

## **Pigment and amylase production in *Penicillium* sp NIOM-02 and its radical scavenging activity**

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### **Abstract**

*Penicillium* sp NIOM-02 was isolated from the marine sediment, produced red pigment. The pigment extracted from this fungus scavenged 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical. *Penicillium* sp NIOM-02 grown in media containing corn steep liquor scavenged 72-88% of DPPH radical. During solid-state fermentation on wheat (S1), the fungus produced more pigment (9.232 OD Units). *Penicillium* sp NIOM-02 grown on sugarcane bagasse scavenged 91% of DPPH radicals. It secreted more amylase (246 U/mg) in culture medium No. 5 and the zymogram analysis revealed its molecular mass (53 kDa). The taka-amylase like character of amylase was determined by acarbose incorporated studies in the culture media. Production of pigment and radical scavenging activity of *Penicillium* sp NIOM-02, suggested its applications in food, pharmaceuticals and nutraceutical industries.

Key words: DPPH, Colour, Antioxidant activity, FTIR, Food use

## Introduction

Filamentous fungi are attractive organisms for production of useful protein and biological active secondary metabolites. These fungi produce high levels of polysaccharide-degrading enzymes and are frequently used for the production of industrial enzymes. Fungi have high secretion capacity and are effective hosts for the production of foreign proteins (Tsukagoshi *et al.*, 2001). Amylases have varied industrial applications and are classified as  $\alpha$ -1-4-glucanase and  $\alpha$ -1-6-glucanase according to specific glucosidic bond cleavage. In brewing industry, the products of starch obtained after enzymatic hydrolysis are used as nutrients in microbial fermentation (Stewart and Russel 1978) for ethanol production (Matsumoto *et al.*, 1982). The major markets for amylases are food industries for the preparation of sweeteners and syrups (Nigam and Singh 1995). During solid-state fermentation *P. restrictum* produced amylase, lipase and protease on basal medium of industrial waste of babassu oil (Palma *et al.*, 2006). A high maltose producing amylase was secreted from *P. expansum* (Doyle *et al.*, 1989) and increased secretion of xylanase and pectinase was observed when grown on xylan and pectin as respective carbon sources (Kimura *et al.*, 2002).

The pigments produced by *Penicillium* (PP-V and PP-R) and *Monascus* (monascorubrine and monascuscorubramine) are structurally similar (Ogihara and Oishi 2002). Lovastatins or monacolins produced by *Penicillium*, *Monascus*, *Aspergillus* and *Rhizopus* (Endo *et al.*, 1979) inhibits cholesterol biosynthesis by binding to catalytic site of HMG-CoA reductase a key enzyme in cholesterol biosynthesis (Albert, 1990) and scavenged DPPH radicals (Aniya *et al.*, 1999; Dhale *et al.*, 2007a & b). Penicillenols secreted by *Penicillium* sp showed biological activity against HL-60 cell lines (Lin *et al.*, 2008). The oceanic environment is a wealthy source to identify new metabolite produced by microbes. The production of amylase, pigment and radical scavenging activity of *Penicillium* sp NIOM-02 are described in this paper.

## Material and Methods

The malt extract agar (MEA), amino acids, soluble starch, 3, 5-dinitrosalicilicacid, glucose, maltose, gelatin, cysteine arginine, casein, peptone, yeast extract, coomassie brilliant blue (CBB) G-250 and R-250 reagents were purchased from Hi-Media Laboratories, Mumbai, India. Tris-buffer, 2, 2, -diphenyl-1-pycrylhydrazyl (DPPH), 4-chloro-1-naphthol, acrylamide, N, N'-methylene-bis-acrylamide, ammonium per sulphate (APS), N, N, N', N'-tetramethylethylenediamine (TEMED), sodium dodecyl sulphate (SDS), dithiothreitol (DDT) and mercaptoethanol were obtained from Sigma Chemicals, USA.

Glucosidase inhibitor acarbose tablets were procured from Bayer Pharmaceuticals, Mumbai, India. The serological reagents goat anti-rabbit IgG tagged biotin and avidin peroxidase were obtained from Bangalore Genei, Bangalore, India. Nitrocellulose membranes were procured from Amersham Pharmacia Biotech, UK.

### **Isolation and culture condition**

The *Penicillium* sp NIOM-02 was isolated from marine sediment sample, was collected from Miramar, India. The 250 ml erlenmeyer flasks containing 50ml of media were sterilized for 15 min at 121°C. The media was prepared with different concentrations of corn flour, corn steep liquor, peptone and yeast extracts as major components (Table 1). The media were inoculated with 7 days old culture spores maintained on MEA. The flasks were incubated at room temperature for 10 days at 118 rpm on rotary shaker. For solid-state fermentation (S1-S6), one ml of spores suspension prepared in 0.5% SDS was used to inoculate the culture medium. The flasks were incubated at inclined position and rolled manually twice a day to ensure acceptable growth.

### **Enzyme assay**

The culture filtrate was used as enzyme source during submerged fermentation and during solid-state fermentation enzyme was extracted using acetate buffer (0.1M, pH 4.3). Amylase activity was determined by using 2% soluble starch prepared in acetate buffer (0.1M, pH 4.3). One ml of soluble starch solution was treated with 0.2 ml of appropriately diluted enzyme in buffer (0.1 M acetate buffer, pH 4.3) and incubated at 37°C for 30 min. The reaction was stopped by adding 1 ml NaOH (4M) and the reducing sugars in the reaction mixture were determined using 3, 5-dinitrosalicylic acid (Miller 1959). One unit enzyme activity corresponded to 1  $\mu$ mol of glucose released per min.

### **Electrophoresis**

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 10% acrylamide gels (Laemmli, 1970). The protein samples were prepared in sample buffer containing dithiothreitol (DTT) for SDS-PAGE. The proteins in the gel were visualized by staining (CBB R-250). Molecular mass of the protein was determined by running the standards alongside of protein sample in SDS-PAGE.

Amylase activities were detected as enzyme zymograms after separating the proteins by SDS-PAGE in 10% acrylamide gel containing 0.1% soluble starch. The samples diluted in the buffer were directly used for electrophoresis without boiling and reducing agent (DTT). After electrophoresis, the amylase proteins were re-natured by incubating the gel in acetate buffer (0.1 M, pH 4.3) at 30°C for 90 min. Amylase activities were detected accordingly Ravi-Kumar *et al.*, (2004) as zone of clearance after staining with iodine/iodide solution (0.6 g I<sub>2</sub> and 6 g KI L<sup>-1</sup>).

### **Immunological methods**

After electrophoresis the proteins were blotted on nitrocellulose membranes (Towbin *et al.*, 1979) were blocked with 10% skim milk solution. The amylase proteins were identified using antibody (10:20000 dilution) and anti-rabbit goat IgG conjugated with peroxidase (10:20000 dilution) in Tris-HCl buffer saline (10 mM, pH 8.0) (Sigma St. Louis, MO, USA). Tris-HCl buffer (50 mM, pH 7.6) containing hydrogen peroxide (0.025%) and 4-chloro-1-naphthol (0.04%) was used as substrate for enzyme reaction. The antibodies used in this study were raised previously to the 125-kDa starch-hydrolyzing enzyme of *Aspergillus niger* (Dubey *et al.*, 2000).

### **Determination of protein concentration**

Protein concentrations were determined by the dye binding method using CBB G-250 (Spector, 1978). Briefly, 200 µl of enzyme preparation was mixed with 1.3 ml of distilled water. To this 1.5 ml of CBB G-250 prepared in 3% of perchloric acid was added. The absorbance was read at 595 nm with appropriate blank.

### **Extraction and quantification of pigments**

The submerged cultures were filtered through membrane (Whatman No.1) and the filtrates were directly used for the quantification of pigment. The pigments from solid cultures (solid-state fermentation) were extracted with methanol (5/20: w/v). The extraction was carried out at 5°C for 60 min in cold room with periodical shaking. The insoluble fragments were removed by filtration. The absorbance of the filtrate was determined spectrophotometrically (UV-Visible 2450, Shimadzu Spectrophotometer, Japan) at 482 nm to quantify the pigments as OD Units. The pigment extracted from submerged fermentation and solid-state fermentation was expressed as OD Units/ml and OD Units/ml/g respectively. The OD units are calculated as follows.

$$\text{OD Units} = \frac{\text{OD} \times \text{Total vol of Solvent} \times \text{Dilution}}{\text{Gram of substrate}}$$

### **DPPH radical scavenging activity**

The DPPH radical scavenging activity (Blois, 1958) was measured using culture filtrate and methanol extract of solid-state culture. The reaction mixture was containing 0.8ml of Tris HCl buffer (100 mM, pH 7.4), 0.2ml of pigment extract and 1 ml DPPH (500  $\mu$ M in ethanol). The mixture was shaken vigorously and incubated at room temperature for 30 min. The absorbance was measured at 517 nm and percentage of radical scavenging activity was calculated as follows.

$$\text{Antioxidant activity}(\%) = \left( 1 - \frac{A_{\text{sample}(517\text{nm})}}{A_{\text{control}(517\text{nm})}} \right) \times 100$$

All the experiments were carried out in triplicates by maintaining appropriate blanks and controls.

### **Determination of total phenolics**

The concentration of phenolics were determined accordingly Singh et al (2002). Two hundred  $\mu$ l of extract was mixed with 1 ml Folin-Ciocalteu reagent (10 fold dilutions) and 0.8 ml sodium carbonate (7.5%) solution. The reaction mixture was incubated for 30 min at room temperature and absorbance was measured at 765 nm. All the experiments were carried out in triplicates by maintaining appropriate blanks and controls.

### **Fourier Transformer Infra Red (FTIR) Spectroscopy**

The *Penicillium* sp NIOM-02 grown on sugarcane bagasse fractionated on silica gel column. The FTIR spectrum of fraction was recorded on a Nicolet 5700 (Thermo Electron Corporation, Madison, WI, US.) spectrometer at room temperature. The fraction was mixed with KBr pellet and scanned in the range of 4000-400  $\text{cm}^{-1}$ . The KBr pellet was used as blank.

### **Results**

The fungus isolated from the marine sediment sample was identified as *Penicillium* sp NIOM-02 by phenotypic and microscopic observations like green coloured spores, phialides and conidia. Secretion of red pigment by this fungus was observed around the colony on MEA plate, suggested its water-soluble characters. *Penicillium* sp NIOM-02 showed increased amylase activity (246 U/mg) in medium No. 5 (initial pH 5.5) after 10 day of inoculation. In the media number 2 and 12 enzyme activity recorded was 64 and 68 U/mg respectively. However, in the media No. 6 and 7 or from 13 to 22, enzyme activity recorded was 36 and 19 or from 2 to 27 U/mg respectively (Table 1). The results could indicate that higher quantities of corn flour and corn steep liquor did not favor the amylase production during submerged fermentation.

Amylase activities in culture filtrate were visualized as zymogram reaction after electrophoresis (non-reducing condition). Zymogram reaction revealed the molecular mass of amylase corresponding to 53-kDa (Fig 1C). Taka-amylase like character was speculated due to secretion of 53-kDa amylase in culture media by this fungus. To confirm the taka-amylase like character, the fungus was grown in the culture media containing different concentrations of acarbose. The zymogram reaction of acarbose incorporated culture filtrate, suggested the secretion of taka-amylase like enzyme (Fig 1 A1-A4). The dot blot reaction of amylase protein (10µg) of *Penicillium* sp NIOM-02 with antibodies raised against purified glucoamylase of *Aspergillus niger*, further confirmed this result and suggested the taka-amylase like character (Fig 1B).

During solid-state fermentation, *Penicillium* sp NIOM-02 was grown on wheat with corn flour (S1) showed increased amylase activity (18 U/mg) and less amylase activity on sugar cane bagasse. The *Penicillium* sp NIOM-02 cultured in a media containing 0.2N HCl, suggested the pessimistic impact of acidic condition for the production of amylase (Table 2). The production of higher quantities of pigment (9.232 OD Units) was observed on medium number S1. The fungus cultivated on media number S3 and S4 produced approximately 55% less pigment compared to medium number S1 (Table 4) and highly sporulated. These results indicated that, combination of wheat and corn flour (S1) is the best substrate for pigment production by solid-state fermentation (Table 4).

A positive correlation was observed between DPPH radical scavenging activity and pigment production during submerged fermentation. The culture grown in corn steep liquor medium induced pigment synthesis and scavenged 70-80% DPPH radical (Table 3). Maximum and minimum DPPH radical scavenging activity was observed in medium number 19 (88%) and 4 (11%) respectively. The

results suggested that DPPH radical scavenging activity was influenced by corn flour and corn steep liquor in growth medium (Table 1 and 3). The *Penicillium* sp NIOM-02 cultured on medium S1, secreted higher pigment during solid-state fermentation (Fig 2) and scavenged 38% of DPPH radicals. *Penicillium* sp NIOM-02 cultured on sugar cane bagasse (S6) scavenged 91% of DPPH radical (Table 4). The IR spectrum (Fig 4) of fractions of methanol extract showed broad OH stretching at 3379  $\text{cm}^{-1}$ .

## Discussion

*Penicillium* sp NIOM-02 grown by submerged fermentation and solid state fermentation produced pigment and amylase and the production of these metabolites varied. The fermentation medium greatly affects the product concentration and yield. During solid-state fermentation, in medium number S1, fungus produced more amylase (Table 2) and pigment (Table 4 and Fig 2) compared to other media (S2-S6). The medium number S1 was autoclaved using water and media numbered S2-S6 were autoclaved with 0.2N HCl. It suggested that acidic condition did not favor the metabolite production, to a certain extent induced the sporulation due to increased acidic stress condition. This could be due to the fact that *Penicillium* sp NIOM-02 was isolated from the marine sediment, where the pH is slightly alkaline. Amylase activity during solid-state fermentation is less compared to submerged fermentation indicated that role of dissolved oxygen and continuous agitation. The oxygen transfer is a major concern of solid-state fermentation and diffusion of oxygen is hampered by mycelia mat on and inside the substrate (Rahardjo, 2002).

The pigment hue produced by the fungi varies by strain, medium composition, and growth condition and is greatly affected by the medium composition (Jung *et al.*, 2003). The pigment production in *Penicillium* sp was influenced by the presence of nitrogen in the medium (Ogihara and Oishi 2002). Formation of red pigment in *Penicillium* (Ogihara and Oishi 2002) and *Monascus* (Hajjaj *et al.*, 1997) is a chemical modification of the precursor pigment by amine group due to Schiff's base reaction (Juzlova and Kein 1996). Apparently the corn steep liquor used in the culture media is rich in nitrogen source was utilized by the fungus for the production of red pigment and suggested similar biosynthetic pathway of pigment synthesis in *Penicillium* sp NIOM-02. It has been established in *Monascus* sp, the over secretion of amylase inhibited pigment production due to alcohol production (Rasheva *et al.*, 2003). However, we did not find any significant difference in pigment yield by *Penicillium* sp NIOM-02 cultured in medium containing a glucosidase inhibitor acarbose.

The UV-visible spectra of pigment extracted from solid-state cultures showed  $\lambda$  max at 482-485 nm indicated the production of a red pigment. The extracts of *P. terrestre* tested by Chen *et al.*, (2008), exhibited cytotoxic effects (HL-60, MOLT-4, BEL-7402, and A-549 cell lines) and moderate DPPH radical scavenging activity. The physiological role of secondary metabolites in the organism producing them is still unclear (Challis, 2000) despite their bioactive properties (Calvo *et al.*, 2002). The culture medium containing corn steep liquor scavenged higher DPPH radicals (Table 1 and 3) and apparently induced the secretion of secondary metabolites. Even during solid-state fermentation the variation in DPPH radical scavenging activity was observed. The medium autoclaved using water (S1) and containing 0.2N HCl (media S2-S6) scavenged 38% and 57-91% of DPPH radicals respectively. These results suggested the variation in the culture medium and conditions during the growth of organism can greatly affect the secondary metabolite production. The estimation of phenolics showed positive correlation with DPPH radical scavenging activity (Fig 3). This suggested DPPH radical scavenging activity was not only due to the pigments because of phenolics also. This can be acceptable by the IR spectrum (Fig 4) showing broad stretching at  $3379\text{ cm}^{-1}$  for hydroxyl group of phenolics. In the IR spectrum stretching frequency were also observed at  $1643$  and  $1414\text{ cm}^{-1}$  assignable to C=C and C-H of the aromatic ring respectively. The *Penicillium* sp NIOM-02 grown on wheat autoclaved using water (S1) secreted more pigment. In the other media (S2-S4), 0.2N HCl was used for autoclave secreted less pigment (Table 4 and Fig 2) and highly sporulated. Apparently, acidic pH inhibited the pigment synthesis and induced sporulation due to stress condition.

Biotechnological industries harness the metabolic activity of compound produced for use in food, pharmaceutical, chemical and health care industries (Parekh *et al.*, 2000). In recent years, production of natural food colourants through microbial fermentation is an extensive area of investigation, since they overcome concerns of unfavorable side effects by synthetic colours (Delgado-Vargas *et al.*, 2000). The production of pigment and amylase by *Penicillium* sp NIOM-02 indicated its importance in food, pharmaceutical and nutraceutical industries.

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Table 1. Amylase production by *Penicillium* sp NIOM-02 during submerged fermentation, initial pH of media was adjusted to 5.5.

Media No	Media Composition (g L <sup>-1</sup> )		Specific Activity (U mg protein <sup>-1</sup> )
1	Corn flour (40)	Peptone (10);Yeast Extract (5)	28.41
2	Glucose (40)	Peptone (10);Yeast Extract (5)	64.87
3	Maltose (40)	Peptone (10);Yeast Extract (5)	39.22
4	Corn flour (40)	(NH <sub>4</sub> ) <sub>2</sub> HPO <sub>4</sub> (15) and NH <sub>4</sub> H <sub>2</sub> PO <sub>4</sub> (15)	17.43
5	Corn flour (20)	Peptone (10);Yeast Extract (5)	246.52
6	Corn flour (60)	Peptone (10);Yeast Extract (5)	35.71
7	Corn flour (80)	Peptone (10);Yeast Extract (5)	19.38
8	Corn flour (60)	Peptone (2);Yeast Extract (5)	31.92
9	Corn flour (60)	Peptone (4);Yeast Extract (5)	29.88
10	Corn flour (60)	Peptone (6);Yeast Extract (5)	12.44
11	Corn flour (60)	Peptone (8);Yeast Extract (0)	20.75
12	Corn flour (40)	Corn steep liquor (20)	68.00
13	Corn flour (60)	Corn steep liquor (30)	6.16
14	Corn flour (60)	Corn steep liquor (40)	2.95
15	Corn flour (60)	Corn steep liquor (50)	6.71
16	Corn flour (60)	Corn steep liquor (60)	2.20
17	Corn flour (60)	Corn steep liquor (80)	4.81
18	Corn flour (60)	Corn steep liquor (100)	2.67
19	Corn flour (60)	Corn steep liquor (40); Gelatin (1)	19.40
20	Corn flour (60)	Corn steep liquor (40); Casein (1)	27.12
21	Corn flour (60)	Corn steep liquor (40); Cystein (1)	8.53
22	Corn flour (60)	Corn steep liquor (40); Arginine (1)	2.93

Table 2. Amylase activity of *Penicillium* sp NIOM-02 during solid-state fermentation.

Media components	Media Number*.					
	S1	S2	S3	S4	S5	S6
Wheat (g)	25	25	25			
Corn flour (g)	0.5	0.5	0.5	25		
Rice grain (g)					25	
Sugarcane bagasse (g)						25
KCl (mg)			25	25	25	25
CuSO <sub>4</sub> .5H <sub>2</sub> O (mg)			0.25	0.25	0.25	0.25
ZnSO <sub>4</sub> .7H <sub>2</sub> O (mg)			0.5	0.5	0.5	0.5
0.2N HCl (ml)		25	25	25	25	25
Distilled water (ml)	25					
Specific Activity (U/mg)	18.46	2.80	14.13	1.49	6.21	1.65

\*S1-S6 represents solid-state culture media.

Table 3. Pigment yield and DPPH radical scavenging activity of the *Penicillium* sp NIOM-02 during submerged fermentation.

Media No*	Pigment yield <sup>@</sup>	DPPH Scavenging (%)
1	0.397	47.88 ± 1.81
2	0.243	65.80 ± 2.11
3	0.310	53.84 ± 5.91
4	0.133	11.61 ± 2.37
5	0.473	23.92 ± 1.89
6	0.648	68.89 ± 5.98
7	1.652	80.25 ± 1.14
8	0.216	62.71 ± 1.94
9	0.252	63.99 ± 0.08
10	0.450	57.83 ± 0.18
11	0.276	47.38 ± 1.59
12	0.671	67.30 ± 1.68
13	1.448	82.77 ± 2.49
14	0.927	85.63 ± 2.15
15	1.267	86.42 ± 1.70
16	1.489	82.45 ± 0.66
17	2.745	71.91 ± 4.38
18	3.154	69.14 ± 7.51
19	1.297	88.05 ± 0.83
20	1.031	87.18 ± 0.59
21	0.587	80.16 ± 2.69
22	1.168	79.60 ± 2.33

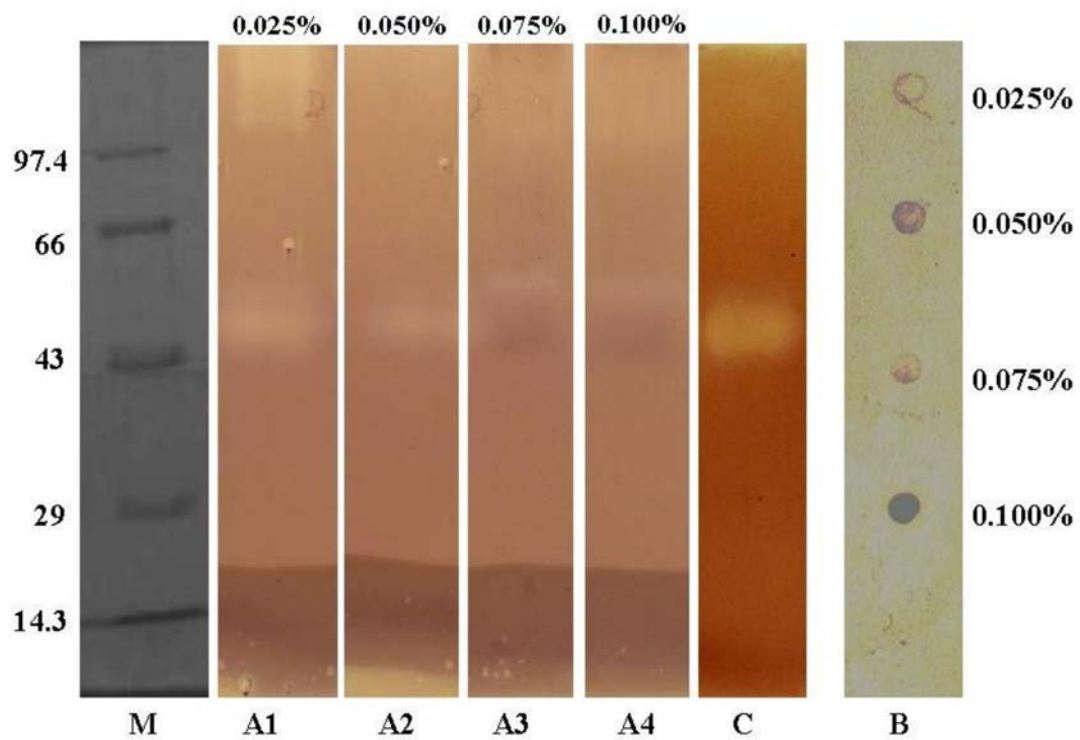
\* Medium composition as in Table 1

<sup>@</sup>Estimated as absorbance at 482 nm

Table 4. Pigment yield and DPPH radical scavenging activity of the *Penicillium* sp NIOM-02 during solid-state fermentation.

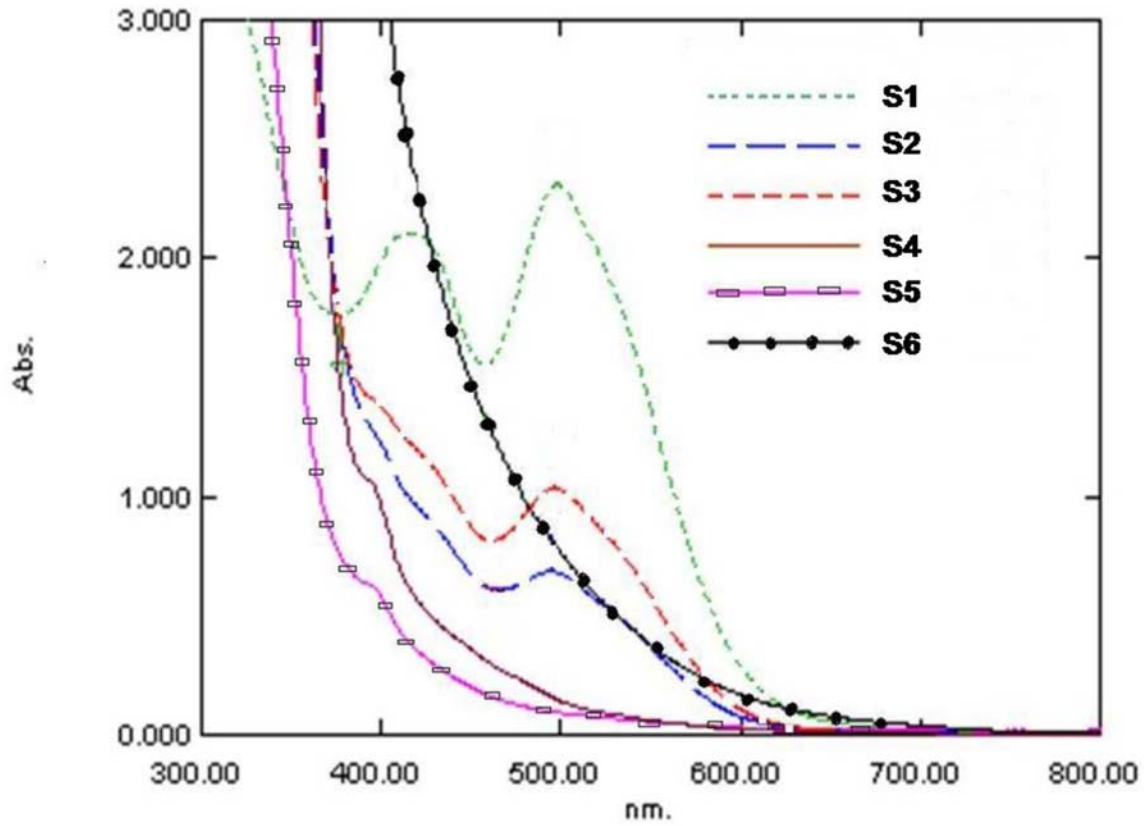
Media No	Pigment yield <sup>@</sup>	DPPH Scavenging (%)
S1	9.232	37.31 ± 0.17
S2	2.632	70.50 ± 0.81
S3	4.14	60.98 ± 1.63
S4	4.14	82.51 ± 0.92
S5	2.054	56.63 ± 1.49
S6	3.856	91.10 ± 0.06

<sup>@</sup>Estimated as absorbance at 482 nm

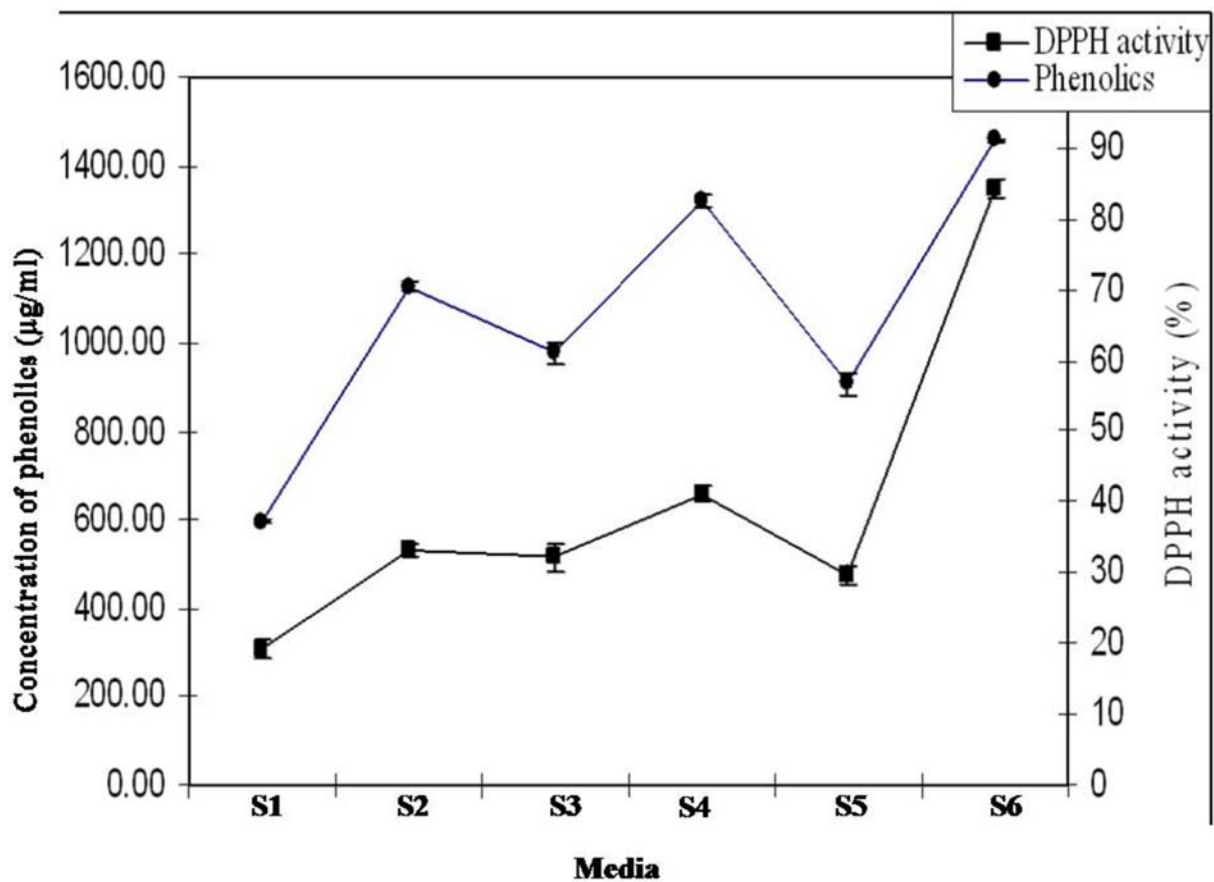


**Figure 1** Culture filtrate proteins (10  $\mu$ g) of *Penicillium* sp NIOM-02 were used for enzyme zymogram reactions with different concentration (0.025–0.1%) of acarbose (A1–A4) and without acarbose (C). Ten microgram of proteins were used for western blot reaction to identify the 53 kDa amylase in culture filtrate (B). Molecular mass standards (M).

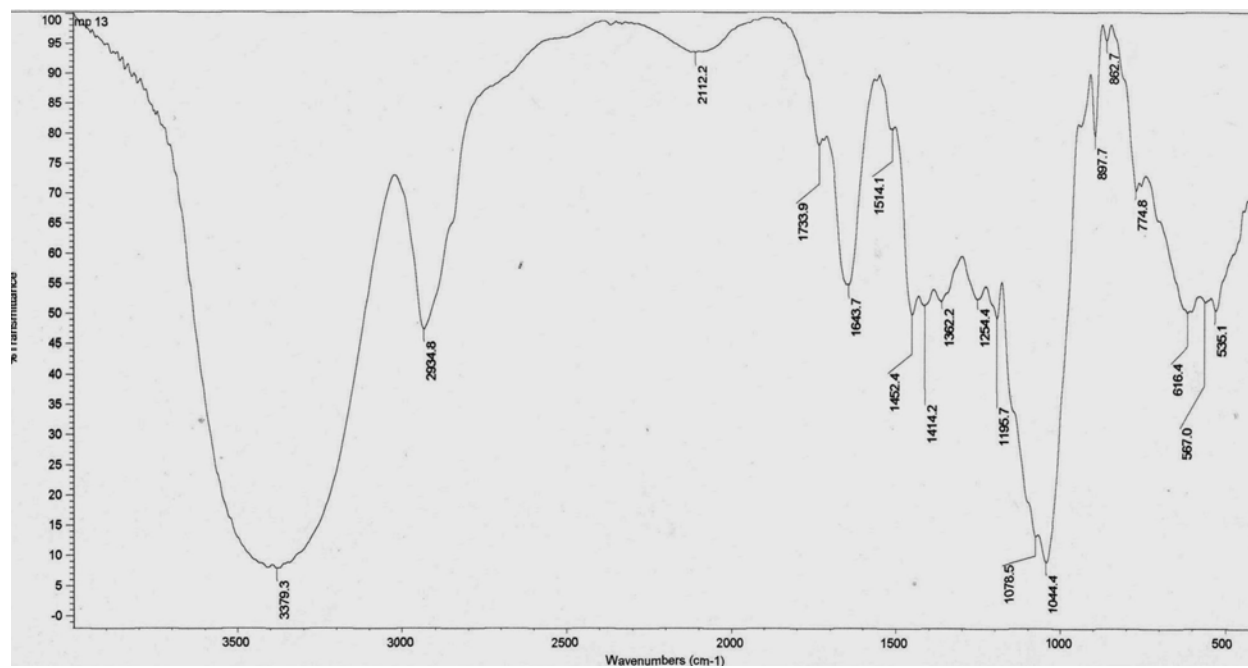




**Figure 2** UV-Visible spectra of pigment produced by *Penicillium* sp NIOM-02 grown on different solid substrate medium (S1–S6).



**Figure 3** A correlation between concentration of phenolics ( $\mu\text{g mL}^{-1}$ ) and antioxidant activity (%) of *Penicillium* sp NIOM-02 extracts grown by solid-state fermentation.



**Figure 4** IR spectrum of *Penicillium* sp NIOM-02 extract cultured on sugarcane bagasse