

Research Paper

Molecular characterization of a Xylanase-producing fungus isolated from fouled soil

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Abstract

Xylanase (EC 3. 2. 1. 8), hydrolyzes xylo-oligosaccharides into D-xylose and required for complete hydrolysis of native cellulose and biomass conversion. It has broad range of applications in the pulp and paper, pharmaceutical and Agri-food industries. Fifty fungal species were isolated from the fouled soil around an oil refinery and screened for the production of xylanase enzyme by enrichment culture techniques. The isolated fungal strain was identified as *Hypocrea lixii* SS1 based on the results of biochemical tests and 18s rRNA sequencing. The phylogenetic tree was constructed using the MEGA 5 software. Further, *Hypocrea lixii* SS1 was tested for the ability to utilize the sunflower oil sludge (waste from the oil industry) as the sole carbon source for xylanase production. The growth characteristics of *Hypocrea lixii* SS1 were also studied and maximum growth was found on the 7th day of incubation. The fungus showed a remarkable xylanase production of 38.9 U/mL. Xylanase was purified using a combination of 0-50% NH₄SO₂ precipitation, DEAE-sepharose and Sephacryl S-200 chromatography. Single peak obtained in RP-HPLC confirms the purity of xylanase. Further the enzyme produced was affirmed as xylanase with its molecular weight (29 kDa) using SDS-PAGE.

Key words: fungal isolate, growth kinetics, *Hypocrea lixii* SS1, fermentation, xylanase, purification.

Introduction

Lignocellulosic wastes are generated from agricultural practices and industrial processes, peculiarly from agro-allied industries such as breweries, paper-pulp and textile industries throughout the world. These wastes generally accumulate in the environment, thereby causing pollution (Okafor *et al.*, 2007). However, these wastes are biodegradable and can be converted into valuable products such as biofuels, chemicals and cheap energy sources for fermentation and ameliorated animal feeds (Howard *et al.*, 2003). Ligno-cellulosic wastes refer to plant biomass wastes that are mainly composed of lignin, cellulose and hemicelluloses. They may be grouped into different categories such as wood residues (including sawdust and paper mill discards), waste paper and agricultural residues including straw, bagasse, domestic wastes (lignocellulose waste), food industry residues and municipal solid wastes (Mtui, 2009). Due to their abundance and renewability, there has been a great deal of concern in utilizing lignocellulosic

wastes for the production and recovery of many value-added products (Pandey *et al.*, 2000; Foyle *et al.*, 2007).

Hemicellulose is the second most abundant plant fraction available in nature after cellulose present agro-industrial wastes. It consist of a mixture of hexosans, pentosans and their monomers are useful in the production of different antibiotics, animal feed and fuels. Xylan is the most abundant of the hemicelluloses which has a linear backbone of β -1,4-linked d-xylopyranose residues which is further substituted, depending on plant sources to a varying degree with glucuronopyranosyl, 4-O-methyl-d-glucopyranosyl, α -l-arabinofuranosyl, as well as linked to feruloyl and coumaryl components of lignin (Shallom and Shoham, 2003). Biodegradation of xylan requires the action of several enzymes, among which xylanases (EC 3.2.1.8) play a key role. Xylanase hydrolyzes xylan fibers to shorter sugar residues which have wide applications in industry (Goulart *et al.*, 2005). Recently, interest in xylanases has markedly increased due to their potential applications in the food and

beverage industries, feedstock improvement and the quality improvement of lignocellulosic residues (Pal and Khanum, 2011). Single enzyme nanoparticles are a recent area of research in industrial enzymes. Nanoparticles are used as an emerging tool for the drug delivery system (Priya VP *et al.*, 2013). Gold nanoparticles can be synthesized by the methods of Arockiya Aarthi Rajathi F *et al.*, 2012; Stalin Dhas T *et al.*, 2012; Venkatachalam M *et al.*, 2013 and Kathick V *et al.*, 2012 and can be used for xylanase immobilization studies. Enzyme immobilization helps in increasing the stability of the enzyme and thus helps in increasing the practical application of enzymes.

Filamentous fungi are useful producers of xylanase because they are capable of producing high levels of extracellular enzymes and can be cultivated very easily. On an industrial scale, xylanases are produced mainly by *Aspergillus* and *Trichoderma spp.* in solid state fermentation (SSF) (Pal and Khanum, 2010). Many reports on xylanases from *Bacillus spp.*, *Streptomyces spp.*, *Aspergillus spp.*, *Trichoderma spp.*, and other microorganisms are available (Krisana *et al.*, 2005; Evstatieva *et al.*, 2010). *Trichoderma harzianum* is present in all soil types and are the most prevalent culturable fungi. This organism has a potential application in the field of biotechnology. Its metabolites stimulate seed germination, plant growth and early flowering and fruit formation. It is also used as a bio-organic fertilizer (Chen LH *et al.*, 2012; Akladios SA *et al.*, 2014), bio-control agent against chickpea (Jayalakshmi SK *et al.*, 2009), reducing root rot disease in tobacco seedlings (Gveroska B and Ziberoski J, 2011), production of industrial enzymes like xylanases (Ahmed S *et al.*, 2012), cellulolytic complexes (Benoliel B *et al.*, 2013), β -1,3-glucanases (Giese EC *et al.*, 2005).

Sunflower (*Helianthus annuus*) is cultivated worldwide mainly for oil extraction. Sunflower oil Sludge (a by-product rendered by the oil industry) has been generated in large quantities and remains as waste. Fractionation of Sunflower oil Sludge results in a lignocellulosic fraction (LCF) - 23.2-25.3%, a proteinaceous fraction (PF) 55.4-57.6% and a soluble fraction (SF) 17.1-21.4% of the dry weight. As evidenced by the growth of different fungi, Sunflower oil Sludge -LCF (obtained after removal of PF) was found to be a suitable fermentation source for SSF (Bautista *et al.*, 1990).

Rapid industrialization for sustaining economic stability leads to the pollution of the environment. Industrialization and urbanization are responsible for air, soil and water pollution. To remove pigments, objectionable odors and non-triglyceride material from crude oil, various methods such as bleaching, deacidification, neutralization and odorization were used (Anderson, 1953). An eloquent amount of by-products such as deodorizer distillates, soap stocks, and acidic water are produced from crude oil refining processes, which are potentially harmful to the environment. If these residues are not disposed properly, it could

lead to environmental damage representing a danger to all forms of life.

There have been many studies on the biodegradation of various aromatic hydrocarbons, using micro-organisms isolated from the oil residues. Researchers have proved the potential of isolated microbes that has the ability to bioremediate oil polluted environments.

The present study is to explore the potential of a fungal species isolated from fouled soil around an oil refinery in a different endeavor. The fungus isolated is being used for the xylanase enzyme production. The principal aim of this study was to isolate, identify, characterize the fungal strain capable of producing xylanase enzyme from fouled soil around oil refinery and the purification of xylanase produced by the fungal strain. Thus, the isolated filamentous fungal strain is used as a potential enzyme producer from the industrial waste sunflower oil sludge.

Materials and Methods

Isolation and identification of fungal strain

To isolate fungal strain, the samples were isolated from soil around oil refinery. 1 g of sample was dissolved in 10 mL of sterilized distilled water, serially diluted upto 10^{-6} and plated onto Potato Dextrose Agar (PDA) medium containing (component g/L) potato infusion (infusion from 200 g potatoes), 4; dextrose, 20; agar, 15. After sterilization, the medium was supplemented with 10 μ g amphotericin/mL and 25 μ g streptomycin/mL (Himedia, Mumbai, India) to inhibit fungal and bacterial contamination respectively. The medium was incubated at 35 °C for 72 h. One plate was kept as an uninoculated control. Pure colonies were isolated by subculturing on PDA. Stock cultures were maintained at 4 °C. Colonies having zone formation were subcultured in potato dextrose broth. The spore morphology was determined by light microscopy and scanning electron microscopy.

Extraction of fungal genomic DNA

The colonies grown on agar plates were recovered from the surface and placed into 1 mL of sterilized water and frozen in liquid nitrogen. Cell disruption was done in an ultra-turrax T25 apparatus. The extraction of DNA and its purification was performed as follows: Precipitation of proteins and polysaccharides was done by the addition of Potassium acetate (5 M). The supernatant was filtered on Whatman filter paper and 1 mL of isopropanol was added to the tube and mixed. Centrifugation at 9600 x g at 4 °C for 30 s was performed. The supernatant was discarded and the pellet (DNA) was dissolved in 70 mL Tris-HCl 50 mM pH 8, EDTA 100 mM pH 8. After purification with phenol, chloroform and isoamyl alcohol (25:24:1), the upper phase was transferred in a separate tube and added 0.10 mL of sodium acetate 3 M and 0.7 mL of isopropanol. After a centrifugation at 9600 x g for 30 s, the supernatant was

carefully removed and the pellet was dissolved in 40 mL of Tris-HCl 50 mM, EDTA 100 mM, pH 8.

Classic and molecular identification of fungal strain

Amplification a 581-bp fragment within the gene coding for the small ribosomal subunit (18S rRNA) of fungi was performed in a Thermal Cycler Gene Amp PCR System 9700 (PE Applied Biosystems, Norwalk, USA) using the fungus specific primers TR1 5'-GTTTCTAGGACCGCCGTA-3' and TR2 5'-CTCAAACCTCCATCGACTTG-3' (Bock *et al.*, 1994). Two thermal amplification cycles were used to amplify the fungal 18S rDNA. The first cycling parameters were as follows: an initial denaturation step of 96 °C for 10 min, followed by 30 cycles of 96 °C for 1 min, annealing at 46 °C for 1 min and extension at 72 °C for 2 min, with a final extension step of 72 °C for 5 min. Cycle 2 parameters were as follows: denaturation step of 95 °C for 3 min followed by 35 cycles of 95 °C for 1 min (denaturation), 53 °C for 1 min (annealing), 72 °C for 2 min (extension) and final extension of 72 °C for 3 min. The amplification products were verified by electrophoresis in 0.8% w/v agarose gel and DNA stained with ethidium bromide.

The sequences of 18S rDNA were obtained using the primer TR2 and ABI PRISMTM dye terminator cycle sequencing kit (PE Biosystem). The reaction products were analyzed using an Applied Biosystem 373A automated DNA sequencer (Perkin Elmer).

Sequence and phylogenetic analysis

The sequences were aligned using CLUSTAL W and CLUSTAL X, which is a graphical user interface program and then the sequences were compared with those from GenBank using BLAST (Thompson *et al.*, 1994). Standard Biochemical tests were also performed to identify the genus. The 18SrRNA sequence analysis of the isolate was also performed. The sequence was analyzed using MEGA 5 and the corresponding phylogenetic tree was constructed (Naveena B *et al.*, 2012).

Growth pattern of fungal strain

Hypocrea lixii SS1 was grown in the growth medium. The temperature and pH of the medium were maintained at 35 °C and 5.5 respectively. Absorbance values of cell suspensions were read at 540 nm at regular intervals of 3 h, over a 192 h period. Cell cultures were shaken well for 60 s before each measurement. The control flask contained only the culture medium. The experiments were carried out as triplicates and their average values were taken into consideration.

Determination of growth kinetics

Samples were collected at every 3 h interval from the culture flask and subjected for centrifugation at 1118 x g separately. The initial weight of the aluminum foil was

taken. The pellet obtained was placed in the foil and kept at 55 °C for 10 min until got dried. The weight of the foil with the dried pellet was measured (Naveena B *et al.*, 2012). From the biomass data obtained at various time intervals, the specific growth rate, μ_m and lag time were calculated using modified Gompertz model.

$$\ln\left(\frac{X}{X_0}\right) = \ln\left(\frac{X_{\max}}{X_0}\right) \exp\left[-\exp\left(\frac{\mu_m e}{\ln\left(\frac{X_{\max}}{X_0}\right)}(\lambda - t) + 1\right)\right] \quad (1)$$

where X_0 , X_{\max} , X , t , μ_m and λ denote initial biomass concentration (mg/mL), maximum biomass concentration (mg/mL) and biomass concentration (mg/mL), incubation time (h), maximum specific growth rate (h^{-1}) and lag time (h), respectively.

Production of xylanase using newly isolated strain

Isolates were screened for the production of xylanase enzyme using the medium containing (component g/L) Peptone, 1.0; KH_2PO_4 , 2.00; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.30; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.30; FeSO_4 , 0.01; $(\text{NH}_4)_2\text{SO}_4$, 1.80; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.0012; $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.0015; Sunflower oil Sludge (sole carbon source), 10 and Agarose, 15. Each plate was supplemented with 0.5 mL of xylan. Fresh fungal spores from the stock culture were inoculated in the plate and incubated at 35 °C, pH 5.5, moisture content 70% for 7 days under static condition.

Enzyme assay and protein determination

Assay for xylanase was performed using 0.5% soluble birchwood xylan (sigma) in 50 mM sodium phosphate buffer, pH 7. The reaction mixture was composed of 1.5 mL substrate and 0.5 mL crude enzyme. The mixture was incubated in water bath at 45 °C for 15 min. The released reducing sugar was measured by the dinitrosalicylic acid (DNS) method (Khanna and Gauri, 1993; Miller, 1959). The absorbance was measured at 540 nm with xylose as the standard. The amount of reducing sugar was calculated from the standard curve based on the equivalent xylose units. One unit of xylanase activity was described as the amount of enzyme producing 1 μmole of reducing sugar per mL medium per min under standard test conditions.

The protein content was determined by the method of Bradford method (Lowry *et al.*, 1954). The protein content in the chromatographic fractions was estimated by measuring the absorbance at 280 nm.

Molecular weight determination of xylanase by SDS-PAGE

The molecular mass of the crude enzyme extract and partially purified xylanase was estimated by SDS-PAGE electrophoresis. SDS-PAGE (12%) was performed as described by Ou *et al.* (2011), using medium range (10 to

100 kDa) molecular weight markers (Banglore Genei Pvt., India). Xylanase and other proteins were visualized by staining the gel with Coomassie brilliant blue.

Four step purification for xylanase enzyme

Xylanase (crude) enzyme was subjected to 0-50% ammonium sulphate precipitation and stored at 4 °C for overnight. This mixture was centrifuged at 10285 x g for a period of 15 min. The precipitate obtained was dissolved in Tris-HCl buffer (20 mM, pH 8.0). The resultant fraction was dialyzed against Tris-HCl buffer (20 mM, pH 8.0) using cellulose tubing (molecular mass cut-off 10 kDa) for a period of 24 h with repeated buffer changes. This step was carried out mainly to make the fraction free from sulphate ions. Further concentration of dialysate was carried out, by dialyzing the resultant fraction against sucrose. This was applied to the DEAE-sepharose column (2.6 * 10 cm). The column was equilibrated with Tris-HCl buffer (20 mM, pH 8.0). Elution of the bound proteins were carried out with the linear gradient of NaCl (0-0.4 M) in the Tris-HCl buffer (20 mM, pH 8.0), with the flow rate of 30 mL/h. 3 mL fractions were collected. These fractions were tested for xylanase activity and those showing xylanase activity were pooled together. The pooled fraction was again subjected to dialysis against Tris-HCl buffer (20 mM, pH 8.0) for 16-18 h. The resultant dialysate was concentrated further and applied to Sephacryl S-200 (1.6 * 80 cm) column. Elution of proteins was carried out using Tris-HCl buffer (20 mM, pH 8.0) + 0.15 M NaCl with a flow rate of 20 mL/h. 2 mL fractions were collected. These fractions were tested for xylanase activity. The fractions showing xylanase activity were pooled together, checked for purity and used for further analysis.

In order to test the purity of xylanase, 0.5 mL of sample was injected into an analytical RP-HPLC (Waters 600 analytical HPLC system) equipped with an analytical column (C4, 25 0.46 cm, 5 µm, pore size 80 Å). Solvents used for RP-HPLC included solvent A (0.1% trifluoroacetic acid (TFA) in water) and solvent B (0.07% trifluoroacetic acid (TFA) in 95% acetonitrile). Xylanase was eluted by means of a linear gradient from 90% A + 10% B to 80% A + 20% B at a flow rate of 2 mL/min for a period of 30 min. This was monitored in a region of 210-400 nm.

Results and Discussion

Screening of the strain

A total of 50 strains was screened from the soil around an oil refinery on PDA for the production of xylanase enzyme. Top ten isolates producing the maximum amount of xylanase is listed in the Table 1. Among those, the strains which produced highest enzyme activity was selected and subjected for further studies. The newly isolated strain produces the maximum xylanase enzyme and its activity was found to be 38.90 U/mL. This is found to be

Table 1 - Different isolates of xylanase production.

Isolate No	Dry weight (mg)	Protein (mg/mL)	xylanase activity (U/mL)
SS1	9.23	3.54	38.90
SS2	6.98	1.09	26.71
SS5	7.90	1.73	24.92
SS7	8.00	0.921	20.08
SS10	3.89	2.93	30.09
SS14	1.07	0.54	15.46
SS20	2.06	0.043	9.23
SS23	4.64	0.0098	6.43
SS33	8.98	0.3956	14.32
SS40	9.54	2.59	16.90
SS42	5.80	1.003	13.04

higher than the enzyme activity obtained from *Jonesia denitrificans* (Nawel *et al.*, 2011), *Streptomyces thermocarboxydus subspecies MW8 strain* (Chi *et al.*, 2013). This is the first report showing the highest yield of xylanase by the isolated new strain to the best of our knowledge.

Identification of new fungal strain

Morphological characteristics

The strain isolated from the soil has the following morphological characteristics: Isolated strain grows at an optimum temperature of 35 °C. The colonies were first found to be transparent and later changed into yellow color. The change in color is mainly due to the pigment production by fungal strain, which is a characteristic feature of fungal species. The fungal strain under microscope was characterized by the effuse conidiation after 3-5 days of growth, whereas the conidiophores aggregations and pustles developed later. Figures 1 and 2 shows the morphological structure of the isolated xylanase producing micro-organism. The microscopical observations were found to be the similar with Castle *et al.* (1988). This confirms that the new species identified belongs to *Hypocrea sp.* However, these methods are problematic as there are different biotypes within a species and time consuming. Hence 18s rRNA sequencing was carried out for getting reproducible and accurate results.

Identification of fungal genome

Genomic identification was carried out by 18s rRNA sequencing, and the sequence was obtained. This was blasted against the entire microbial genome database in National Center for Biotechnology Information (NCBI). The BLAST result showed only 93% similarity with other existing fungal species and thus was found to be a new strain (named SS1). Hence, this sequence was deposited in the



Figure 1 - Morphological structures of isolated *Hypocrea lixii* SS1.

GenBank and was provided with a new accession number (JN687587).

Phylogenetic tree

Phylogenetic tree was constructed from neighbor-joining program, using bootstrap consensus test with 500 in MEGA 5 and the branch lengths are in the same as those of the evolutionary distances used to infer the phylogenetic tree. The newly isolated strain was closely related to *Hypocrea lixii* with 96% similarity. Based on this similarity the isolated new fungal strain was identified as a *Hypocrea lixii* strain SS1 shown in Figure 3.

Growth pattern of fungal strain

The growth kinetics of the *Hypocrea lixii* SS1 obtained by their cultivation from 0 to 192 h was shown in Figure 4. The biomass data obtained from the growth analy-

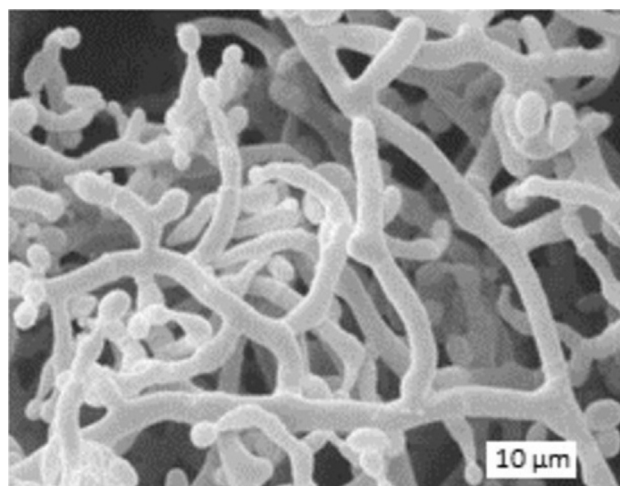


Figure 2 - SEM image of the isolate, *Hypocrea lixii* SS1.

sis was modeled using modified Gompertz model which was denoted by Eq. (1). The experimental data obtained for the fungal isolate fits into the model using Matlab 7.0. The specific growth rate and the lag time obtained for the fungal isolate was given in Table 2. The exponential growth was observed from 96 h to 168 h after which it attains a steady state around 192 h. This implies that the maximum growth observed on the 7th day of incubation. And there was no growth in the uninoculated control which indicates that the biomass obtained on the inoculated medium was only due to the fungal growth.

Production of xylanase enzyme by newly isolated strain

The newly isolated strain *Hypocrea lixii* SS1 was able to produce xylanase enzyme using Sunflower oil Sludge as the sole carbon source in the production medium. The strain *Hypocrea lixii* SS1 utilizes the lignocellulosic waste (Sunflower oil Sludge) with the help of xylanase produced on its own and convert them into xylose units, as described in the biochemical pathway shown in Figure 5 and is similar to re-

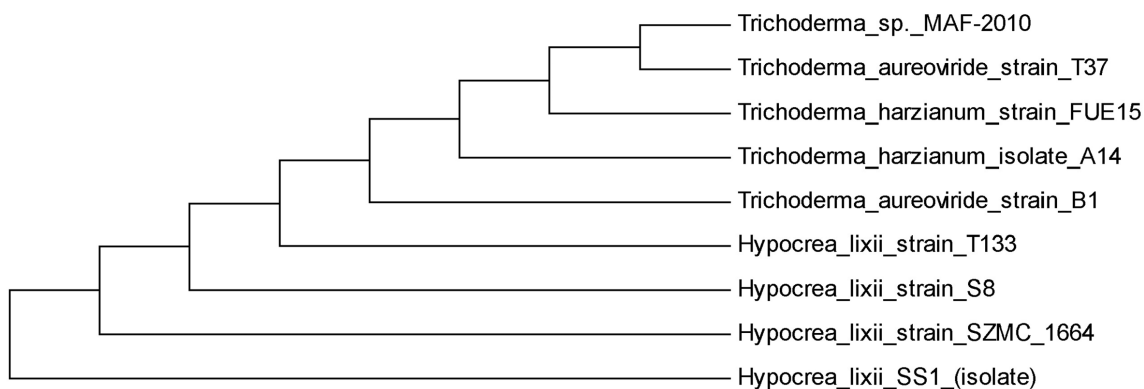


Figure 3 - Neighbour-joining tree based on nearly complete 18S rDNA gene sequences showing relationships between *Hypocrea lixii* SS1 (isolate) and the related *Hypocrea* species strain.

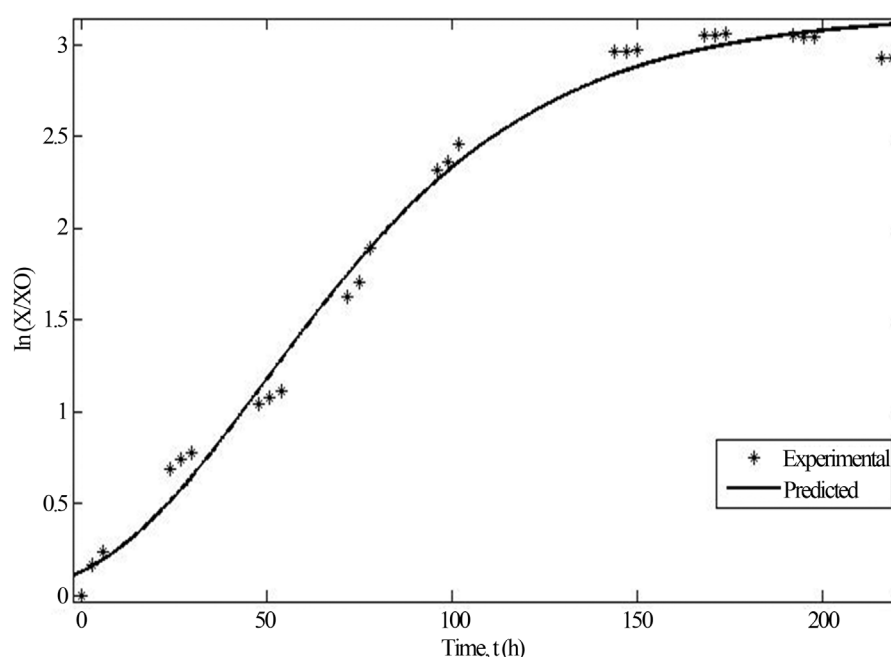


Figure 4 - Growth Kinetics of fungal *Hypocrea lixii ss1*.

Table 2 - Determination of Growth Kinetic parameters of *Hypocrea lixii SS1*.

Fungal isolate	Specific growth rate, μ_m (h^{-1})	Lag time, λ (h)	Coefficient of determination, R^2
<i>Hypocrea lixii SS1</i>	0.032	6.932	0.989

view by Polizeli MLTM *et al.*, 2005. A maximum yield of 38.90 U/mL of xylanase was obtained by the isolated strain *Hypocrea lixii SS1* in production medium. Singh R *et al.* (2012) reported a maximum xylanase yield of 30.15 IU/mL from thermophilic *actinomycetes* using various agro industrial wastes. Moreira LRS *et al.* (2012) reported a maximum yield of 0.978 IU/mL xylanase using a combination of *Aspergillus niger* on sugarcane baggasse and *Emericela nidulans* on dirty cotton residue. Gottschalk LMF *et al.* (2013) reported a maximum yield of 44880 U/L xylanase along with other enzymes - xylosidase and ferulic acid esterase from *Aspergillus awamori 2B.361 U2/1*. Moretti MMS *et al.* (2012) reported a maximum yield of xylanase 1040 U/g from *Aspergillus fumigates M.7.1* and 1292 U/g from *Myceliophthora thermophila M.7.7* using the wastes wheat bran and sugarcane baggasse.

Molecular weight determination of xylanase by SDS-PAGE

The extracellular enzyme produced by the isolated *Hypocrea lixii SS1* was found to be xylanase. This was further confirmed and characterized by SDS-PAGE. The supernatant (crude enzyme) and gel filtration active fraction of supernatant (representing partially purified enzyme)

from *Hypocrea lixii SS1* culture were used to perform the zymogram analysis. Figure 6 shows the protein content on the SDS-PAGE (Lanes 1, 2, 3). Lane 1 & Lane 2 contain the crude enzyme extract and Lane 3 contains the product after the gel filtration procedure. The gel-filtration procedure is proved to be effective for xylanase enzyme purification since Lane 3 shows the predominant band. The results indicate that *Hypocrea lixii* strain SS1 has the ability to produce xylanase enzyme and its molecular weight was found to be 29 kDa. The results obtained were similar to Kamble and Jadhav (2012).

Purification of xylanase enzyme

Xylanase from *H.lixii SS1* was purified using ammonium sulphate precipitation followed by a two-step chromatographic procedure. The results of purification procedures are represented in Table 3. The crude enzyme had a total activity of 21,580 U. After ammonium sulphate precipitation, the enzyme was purified 6.38 fold with 86.32% recovery. The enzyme was then subjected to DEAE-sepharose ion exchange chromatography. In this step, xylanase was purified 24.67 fold with 69.54% recovery. Finally, xylanase was subjected to Gel filtration chromatography. In this step, xylanase was purified 41.23 fold with 47.9% recovery. The results obtained in the current study, were found to be better when compared to the studies by others in various microorganisms. Pal A *et al.* (2011) reported a yield of 38.9% with a purification fold of 36.97 recovery from *Aspergillus niger DFR-5* using ion exchange and gel filtration chromatography. Gaffney M *et al.* (2009) reported 7.6% recovery with a purification fold of 9.3 from *Thermomyces lanuginosus* using a combination of ultra-

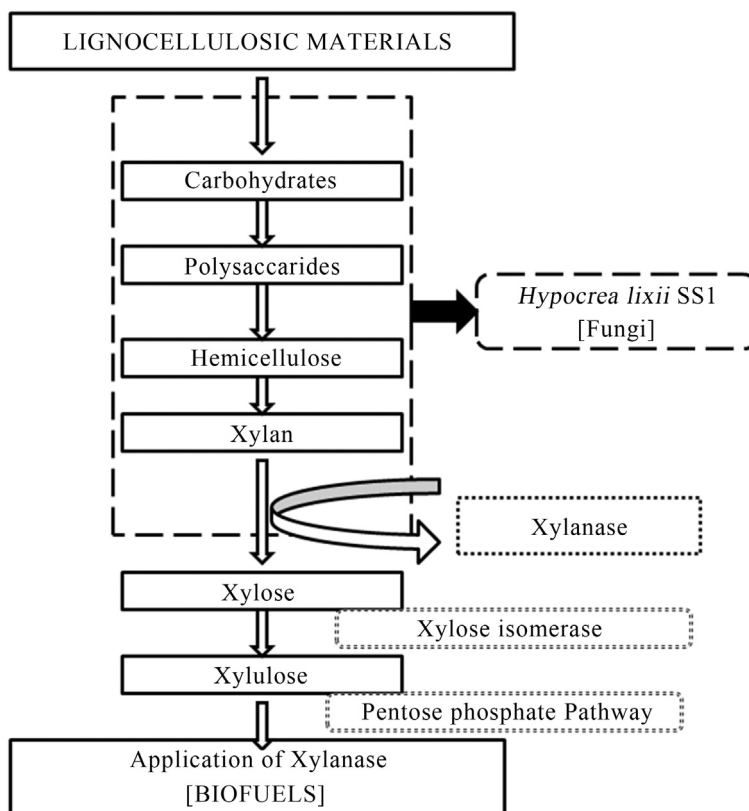


Figure 5 - Biochemical pathway of Xylanase production.

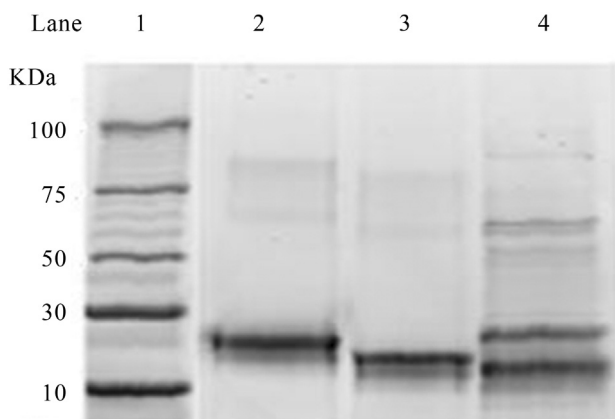


Figure 6 - Estimation of xylanase molecular weight using SDS-PAGE.

filtration, anion exchange, gel filtration chromatography. Yang S *et al.* (2008) reported 34.5% recovery with a purification fold of 5.8 from *Paecilomyces thermophile* using a combination of 20-60% (NH₄)SO₂ concentration, Supradex 75 column chromatography and DEAE 52 column chromatography. Ninawe S *et al.* (2008) reported a purification fold of 2.25 with 43.62% recovery from *Streptomyces cyaneus* SN32 using a combination of ammonium sulphate concentration and DEAD-sepharose chromatography.

Both crude enzyme and purified xylanase was subjected RP-HPLC method to confirm the purification procedure. The results are represented graphically Figure 7a and 7b. Figure 7a, has numerous peaks, showing that the crude extract is heterogeneous containing various protein components along with it. Figure 7b, shows a unique peak at 280 nm (Retention time 16.60 min), confirming the purified xylanase is homogeneous indicating the absence of other impurities, thus confirming the purity of xylanase.

Table 3 - Four Step Purification of xylanase.

Fraction	Total activity (U)	Total protein (mg)	Purification fold	Yield (%)
Crude extract	21,580	5681	1	100
Ammonium sulphate	17,810	1082	6.38	86.32
Ion-exchange chromatography	13,560	148	24.67	69.54
Gel permeation chromatography	6,480	56	41.23	47.90

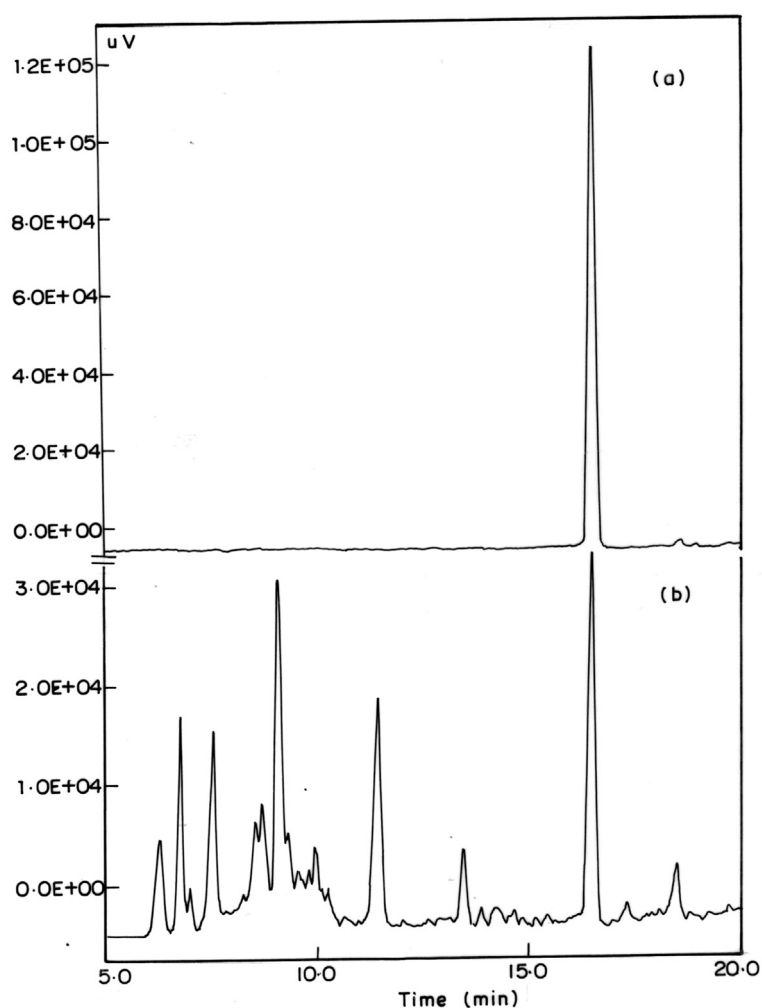


Figure 7 - RP-HPLC analysis of (a) purified enzyme and (b) crude enzyme.

Conclusion

The present study showed that soil near oil refineries could be a rich source of xylanase enzyme producing fungi using Sunflower oil Sludge as the waste from the oil industry. The following conclusions were made from this study:

Out of various microorganisms isolated from the fouled soil, fungal strain *SS1* was found to be high potential for the production of xylanase enzyme.

The genus of the fungal isolate was identified as *Hypocrea* by performing the morphological and biochemical tests. Furthermore the 18srRNA and the phylogenetic analysis reasserted the isolate as *Hypocrea lixii SS1*.

A maximum yield of 38.90 U/ml xylanase was obtained using the isolated strain *Hypocrea lixii SS1*.

The enzyme was further affirmed by SDS-PAGE with their standard molecular weight (29 kDa).

Xylanase from *Hypocrea lixii SS1* was purified to absolute homogeneity using a combination of ammonium sulphate precipitation, DEAE-sepharose ion exchange chromatography and gel filtration chromatography. Single peak

was obtained when this preparation was subjected to RP-HPLC, confirming the purity of xylanase.

Acknowledgments

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