JOURNAL OF PURE AND APPLIED MICROBIOLOGY, Sept. 2012. Vol. **6**(3), p. 1265-1271

Screening and Selection of *Aspergillus flavus* Strain for Alkaline Protease Production by Submerged Fermentation

M. Roja Rani[1]* and N.N. Prasad[2]

[1]Department of Biotechnolgy, Amara Institute of Engineering & Technology (AIET), JNTUK, Satuluru, NRT, Guntur (Dist), - 522 006 India.
[2]Principal,Vignan School of PG Studies, Guntur, Andhra Pradesh, India.

(Received: 28 December 2011; accepted: 18 February 2012)

20 fungal strains were screened from alkaline soils such as black cotton soil, groundnut field, milk processing unit, Kotappakonda hill area in Guntur Dist and Tirumala in Chittur District, Andhrapradesh, India. KS2 exhibited the prominent zone of clearance on modified seed medium at pH 9.0. The isolated species were identified basing on colony morphology,microscopic morphology,growth presence on temperature and pH of test fungal organism were identified as *Aspergillus flavus*. The alkalophillic isolate *Aspergillus flavus* shows maximum alkaline protease enzyme activity was (2000 U/mL) at pH 8.5, 40°C for 72 hr. These characteristics its potential use in alkaline protease production.

Key words: Alkaline protease, Isolation, Screening, *Aspergillus flavus.*

Proteases are the most important industrial enzymes that secreate a wide variety of functions and have various important biotechnological applications[1]. Proteases are the single class of enzymes which occupy a pivotal position with respect to their application in both physiological and commercial fields and account for about 60% of the total worldwide sale of enzymes. Proteases are useful in the field of medicine also where they have some diagnostic and therapeutic applications. Among the different types of microbial proteases the most commercially important are the alkaline proteases, especially those from the fungal sources. Numerous moulds especially belonging to the genera *Aspergillus* species such as *A.melleus*[3]. *A.fumigatus*[4-5], *A.flavus*[9], *A.sojae* [7]. *A.sydowi* [8]. However, the main drawback with production of bacterial proteases is the requirement of cost-intensive procedures for separation of mycelium can easily be removed from the final product by simple filtration. Besides the fungus can be grown on inexpensive substrates. Commercially important fungal alkaline protease were obtained from *Aspergillus sydowi*[8], *A.mellieus*[9] and *Aspergillus oryzae*[6]. The commercial superiority of alkaline proteases is due to their suitability for use in the field of detergent industry and in the leather industry. Enzymatic depilation has been widely accepted as a sound alternative to the chemical process.[10].

The naturally occurring alkaline environments comprise alkaline soils, soda lakes, alkaline springs etc. Isolation and screening of fungi from these natural environments can be supposed to be useful for obtaining fungal strains

* To whom all correspondence should be addressed.
Mob.: +91-9848735112.
E-mail: microroja.13@gmail.com

with the potential of yielding alkaline protease. The commercial superiority of alkaline proteases is due to their suitability for use in the field of detergent industry.Therefore,a need was felt to explore new fungal isolate, capable of producing alkaline protease,which can withstand high temparatue and the pH at strain has been exploited to improve enzyme production.

The aim of this study was isolation and screening of high yielding alkaline producing fungi from different alkaline environments. The promising strain in this study may be used in various economic industrial applications.

MATERIALS AND METHODS

Isolation of fungal Strains

Proteolytic fungi were isolated from alkaline soil samples collected from black cotton soil, groundnut field, milk processing unit, Kotappakonda hill area in Guntur Dist, and Tirumala in Chittur District, Andhrapradesh, India.

These soils were enriched with a protein source namely soyabean meal flour and incubated at 30^{0}C for 15 days. After the incubation period enriched soil samples were taken from each treatment, serial dilutions were made upto 10^{-6}in sterile water and 0.1 ml of dilution having the modified seed medium [11] in at pH8.5 was added to petriplate.

Composition of Modified seed medium [11]

Component	Amount (GL^{-1})
Glucose	2.5
Casein	5
Ampicillin	0.05
pH	9.0

The plates were incubated at 45^{0}C for 72 hr. A clear zone of casein hydrolysis around the colonies indicated as alkaline protease production by the organism. The fungal culture showing zone of hydrolysis around the colony (Fig.1) intowere picked up, purified by single spore method / single hyphal tip method and inoculated intoRose Bengal agar slants . More than twenty isolates were thus collected and maintainted in Potato Agar slants (Table 1).

Composition of Potato Dextrose agar medium

Component	Amount(GL^{-1})
Potatoes(Peeled & Sliced)	200g
Glucose(Dextrose)	20g
Agar	20g
pH	5.5

Screening of the isolates for alkaline protease production

The proteolytic fungal strains were screened for the yield of alkaline protease by submerged fermentation.The results are summarized in Table 2. The isolates were screened using the following medium for protease production.

Composition of production medium [12]

Component	Amount (GL^{-1})
Glucose	10
Casein	5
Yeast Extract	5
K_2HPO_4	1
$Mg\ SO_4\ 7H_2O$	0.2
$Na_2\ CO_3$	10
pH was maintained at	8.5
Ampicillin	0.05

50 ml of the production medium were taken into 100 ml Erlenmayer flasks were inoculated with fresh fungal cultures and incubated at 40^{0}C for 72 hr.

Preparation of Crude extract and Inoculum

Table 1. Isolation of alkaline protease producing fungal strains

S.No.	Source	Isolate number
1	Kotappa Konda Soil	KS1
2	Kotappa Konda Soil	KS2
3	Kotappa Konda Soil	KS3
4	Kotappa Konda Soil	KS4
5	Tirumala soil	TS1
6	Tirumala soil	TS2
7	Tirumala soil	TS3
8	Tirumala soil	TS4
9	Milk Processing unit	MS1
10	Milk Processing unit	MS2
11	Milk Processing unit	MS3
12	Milk Processing unit	MS4
13	Milk Processing unit	MS5
14	Groundnut soil	GS1
15	Groundnut soil	GS2
16	Groundnut soil	GS3
17	Black Cotton Soil	BS1
18	Black Cotton Soil	BS2
19	Black Cotton Soil	BS3
20	Black Cotton Soil	BS4

Preparation

Five ml of sterile water was added to 48 hr old isolates in PDA agar slants (pH 8.5). The cells were scrapped from the slant into sterile water and resultant cell suspension was transferred at 10% level aseptically into 250 ml Erlenmeyer flasks containing 45 ml of sterile inoculum medium. The composition of inoculum medium is Glucose 10 g, Casein 5g, Yeast extract 5g, K_2HPO_4 1g, $MgSO_4$ $7H_20.2$, Na_2CO_3 10g,Ampicillin 0.04 and distilled water 1000ml. pH was maintained at 8.5 and incubated at 40^0C for 72 hr. In inoculated flasks the cell pellet is inoculated into the production medium and andincubated at 40^0C for 72 hr. for submerged fermentation by using rotary shaker. After completion of incubation period the flasks were centrifuged at 150 rpm for 10 minutes and the culture broth was filtered by using what mann No. 1 filter paper to remove particulate matter. Then supernatant was centrifuged at 8,000Xg for 10 minutes. The Supernatant was used as a crude source of alkaline protease. The supernatant was decanted. The cell pellets were washed thoroughly with sterile saline followed by sterile distilled water. Finally the cell mass was suspended in sterile saline

Table 2. Screening of the isolates for the alkaline proteases production

S.No	Isolate number	Enzyme activity UmL^{-1}
1	KS1	1800
2	KS2	2000
3	KS3	1400
4	KS4	1900
5	TS1	1300
6	TS2	900
7	TS3	1000
8	TS4	1100
9	GS1	600
10	GS2	600
11	GS3	400
12	MS1	800
13	MS2	1000
14	MS3	1400
15	MS4	900
16	MS5	1700
17	BS1	900
18	BS2	600
19	BS3	700
20	BS4	500

Table 3. Physiological tests for KS2 Fungal Culture

Tests	Results
Growth at pH	-
3.5	-
4.0	-
4.5	-
5.0	+
5.5	+
6	+
6.5	+
7.0	+
7.5	+
8.0	+
8.5	+
9.0	-
9.5	-
10	-
10.5	-
11	-
Growth on NaCl(%)	
2.5	+
5.0	+
7.0	+
8.5	+
10.0	-
Growth under anaerobic conditions	±
Growth at Temp.	KS2
25^0C	-
30^0C	+
35^0C	+
40^0C	+
45^0C	+
50^0C	+
55^0C	+
60^0C	-
65^0C	-
70^0C	-
75^0C	-
80^0C	-
Acid Production from carbohydrates	
Adonitol	-
Arabinose	+
Cellobiose	-
Dextrose	+
Dulcitol	+
Fructose	+
Galactose	-
Inositol	-
Lactose	+
Maltose	+
Mannitol	-
Melibiose	-
Raffinose	-
Rhamnose	+
Salicin	-
Sorbitol	-
Sucrose	+
Trehalose	+
Xylose	-

and used as inoculum for subsequent experiments.

Enzyme Assay

According to[13], the enzyme was assayed in the reaction mixture containing 2.0 ml of 0.5% casein solution in 0.1M Carbonate –Bicarbonate buffer pH 9.5 and 1ml enzyme solution in a total volume of 3.0ml. Reaction mixture was incubated for 5 min at 40^0C. The reaction was terminated by adding 3ml of 10% ice-cold trichloroacetic acid.

The tubes were incubated for one hour at room temperature. Precipitate was filtered thorough whatman no.1 filter paper and the filtrate was collected. For the color development for the assay of tyrosine in the filtrate, 5ml of 0.4 M Sodium carbonate and 0.5 ml of Folin phenol reagent were added to 1ml of filtrate. vortexed immediately and incubated for 20 min at room temperature O.D was taken at 660 nm. Concentration of tyrosine in the filtrate was read from a standard curve for tyrosine already prepared.

One unit enzyme activity was taken as the amount of enzyme producing 1µg of tyrosin under standard assay conditions and expressed as Units mL^{-1} enzyme.

RESULTS AND DISCUSSION

Monitoring the stability of the highest yielding strain

The stability of the highest yielding strain viz., that is KS2 was assessed by subculturing and testing the yield at monthly intervals. The yield was determined using Reese medium as mentioned earlier and the strain was tested after first and second subcultures.

Identification of the highest yielding strain

The fungal strains selected among 20 isolates for high protease activity viz., that is KS2 was identified as *Aspergillus flavus*. Various physiological, morphological and microscopic properties of the high yielding strain were studied.

Identification of KS2 Fungal Culture

The high yielding fungal culture viz., KS2 was identified as *Aspergillus flavus* based on the following physiological, colony morphology and microscopic observations.

Physiological tests for KS2 Fungal Culture

The isolated fungal strain was inoculated into Potato Dextrose browth and the culture was incubated at different pH (3.5-11),NaCl concentration (2.5-8.5),[14] and Incubated at different

Table 4. Microscopic observation of observation of *Aspergillus flavus*

Colony	Size	Structure	Colour
Hyphae	45^0angle	Branching	Pale Green
Conial heads	Uni & biseriate	Radiate to loosely columnar with age.	Pale Green
Conidiophores	800um long X15-20um wide	-	Lime green colonies with rough conidiophores
Vesicles	(20-45um)	Spherical	Lime green colonies with rough conidiophores
Metulae	(8-10X5-7um)	-	Lime green colonies with rough conidiophores
Phialide	8-12x3-4um	-	Lime green colonies with rough conidiophores
Conidia	3.5-6um	smooth to very finely roughed conidia distinguish from *Aspergillus parasiticus*	Pale green & Lime green colonies with rough conidiophores

temparatures (25-80^{0}C).Acid production from Carbohydrates. After 96 hr of incubation growth was observed at different PH (5.0-8.5),Nacl concentration(2.5-8.5),Temparature(30-55^{0}C).Acid production from Carbohydrates (Dextrose,Dulcitol, Fructose, Lactose, Maltose, Sucrose, Trehalose) Results were shown in (Table 3).

Colony Morphology

2,3 *Aspergillu flavus* colonies are byellowish-green,consisting of a dense felt of conidiophores or mature vesicles bearing phialides over their entire surface. Colonies on potato dextrose agar at 25°C are olive to lime green with a cream reverse, rapid growth.Texture is woolly to cottony to some what granular. Sclerotia, when present, are dark brown. A clear to pale brown

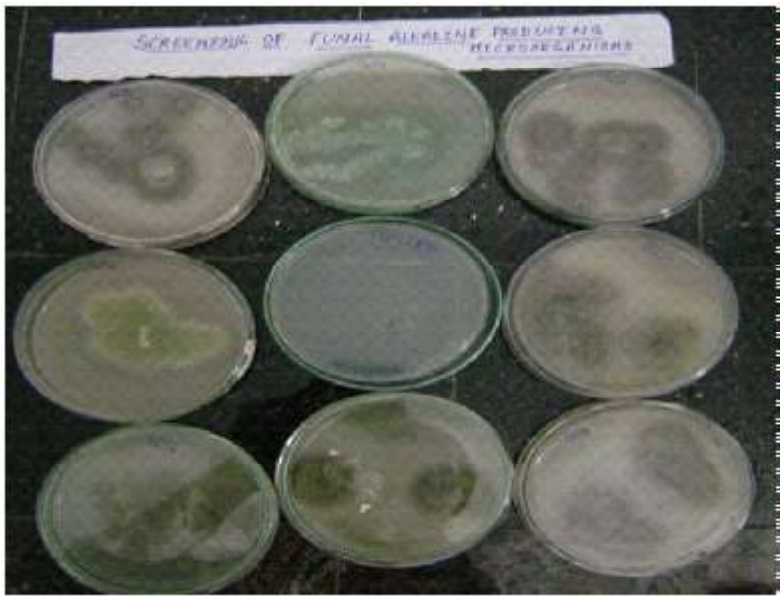

Fig. 1. Screening of the isolates for the alkaline protease production

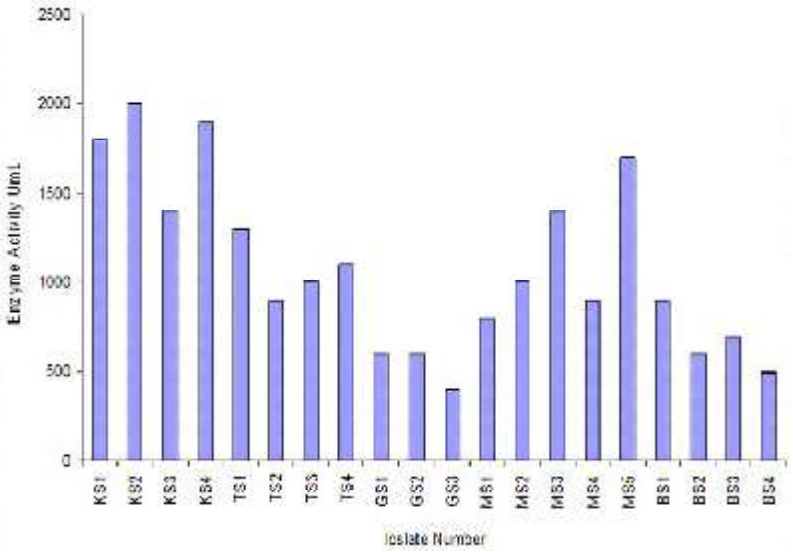

Fig. 2. Screening of the isolates for the alkaline proteases production

exudate was present. Effuse, lime green colonies with rough conidiophores and smooth to very finely roughed conidia distinguish *A. flavus* from the similar *Aspergillus parasiticus* that produces very rough conidia. (Fig. 3.)

Colony Morphology

Medium	Temparature	Color
PDA	25°C	Olive to lime green with a Cream reverse

Colony structure

Medium	Temparature	Color
PDA	25°C	It is wooly to cottony to some what granular

Microscopic observation

Aspergillus hyphae are septate and show dichotomous, 45° angle branching. Larger hyphae may resemble hyphae of the Zygomycetes. Conidial heads are radiate to loosely columnar with age. Conidiophores are coarsely roughened, uncolored, up to 800 µm long X 15-20 µm wide, vesicles spherical (20-45µm),metulae (8-10 X 5-7 µm) covering nearly the entire vesicle in biseriate species. Conidial heads radiate, uni- and biseriate; however, some isolates may remain uniseriate, producing only phialides (8-12 X 3-4 µm) covering the vesicle. Nd Conidia are pale green and conspicuously echinulate, smooth to very finely roughed, (sub) spherical, 3.5 - 6 µm in diameter. The results were shown in (Table 4 and Fig. 4).

CONCLUSION

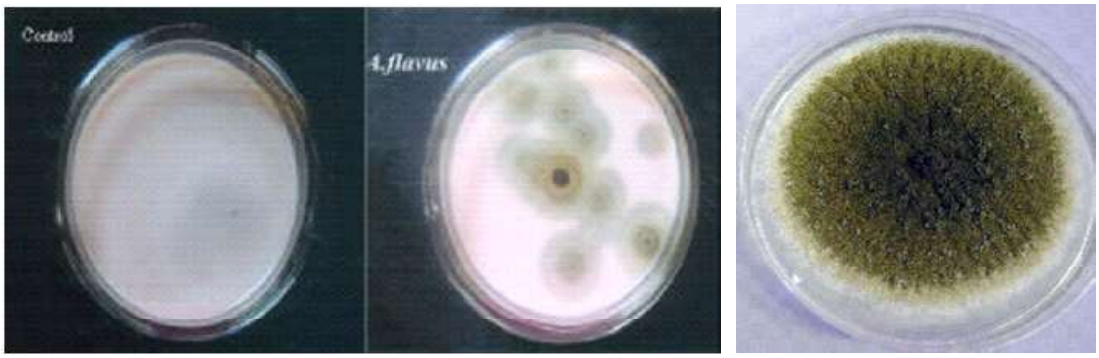

Fig. 3. Colony Morphology of *Aspergillus flavus*

Fig. 4. Microscopic observation of *Aspergillus flavus*

From the results, it could be concluded that the isolated strain of *Aspergillus flavus* KS2 produce the alkaline protease enzyme. Ability of the fungus to grow on cheap and environmental friendly substrates and it secreate a high yields of alkaline protease makes it is a potential candidate for alkaline protease production.

REFERENCES

1. Mohan F N, Dileep D, Deepthi D Potential application of protease isolated from Pseudomonas auriginosa PD 100. *Biotechnol. Ind*., 2005; **8**: 19.
2. Ward, O.P. Proteolytic enzymes. In: Blanch, H. W., Drew, S. and Wang, D. I. C. (eds.), The Principles, Applications and Regulations of Biotechnology in Industry Agriculture and Medicine. Pergamon Press, New York. *Comprehensive Biotechnology.*, 1985; **3**: 789-818.
3. Luisetti M piccioni PO Dyne k Donni M Bulgheroni A Pasturenzi LDonnetta AM Peona V.Some properties of the alkaline proteinase from *Aspergillus mellus*. *Int J Tissue React* ., 1991; **13**: 187-92.
4. Monod M Togni G Rahalison L frenk E. Isolation and characterisation of an extracellular alkaline p). rotease of *Aspergillus fumigates*. *J Medical Microbiol*., 1991; **35**: 23.
5. Larcher G Bouchara J PAnnaixV Symeoens F, Chabasse D Tronchin G) purification and characterization of a fibrinogenolytic serine proteinase from *Aspergillus fumigatus* culture filtrate. *FEBS Lett.*, 1992; **308**: 65-69.
6. Malathi, S. and Chakraborthy, R. Purification of alkaline protease by a new *Aspergillus flavus* isolate under solid substrate fermentation conditions for use as a depilation agent. *Appl Environ Microbiol.*, 1991; **57**: 712-716.
7. Hayashi., K.Fukushima.D .Mogi.K.Isolation of alkaline proteinase from *Aspergillus sajae* in homogeneous from *Agric Biol Chem* ., 1967; **31**: 1237-41.
8. Danno,G&YoshimuraS. Studies on an alkaline proteinase of *Aspergillus sydowi* part 1:Purification and some properties of the proteinase. *Agri Bio chem*., 1967; **31**: 1151-1158.
9. Ito.,M &Sugira M Purification and characterization of alkaline protease from *Aspergillus oryzae*.*Yakaguku Zasshi*, 1968; **88**: 1576-1582.
10. Taylor,M.M.,D.G.Bailey, and S.H.Feair heller.A Review of the uses of enzymes in the tannary. *J.Am.Leather Chem.Assoc*., 1987; **81**: 85-102.
11. Reese,E.T.,Siu,R.G.H.and Levinson, H.S. The biological degradation of cellulose derivatives and its regulation to the mechanism of cellular hydrolysis. *Bacterias*., 1950; **59**: 485-489.
12. Nehra, K.S., Dhillon, S., Kamala Chowdhary and Randhin singh. Production of alkaline protease by Aspergillus sp under submerged and solid state fermentation. *Indian J Microbial*., 2002; **42**: 43-47.
13. Udandi Boominadhan, Rajendran Rajakumar. Optimization of protease enzyme production using *Bacillus* sp. Isolated from different wastes. *Bot Res Int*., 2009; **2**(2): 83-87.
14. AnejaK.R.,Experments in Microbology,Plant pathology,Tissue culture and Mushroom production Technoloyg; New Age Intenational publishers;Third Edition. 2003; 221-240.