum and P.funculosum gave highest production of protease when incubated at 30℃ and reported that fungal proteases are usually thermolabile and show reduced activities at high temperatures (Sharma et al.,1980)[62]. Alkaline protease isolated from Tritirachium albumlimberand Thermomyces lanuginose (Samal et al.,1991) [63]; Aspergillus flavus 50℃ (Chinnasamy Muthulakshmi et al., 2011)[25] in optimum temperature of 50˚ C. The high yield production of protease by P.griseoroseum was obtained at an incubation temperature of 30℃ (Haq et al., 2004) [29]. Paranthaman etal.,2009[11] using different varieties of rice broken, among all tested varieties of rice broken, Aspergillus niger produced maximum yield of protease production during at 35˚C.

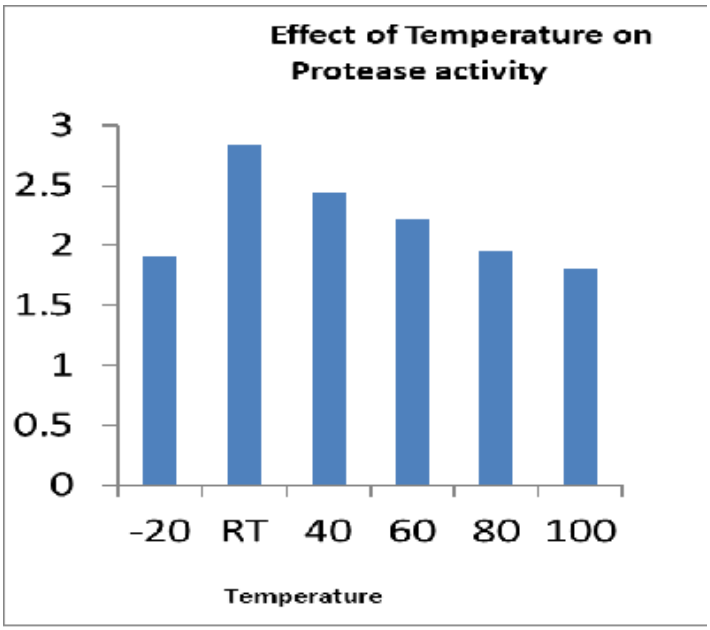

Fig .3. *Effects of Temperature on protease activity*

4. CONCLUSION

During this study 6 fungal strains were isolated from leaves of mangrove plants Suaedo monoica . The fungal strain was further characterized and screened for the presence of different medium. Warcupiella spinulosa to possess more activity after screening and were observed under microscope by using molecular identification method. The growth and metabolic production of a microbial population is based on the selection of Fermentation medium, Suitable substrates, Incubation time, Inoculum size, Nitrogen sources, Metal ion concentration, pH and Temperature. Protease production below Solid-state fermentation was considered using isolated Warcupiella spinulosa. The results displayed that the optimum conditions for maximum protease production were found to be 7th day of incubation, Sugarcane bagasse as the suitable substrate, Inoculum size 3%, and Beef extract as Nitrogen source(1%), Metal ions concentration as Fe_2SO_4 (0.1%) at pH 7.0 and Temperature at room temperature

3.11 Solid state fermentation combined conditions

Table. 7. *Solid state fermentation combined conditions*

S. No	Substrates	Acid protease (U/ml)	Neutral protease (U/ml)	Alkaline protease (U/ml)
1.	Sugarcanebagasse,3%, Beefextract (1%), Fe_2SO_4 (0.1%), pH(7), Temperature (RT) Day-7	13,440	20,366	144.44

Solid state fermentation was carried out in 250ml conical flask at various selected substrates (sugarcane bagasse with salt solution, Nitrogen source of Beef extract (1%), Metal ion of Fe_2SO_4 (0.1%)), were added to the fermentation media. After sterilization, the flasks were inoculated with 3% of fungal spore solution and incubated at 30˚C for seven days in an incubator shaker at 125 rpm (Chinnasamy muthulakshmi et al., 2011). [25]. on protease activity is shown in (Table 7). The enzyme production was gradually increased and highest Neutral protease activity (20,366 U/ml).

IV. CONCLUSION

During this study 6 fungal strains were isolated from leaves of mangrove plants Suaedo monoica. The fungal strain was further characterized and screened for the presence of different medium. Warcupiella spinulosa to possess more activity after screening and were observed under microscope by using molecular identification method. The growth and metabolic production of a microbial population is based on the selection of Fermentation medium, Suitable substrates, Incubation time, Inoculum size, Nitrogen sources, Metal ion concentration, pH and Temperature. Protease production below Solid-state fermentation was considered using isolated Warcupiella spinulosa. The results displayed that the optimum conditions for maximum protease production were found to be 7th day of incubation(10,833 U/ml), Sugarcane bagasse (10,616 U/ml)as the suitable substrate, Inoculum size 3%(13,216 U/ml), and Beef extract as Nitrogen source(1%)(10,216 U/ml), Metal ions concentration as Fe_2SO_4 (0.1%)(14,083 U/ml), at pH 7.0 and Temperature at room temperature. Solid state fermentation combined conditions the highest Neutral protease activity (20,366 U/ml).

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