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Optimization of Solid State Fermentation and Leaching Process Parameters for Improvement Xylanase Production by Endophytic *Streptomyces* sp. ESRAA-301097

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Abstract

In the course of our searching program on the microbial endophytes of medical plants (*Cympobogon proximus*, *Anethum graveolens*, *Artemisia judaica* and *Corchorus olitorius*), the endophytic strain *Streptomyces* sp. ESRAA-301097 derived from *Cympobogon proximus* proved to be the hyper xylanase producer. Screening of various locally available agro-industrial residues as substrate support for xylanase production under SSF exhibited a mixture of wheat bran (WB); sugarcane bagasse (SCB) with corncob (CC) at a ratio of 0.5:1:1 as the efficient inducer for the induction of ESRAA-301097 xylanase production as it gave the highest enzyme productivity (2364 $Ugds^{-1}$) at the 4th day of fermentation when compared to individual WB, SCB or CC (1167, 1241 or 1404 $Ugds^{-1}$) after 3, 4 and 4 days of incubation. Xylanase production was enhanced to 3819 $Ugds^{-1}$ after optimizing the physical process parameters including temperature 30-40°C, pH 7.0, an inoculum level of 10^7 spore gds^{-1}, 80-85 % initial moisture content and substrate particle size of 800 µm. An overall 23.96 % increase in enzyme production was attained with a mixture of soybean and corn steep solid as a nitrogen source but no enhancement was obtained with any of carbon or metal supplementation. Whereas xylanase yield was elucidated to 5709.2 $Ugds^{-1}$ by adding Tween 20, SDS repressed its production to 750.29 $Ugds^{-1}$. The optimized leaching parameters for effective extraction of xylanase (6312.45 $Ugds^{-1}$) from the fermented solid mixture were found to be citrate buffer (0.1 M, pH 4.0) containing 0.2% Tween 80 as leaching agent, extractant volume 1:8 - 1:10 (w/v), soaking time 120 min, leaching pH 4 and leaching temperature 50°C under agitation at 150 rpm. The overall level of 44.61-fold purification of *Streptomyces* sp. ESRAA-301097 and xylanase recovery 32.52% were achieved with specific activity of 493.48 Umg^{-1}. The purified enzyme showed a single protein band on SDS-PAGE indicating the monomeric nature of the enzyme with molecular weight ~31.5 kDa. Furthermore, whereas the inhibitors of cysteine protease (1, 10-phenanthroline and Dithiothreitol), metaloprotease (EDTA and EGTA) and thioprotease (iodoacetamide and p-chloromercuribenzoate) had no to minor effects on xylanase activity, the serine protease inhibitor (PMSF) markedly decreased it.

Keywords: Endophytic *Streptomyces*; Xylanase; Optimization; Purification; Characterization

Introduction

Xylan constitutes 20 to 40% of higher plants and agricultural wastes dry weight. Microbial xylanases are of increasing interest due to their potential in biotechnological applications as converting of lignocelluloses in industry to sugar; ethanol or other useful substances, improving the nutritional quality of silage or green feed, deinking processes of waste papers and liquefying the fruits and vegetables [1]. The multifunctional xylanolytic enzyme system is wide spread among fungi [2] and bacteria but great potential of xylan assimilating *Actinomycetes* can be attributed to highly activity, thermo-stability and free of substantial cellulase activity [3]. One relatively unexplored and new microbial niche is the inner tissues of higher plants, creating an enormous biodiversity that can be isolated after surface sterilization [4,5]. However *in vitro*, various endophytes exhibited high ability to produce various enzymes of biotechnological importance with new characters such as endophytic *Micromonospora* sp. Aya 2000, the recombinant strain Tahrir-25, *Aspergillus* sp. Jan 25, and *Aspergillus* Sp. NRCF5 that have been reported as potent producer for the highly active keratinase, cellulase, glucoamylase, and xylanase enzymes with new characters [6-9]. In spite of the enormous industrial importance, the production of xylanase was hindered by the high cost of production [10]. In order to curtail the production cost, one should use inexpensive substrates and follow an efficient fermentation process as solid state fermentation (SSF), which features by higher productivity with better exploitation of agro residues as substrates to achieve the economic viability of these otherwise waste resources as well as safeguard the environment [10]. The goals of the recent study are: i) Evaluation of some Egyptian medical plants as enormous source for endophytic actinobacteria, especially those with xylanolytic activity ii) Cost effective production of xylanase by the hyper endophytic producer, *Streptomyces* sp. ESRAA-301097 iii) Optimization of SSF and leaching process parameters for maximum yield of xylanase iv) Purification and characterization of xylanase produced by *Streptomyces* sp. ESRAA-301097.

Materials and Methods

Birchwood xylan (Sigma Co.) was used for enzyme assay. Each fermented matter as wheat bran (WB), rice bran (RB), sugarcane

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bagasse (SCB), corncob (CC), rice straw (RS), wheat straw (WS), barely bran (BB), banana stalk (BS), sorghum stalk (SS) and maize stalk (MS) was obtained from local supplier, stripped, dried, grinded, sieved and evaluated as substrate support.

Enzyme assay

Xylanase activity was assayed according to the method of Bailey et al. [11] using 1% birchwood xylan in 0.01 M phosphate buffer (pH 7.0). The release of reducing sugars was determined using the 3, 5- dinitrosalicylic acid (DNS) method [12]. One unit (U) of xylanase was defined as the amount of enzyme required to liberate 1 µmol of xylose from xylan in 1 min under the assay conditions. All experiments have been performed in triplicates. Xylanase production in SSF was expressed as U/g dry solid substrate ($Ugds^{-1}$) but in submerged fermentation (SMF) in terms of U/ml (Uml^{-1}).

Isolation of plant-derived endophytic *Actinomycetes*

Each organ (roots, stems and leaves) of the collected Egyptian medicinal plants *Cympobogon proximus* (halfabar), *Anethum graveolens* (dill), *Artemisia judaica* (shih balady) and *Corchorus olitorius* (malukhiyah) were surface sterilized and sectioned into small fragments as previously described [5]. These surface sterilized tissue segments were plated onto three different isolation media, *actinomycetes* isolation agar (AIA), dextrose yeast extract malt extract agar (DYMA) [13] and xylan agar medium (XAM) [14], which incubated at 28°C for 3 weeks until the selected single colonies that exhibited similar morphological features of *Actinomycetes* growing out the plated segments. Endophytic actinobacterial isolates obtained were maintained at 4°C in the Chemistry of Natural and Microbial Products Department at National Research Center.

Screening of xylanase-producing *Actinomycetes*

Xylanolytic isolates were detected by growing on selective xylan-agar medium at pH 7.0 and 28°C for 5 days and then stained with Congo red solution [14]. Xylanolytic isolates were evaluated on the basis of the diameter of the xylan digestion halo zone as: weak xylanase producer (8–14 mm); moderate xylanase producer (15–24 mm) and high xylanase producer (25–35 mm). The strains displaying the biggest xylan digestion halo were secondary screened in 250 ml Erlenmeyer flasks containing 50 ml of xylan broth medium at 30°C and pH 7.0 in a rotary shaker at 180 rpm for five days. The endophytic isolate demonstrating the highest xylanase activity in primary and secondary screening was selected for further studies.

Characterization of xylanase hyper-producer isolate

Characterization of the hyper-xylanase producing isolate, ESRAA-301097, was done through polyphasic characterization (phenotypic, chemotypic and genotypic features).

Phenotypic and chemotypic characterization

The analysis of phenotypic and chemotypic characteristics was done according to the diagnostic key of Szabo et al. [15], Williams et al. [16] and Shirling and Gottlieb[17]. Determination of the isomer of diaminopimelic acid (DAP) and the whole-cell sugar pattern was carried out as described by Hasegawa et al. [18], but fatty acid methyl esters were prepared by the trimethylsulphonium hydroxide method [19]. Phospholipids analysis was determined according to Lechevalier et al. [20] and Minnikin et al. [21]. The base composition of genomic DNA was determined by the method of Mandel and Marmur [22].

Genomic DNA preparation and 16S rDNA sequencing

Genomic DNA was extracted and purified using the QIAGEN DNeasys Tissue Kit following the manufacturer's protocol for Gram-positive bacteria. Amplification of ribosomal DNA was performed using puReTaq™ Ready-To-Go™ PCR Beads (GE Healthcare). For amplification of the nearly complete 16S rRNA gene the eubacterial primers 27f and 1492r were used [23]. The conditions for this PCR were applied according to El-Bondkly et al. [24]. PCR products were checked for correct length on a 1% Tris-borate-EDTA (TBE) agarose gel (1% agarose, 8.9 mM Tris, 8.9 mM borate, 0.2 mM EDTA), stained with ethidium bromide and visualized under UV illumination. Purification of PCR products and determination of sequences using the 16S rDNA-specific primers 342f, 534r, 790f and 1492r were done. Sequence data were edited with Lasergene Software SeqMan (DNAStar Inc.). Next relatives were determined by comparison to 16S rRNA genes in the NCBI GenBank database using BLAST (Basic Local Alignment Search Tool, http://www.ncbi.nlm.nih.gov website) to create a matrix using MEGA6 and ClustalW programs. The tree topologies were evaluated by bootstrap analyses based on 1,000 replications with MEGA6 and phylogenetic trees were inferred using the neighbor-joining method. The complete 16S rDNA sequences of the hyper-xylanase producing strain ESRAA-301097 have been deposited in GenBank database under the Accession numbers KF877333.

Xylanase production under submerged fermentation using agro industrial residues

The production media (ISP4) supplemented with 1% wheat bran (WB), rice bran (RB), sugarcane bagasse (SCB), corncob (CC), rice straw (RS), wheat straw (WS), barely bran (BB), banana stalk (BS), sorghum stalk (SS) or maize stalk (MS), individually instead of starch were inoculated with 10^6 spores ml^{-1} at pH 7.0, 28°C and 180 rpm for 7 days. After fermentation, the culture broth was centrifuged at 4°C for 20 min at 10,000 rpm and the cell free supernatant was used as enzyme source.

Optimization of solid state fermentation (SSF) process parameters

Solid state fermentation (SSF) process parameters were optimized by adopting search technique varying each parameter independently at the time and subsequently once optimized fixed for each subsequent experimental run as described by El-Gendy [8].

Selection of solid substrate support

Ten grams of such agro industrial residue mentioned before with a particle size of 600 µm moistened at 60% (v/w) with phosphate buffer (pH 7.0) were inoculated with 10^6 spores g^{-1} and incubated at 28°C in 250 ml Erlenmeyer flasks to evaluate the impact of each substrate support on xylanase production by *Streptomyces* sp. ESRAA-301097 versus different incubation periods (1-7 days) under SSF. Furthermore, the impact of mixtures of the best inducers (CC, SCB and WB) with different concentrations on xylanase production was determined. At the end of each experiment the homogenized fermented substrates were suspended in 100 ml of citrate buffer (0.1 M and pH 4), shaking at 150 rpm for 120 min at 45°C, then centrifuged at 10,000 rpm and 4°C for 15 min and the cell free supernatant was used as enzyme for analysis.

Optimization of physical and nutritional process parameters in SSF

In this study the optimized physical process parameters were

incubation temperature (25, 28, 30, 35, 40 and 45°C), initial pH (3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 9.0 and 10), inoculum level ($1x10^3$, $1x\ 10^4$, $1x\ 10^5$, $1x\ 10^6$, $1x\ 10^7$, $1x\ 10^8$, $1x\ 10^9$ and $1x\ 10^{10}$ spores g^{-1}), initial moisture content (40, 50, 60, 70, 75, 80, 85 and 90%) and substrate particle size of 200, 400, 600, 800, 1000 and 2000 µm. On the other hand, the impact of adding various supplementations to the solid substrate support as carbon sources (0.5% w/w of glucose, fructose, mannose, maltose, lactose, sucrose, arabinose, galactose and starch), nitrogen sources (0.2% w/w, in terms of available nitrogen) of organic sources (yeast extract, peptone, soybean meal, corn steep solid, casein, urea, phenylalanine, arginine, glutamine and tryptophan) or inorganic nitrogen (NH_4NO_3, $NaNO_3$, $(NH_4)2SO_4$ and NH_4Cl), 0.1% of metal source ($CaCl_2$, NaCl, KCl, $MgCl_2$ and K_2HPO_4) and detergent additives (Triton X-100, Tween 20, Tween 80, sodium dodecylsulfate, sodium tetraborate and Polyethylene glycol) on xylanase production was attempted.

Optimization of leaching process parameters for xylanase from fermented solids

The leaching parameters were optimized by adopting search technique varying parameters one at the time as described by El-Gendy [8].

Optimization of leaching agent

The leaching out of xylanase from the fermented mixture was carried out with different extractants, as water, methanol, acetone, butanol, glycerol, NaCl (1%), citrate buffer (0.1 M at pH 4.0 and pH 6.0), phosphate buffer (0.1 M, pH 7.0), glycine-NaOH buffer (0.1 M, pH 10.0), Tween 80 (0.1, 0.2 and 0.3%) followed by leaching out by citrate buffer (pH 4.0, 0.1 M) contain 0.2% tween 80 (the best leaching agents) at a ratio of 1 fermenter substrates:10 leaching agent (w/v), 45°C and 150 rpm for 120 min.

Optimization of leaaching agent volume, soaking time, temperature, pH and physical state of the leaching process

The leaching parameters including the ratio of leaching agent to the fermented substrate (from 1:2 to 1:20 w/v), soaking time (30, 60, 90, 120, 150 and 180 min), leaching pH (3, 4, 5, 6, 7, 8, 9 and 10), leaching temperature (30, 40, 45, 50, 55, 60 and 70°C) and physical state of extraction process (static and agitation at 150 rpm) were optimized for maximum xylanase leaching out from the fermented solid substrates.

Purification and electrophoresis

Xylanase of *Streptomyces* sp. ESRAA-301097 was maximized and leached as described before, the cell debris was removed by filtration under vacuum and the cell-free supernatant was precipitated by 70% (w/v) saturated ammonium sulphate, centrifuged at 10000 rpm for 20 min at 4°C and the collected precipitate was resuspended in 50 mM phosphate buffer (pH 7.0), dialyzed against the same buffer for 24 h at 4°C and the desalted ammonium sulfate fraction was lyophilized for further purification by chromatography. The lyophilized material was dissolved in 10 ml of phosphate buffer (pH 7.0) and loaded on to a DEAE-cellulose chromatographic column (2.5×40 cm) that had been equilibrated and eluted with 50 mM phosphate buffer containing 0.5 M NaCl, at a flow rate of 30 ml/h. The xylanase fractions were pooled, concentrated, dialyzed against the same buffer, lyophilized, dissolved in 5.0 ml of the same buffer and loaded into a Sephadex G-200 column (1.5×60 cm) that equilibrated and eluted with 50 mM phosphate buffer (pH 7.0). Fractions of 2 ml were collected at a flow rate of 10 ml/hour. The pooled and concentrated active xylanase fractions were loaded onto the Sephadex G-100 column (1.5×50 cm) equilibrated and subsequently eluted by using the same buffer at a flow rate of 10 ml/hour. The resulting active fractions were pooled and used as the purified xylanase.

Protein estimation

During purification, protein was estimated by the method of bicinchoninic acid with bovine serum albumin (Sigma Co.) as a standard [25]. The protein content of eluants was measured by monitoring the optical density at 280 nm.

Molecular mass determination

The molecular mass of the purified xylanase was estimated by SDS–PAGE electrophoresis (12%) as described by Laemmli [26] using medium range molecular weight markers (14.4 to 97.4 kDa, Sigma). Proteins were visualized by staining with Coomassie Brilliant Blue R-250.

Characterization of ESRAA-301097 xylanase

The optimum temperature for xylanase activity was determined by measuring the activity at various temperatures (25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 and 100°C) and its thermal stability was estimated by incubating the enzyme at these different temperatures for 1 h and then the residual activity determined at the optimum assay temperature. Similarly, optimum pH for the purified enzyme was determined at optimized temperature using 0.1 M buffers of different pH values such as citrate phosphate (pH 4.0–6.0), sodium phosphate (pH 7.0), Tris-HCl (pH 8.0–9.0), and glycine NaOH (pH 10.0–11.0) and its pH stability was determined by incubating the enzyme in the pH range 4–11 for 24 hours at 30°C and then the residual activity was measured. The effect of different xylan concentrations (1, 2, 3, 4, 5 and 6% of birchwood xylan) as well as the substrate specificity of xylanase toward 1% of birchwood xylan, xylan oat splet, CM-cellulose and filter paper was evaluated. Moreover, the purified enzyme was incubated with 10 mM of different salt solutions (Mn^{2+}, Cu^{2+}, Mg^{2+}, Zn^{2+}, Fe^{3+}, Na^{+}, Ba^{2+}, Hg^{+2}, Co^{+2}, Cd^{2+}, Pb^{+2}, Ca^{2+}, Ni^{+} and Li^{+}), 1% v/v of different detergents (Triton X-100, sodium dodecyl sulphate, sodium tripolyphosphate, sodium tetraborate, Tween 20 and Tween 80), protease inhibitors, (phenyl methyl sulphonyl fluoride PMSF, iodoacetamide, 1,10-phenathroline, dithiotheitol DTT, p-chloromercuribenzoate PCMB, EDTA and EGTA at a concentration of 10 and 50 mM as well as with different organic solvents (1-propanol, propyleacetate, benzene, toluene, n-hexane, decanol, isooctane, tetradecane, n-hexadecane and ethyl acetate, 50% (v/v) for 1 h at 30°C and the residual activity of the purified xylanase of such chemical additive was determined and compared with the control (without inhibitors as 100%).

Results and Discussion

Isolation of endophytic *Actinomycetes* from different Egyptian medical plants

Four of the most important Egyptian medical plants namely, *Cympobogon proximus* (halfabar) which used In Egyptian folk medicine as an effective renal antispasmodic, diuretic and antispasmodic agents [27], *Artemisia judaica* (shih balady) that used as antiseptic agent or tinctures applied for the relief of rheumatic pains [28]; *Anethum graveolens* (dill), which has antimicrobial, antihyperlipidaemic, antispasmodic, antihypercholesterolaemic activities [29] and *Corchorus*

olitorius (malukhiyah) that exhibits several antifertility, anti-convulsive, antioxidants, anti-inflammatory, anti-proliferative, antimicrobial and antitumor activities with gastro-protective effect [30] were selected and tested for their endophytic actinobacteria. *Cympobogon proximus* hosted in its leaves; stems and roots 18, 11 and 24 endophytic isolates of actinobacteria among them 7, 4, and 10 isolates, respectively are xylanolytic strains (Table 1). Stems and roots of *Artemisia judaica* were colonized by 19 and 10 actinobacterial isolates out of them 7 and 3 isolates have xylanolytic activity. Interestingly, among the tested plants, *Corchorus olitorius* proved to be the best host for endophytic actinobacteria by noticing the growing number of derived isolates (29, 40 and 48 isolates) with the highest number of xylanolytic isolates (14, 16 and 21 isolates) from its leaves, stems and roots, respectively (Table 1). On the other hand, no actinobacteria were isolated from all organs of *Anethum graveolens* due to several endophytes can be isolated from different host but at the same time they are reported to be host specific [31]. Also whereas actinobacterial isolates were not derived from the leaves of *Artemisia judaica*, they were detected from its stems and roots due to many endophytes appear specialized to particular host tissues as reported previously by Suryanarayanan et al. [31]. Thus, such host specific/organ specific endophytes have been observed in the plants used in the present work. These data are sufficient for the Egyptian medical plants to be underexplored reservoirs of *Actinomycetes* especially those with xylanolytic activity.

The growth of microbes in the laboratory is dependent on the composition of the media and the cultivation conditions that are applied [32]. However maximum endophytic *actinomycetes* (86 isolates) were obtained in *actinomycetes* isolation agar medium followed by xylan agar medium (82 isolates) and minimum (31 isolates) detected in dextrose yeast extract malt extract agar (DYMA) (Table 1). Qin et al. [4] reported that high nutrient concentration medium (as in DYM) allowed fast growing bacteria to overgrow slower growing microorganisms but some media composed of amino acids as nitrogen sources (as in AIA) or cellulose and xylan as carbon sources (as in Xylan agar medium) had prominent isolation effectiveness for actinobacterial genera.

Screening of the hyper xylanase producing actinomycete isolate

Among 80 xylanolytic actinomycete isolates obtained in this study, 26; 33 and 21 isolates were detected as weak (8–14 mm); moderate (15–24 mm) and hyper xylanase producers (25–35 mm), respectively on xylan-agar plates. Further xylanase evaluation in xylan liquid medium supported endophytic actinomycete isolate ESRAA-301097 of *Cympobogon proximus* as the hyper xylanase producer. It displayed xylan digestion halo diameter of 35 mm in primary screening with enzyme activity equal to 52.06 Uml^{-1} in secondary screening (Table 2), thus it was selected for further studies.

Host plant name	Local name	Medicinal activities	Organ	Media used*	No. of derived Actinomycete isolate
Cympobogon proximus	Halfa barr	In Egyptian folk medicine as an effective renal antispasmodic, diuretic and as antispasmodic agent.	Leaves	(AIA)	8
				(DYM)	3
				(XA)	7
			Stems	(AIA)	6
				(DYM)	1
				(XA)	4
			roots	(AIA)	10
				(DYM)	4
				(XA)	10
Anethum graveolens	Dill	antimicrobial, antihyperlipidaemic, antispasmodic, antihypercholesterolaemic agent and for some gastrointestinal ailments such as flatulence, indigestion, stomachache and colic	Leaves	(AIA)	0
				(DYM)	0
				(XA)	0
			Stems	(AIA)	0
				(DYM)	0
				(XA)	0
			Roots	(AIA)	0
				(DYM)	0
				(XA)	0
Artemisia judaica	Shih balady	used as antiseptic agent or tinctures applied for the relief of rheumatic pains	Leaves	(AIA)	0
				(DYM)	0
				(XA)	0
			Stems	(AIA)	9
				(DYM)	3
				(XA)	7
			Roots	(AIA)	5
				(DYM)	2
				(XA)	3
Corchorus olitorius	Malukhiyah	Exhibits several antifertility, anti-convulsive, antioxidants, anti-inflammatory, anti-proliferative, antimicrobial and antitumor activities with gastroprotective effects	Leaves	(AIA)	11
				(DYM)	4
				(XA)	14
			Stems	(AIA)	16
				(DYM)	8
				(XA)	16
			roots	(AIA)	21
				(DYM)	6
				(XA)	21

**Actinomycetes* isolation agar (AIA), dextrose yeast extract malt extract agar (DYM) and xylan agar (XA) media

Table 1: Medicinal plants selected for endophytic *actinomycetes* isolation using different cultivation media.

isolate	Primary screening (diameter of xylan digestion zone, mm)	Secondary screening (Uml^{-1})
Esraa 300097	29	38.96
Esraa 300197	34	45.08
Esraa 300297	31	43.15
Esraa 300397	30	42.21
Esraa 300497	26	38.49
Esraa 300597	25	31.57
Esraa 300697	27	35.00
Esraa 300797	30	40.12
Esraa 300897	30	39.75
Esraa 300997	34	45.04
Esraa 301097	35	52.06
Esraa 301197	26	34.73
Esraa 301297	28	39.00
Esraa 301397	27	33.98
Esraa 301497	26	30.00
Esraa 301597	33	40.19
Esraa 301697	32	40.00
Esraa 301797	35	45.90
Esraa 301897	27	36.43
Esraa 301997	30	40.18
Esraa 302097	35	48.00

Table 2: Xylanase activity (Uml^{-1}) of hyper xylanolytic endophytic actinobacteria after primary and secondary screening at 30°C for 5 days in selective xylan medium.

Identification of the endophytic xylanolytic isolate ESRAA-301097

The aerial mycelium of ESRAA-301097 strain formed spiral spore chains with spiny spore surface (Figure 1). It was developed well and ranged in color from reddish olive to dark gray but the substrate mycelium was ranged between deep red to dark brown (Table 3). As shown in Table 4 tests for diffusible pigments formation on ISP2; ISP3; ISP4 and ISP5, melanin formation, H_2S production, gelatin liquefaction, milk coagulation, milk peptonization and nitrate reduction are positive (Table 4). Chemotaxonomic analysis showed that the cell wall of the xylanolytic isolate, ESRAA-301097, contains LL-diaminopimelic acid in addition to lysine, glutamic acid and glycine but the whole-cell sugar analysis reveals the presence of the diagnostic sugars glucose, mannose, galactose and xylose (cell-wall type I) (Table 4). Whereas the phospholipid pattern of endophytic ESRAA-301097 isolate showed characteristic phospholipids of chemo-type I that possess PE, DPG, PG, PIMS and PI, the major fatty acids components found are Iso- C14:0 (1.20%), C14: 0 (0.52%), Anteiso-C15:0 (15.10%), Iso- C15:0 (2.82%), C15:0 (12.43%), Iso- C16:0 (25.0%), C16:0 (15.72%), Anteiso-C16:0 (3.10%), Anteiso-C17:0 (14.43%), C17:0 (5.74%), C18:0 (2.55%), Iso-C18:0 (1.18%) and C18:2 (0.21%). Data in Table 4 refers to ESRAA-301097 quinone system of the predominant menaquinone MK-9(H6) (31%); MK-9(H8) (24 %) followed by MK-10(H6) (22%), moderate amounts of MK-10(H8) (10%) and MK-9(H10) (8%) beside minor amounts of MK-8(H8) (2%); MK-10(H4) (2%) and MK-10(H10) (1%). The G+C content of ESRAA-301097 genomic DNA was determined to be 70.4% (Table 4). According to Kim et al. [33], the major menaquinones of the genus *Streptomyces* are MK-9(H6) and MK-9(H8), thus it was interesting that strain ESRAA-301097 contained in addition to MK-9(H6) and MK-9(H8) unusual quinone systems. Data in Table 4 showed that all carbon and nitrogen sources tested with the exception of rhamnose, sorbitol and inositol are utilized by ESRAA-301097. Moreover, it has degradable activity towards chitin, aesculin, citric acid, succinic acid, malonic acid, pectin, malic acid, starch, cellulose, inulin, xylan and gelatin. On the other hand, ESRAA-301097 showed resistance against clindamycin, furazolidone, amikacin, penicillin G, kanamycin, furazolidone, streptomycin, erythromycin, cefazolin and rocephin but it was sensitive to gentamycin, tetracycline, lincomycin, vancomycin, chloromycetin, rifampicin and tobramycin (Table 4). Furthermore, ESRAA-301097 strain was grow well in pH range 4-11 and temperature range 15-45°C and NaCl up to 18%.

Molecular identification of hyper-xylanase producing strain ESRAA-301097 through 16S rRNA gene sequencing

The 16S rDNA region of the producing strain (ESRAA-301097) was amplified, sequenced, and submitted to GenBank (Accession no. KF877333). The obtained sequences were compared with those in the National Center for Biotechnology Information (NCBI) Nucleotide Sequence Database by using the Basic Local Alignment Search Tool (BLAST) algorithm. A comparative analysis by MEGA6 and ClustalW software demonstrated that 16S rDNA sequence from hyper-xylanase producing strain ESRAA-301097 had a significant identity to a number of *Streptomyces* sp. The comparison of xylanolytic strain ESRAA-301097 with sequences of the reference species of bacteria contained in genomic database banks exhibited a similarity of 100, 100, 100 and 99 % with *S. variabilis* NRRL B-3984, *S. vinaceus* NBRC 3406, *S. griseoincarnatus* NBRC 12871 and *S. labedae*, respectively. The phylogenetic tree obtained by applying the neighbor joining method is illustrated in Figure 2. According to the analysis of 16S rDNA sequence, together with their morphological and biochemical characteristics, hyper-xylanase producing strain ESRAA-301097 was identified as *Streptomyces* sp. and designated as *Streptomyces* sp. ESRAA-301097. Manfio et al. [34] and El-Bondkly et al. [24] reported that the

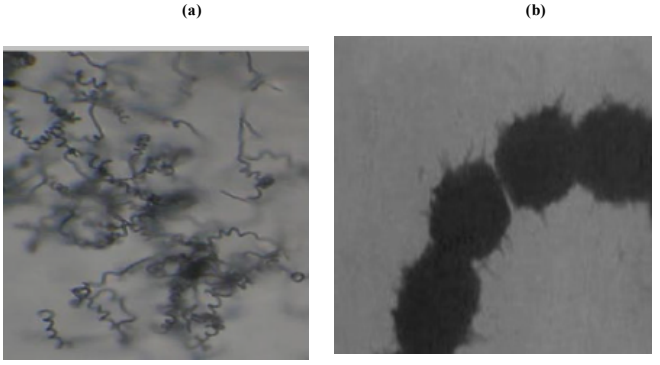

Figure 1: Scanning electron micrographs of endophytic hyper xylanolytic producer strain ESRAA-301097: (a) Spiral spore chains; (b) spiny spore surface ornamentation.

Medium*	Growth	Sporulation	Aerial mycelium	Substrate mycelium
ISP 2	Abundant	Good	Gray	Dark brown
ISP 3	Good	Good	Dark gray	Reddish brown
ISP 4	Abundant	Good	Reddish olive	Brown
ISP 5	Abundant	Good	Whitish gray	Grayish brown
ISP 7	Good	Good	Dark gray	Grayish brown
Czapek's agar	Moderate	Poor	Light gray	Brownish red
Starch casein agar	Abundant	Good	Gray	Brownish red
Bennett's agar	Good	Good	Whitish gray	Light brown
Hickey &Tresner agar	Good	Good	Gray	Deep red
Nutrient agar	Poor	Poor	Gray	Brown

*ISP 2, yeast extract agar; ISP 3, oat meal agar; ISP 4, inorganic salt starch agar; ISP 5, glycerol asparagines agar and ISP 7, tyrosine agar

Table 3: Phenotypic characteristics of the hyper xylanolytic producer strain ESRAA-301097.

Chemotypic characters of the hyper xylanolytic producer strain Esraa 301097			
Spore surface	Spiny	Inulin	+
Spore chain morphology	straight	Xylan	+
Spores / chain	>10	Gelatine	+
Melanin production	+	**Carbon utilization (1%)**	
H2S production	+	D- Glucose	+
Soluble pigment on		D-Ribose	+
ISP 2	+	L-Arabinose	+
ISP 3	+	Fucose	+
ISP 4	+	D- xylose	+
ISP 5	+	Rhamnose	-
Milk coagulation	+	D-Mannitol	+
Milk peptonization	+	Adonitol	+
$NaNO_3$ reduction	+	Glycerol	+
Cell wall amino acids	L, L-diaminopimelic acid, lysine, glutamic acid, glycine	Sorbitol	-
Whole cell sugars	Glucose, mannose, galactose, xylose	Lactose	+
Major fatty acids (%)		sucrose	+
Iso- C14:0	1. 20	Maltose	+
C14:0	0.52	Fructose	+
Anteiso- C15:0	15.10	D-mannose	+
Iso- C15:0	2.82	Raffinose	+
C15:0	12.43	L-Inositol	-
Iso- C16:0	25.0	**Nitrogen source utilization**	
C16:0	15.72	Casein	+
Anteiso-C16:0	3.10	L-Histidine	+
Anteiso-C17:0	14.43	L-phenylalanine	+
C17:0	5.74	Dl-Methionine	+
C18:0	2.55	L-Serine	+
Iso-C18:0	1.18	L-Valine	+
C18:2	0.21	Urea	+
Characteristic phospholipids	PE, DPG, PG, PIMS and PI	**Response to antibiotics**	
Major menaquinones		Clindamycin (2 mg/disc)	R
MK-8(H8)	2 %	Furazolidone (15 mg/disc)	R
MK-9 (H6)	31 %	Gentamycin (10 mg/disc)	S
MK-9(H8)	24 %	Amikacin (30 mg/disc)	R
MK-9(H10)	8 %	Penicillin G (10 mg/disc)	R
MK-10(H4)	2 %	Kanamycin (30 mg/disc)	R
MK-10(H6)	22 %	Furazolidone (15 mg/disc)	R
MK-10(H8)	10 %	Tetracycline (30 mg/disc)	S
MK-10(H10)	1 %	Lincomycin (2 mg/disc)	S
Hydrolysis activity (0.1 %)		Streptomycin (10 mg/disc)	R
Chitin	+	Vancomycin (30 mg/disc)	S
Aesculin	+	Chloromycetin (30 mg/disc)	S
Citric acid	+	Erythromycin (15 mg/disc)	R
Succinic acid	+	Cefazolin (30 mg/disc)	R
Malonic acid	-	Rocephin (30 mg/disc)	R
Pectin	+	Rifampicin (5 mg/disc)	S
Fumaric acid	-	Tobramycin (10 mg/disc)	S
Malic acid	+	pH range for growth	4 - 11
Starch	+	Temp. range for growth (°C)	15 - 45
Cellulose	+	NaCl tolerance (%)	Up to 18 %
		DNA G+C content (mol %)	70.4

PE, phosphatidylethanolamine; DPG, diphosphatidylglycerol; PG, phosphatidylglycerol; PI, phosphatidylinositol; PIMS, phosphatidylinositolmannosides; +, utilized or reaction positive; -, not utilized or reaction negative, R, resist; S, sensitive

Table 4: Chemotypic characteristics of the hyper xylanolytic producer strain ESRAA-301097.

description of *Streptomyces* species must be based on a combination of genotypic and phenotypic data and if sufficient evidence is provided that an unknown is clearly different in both genotypic and phenotypic features, novel species can be described.

Xylanase production in submerged fermentation

As shown in Table 5 wheat bran; corncob and sugarcane bagasse resulted in 33.19, 46.12 and 47.30 Uml^{-1} of xylanase at the 5th, 5th and 6th day of fermentation by *Streptomyces* sp. ESRAA-301097, respectively and then gradually declined. The reduction in xylanase yield after optimum period was probably due to the depletion of available nutrient or due to proteolysis [2]. When Data in Table 5 compared with those in Figures 3 and 4, it is obviously indicated to SSF as an attractive tool for the production of xylanase over submerged fermentation due to higher productivity. Previously, xylanase production was achieved by *Streptomyces* species in different technique but SSF presents some advantages over SmF concerning it is a fit technique for using natural substrates (agro-industrial residues) as nutritional support [3,10,35].

Optimization of solid state fermentation (SSF) process parameters

Substrate support (agro-industrial residues) versus incubation time:

There is an intense focus on the valorization of agro-industrial residues for production of value added products. Data in Figure 3 indicating that all agro industrial residues used in this study could function only as nutrient support as well as inducer for xylanase production by *Streptomyces* sp. ESRAA-301097 in the range from 370 to 1404 $Ugds^{-1}$ (with banana stalk and corncob, respectively). Moreover, data in Figure 4 indicating the role of inducers for effective induction of xylanase, xylan containing substrate such as corn cob

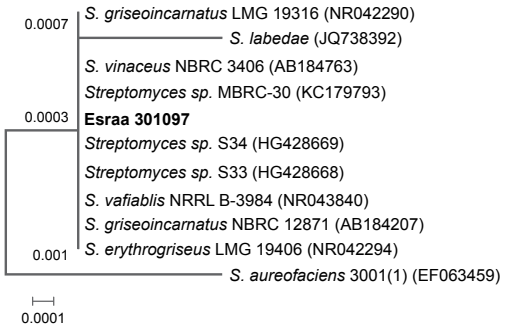

Figure 2: Phylogenetic relation of endophytic marine *Streptomyces* sp. ESRAA-301097 strain sequence with 16S rDNA of the highest similar bacteria. The dendogram was generated by the neighbor-joining method using MEGA6 software.

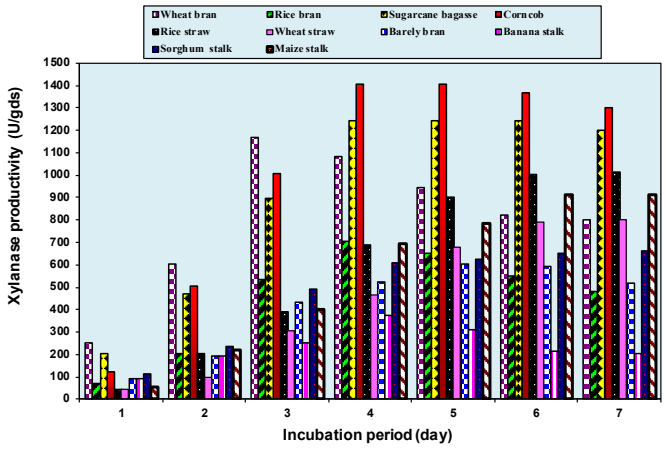

Figure 3: Screening and optimization of solid agro residue substrates for ESRAA-301097 xylanase production (Ugds^{-1}) in SSF at different incubation periods.

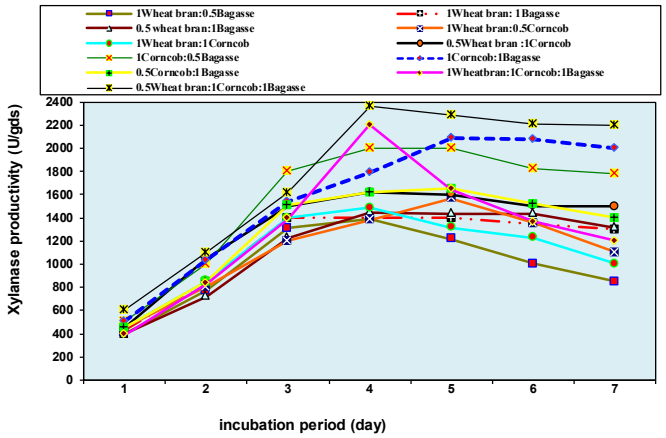

Figure 4: Effect of different combinations at different concentrations of agro residues for hyper production of ESRAA-301097 xylanase (Ugds^{-1}) in SSF at different incubation periods.

(23% xylose and 28% xylan) and sugarcane bagasse (28-30% xylan) gave the highest level of xylanase production (1404 and 1241 Ugds^{-1}) after the 4th day of fermentation and then declined till day 7 reaching 1299 and 1200 Ugds^{-1}, respectively. However, Li et al. [36] reported that corncobs xylan supported the highest xylanase activity to 334.34 U/ml after 7 days of cultivation in *S. chartreusis* L1105, Ninawe and Kuhad [3] reported wheat bran and corn cob as an enhancer for xylanase production by *Streptomyces cyancus* SN32, Techapun et al. [37] supported cane bagasse as inducer for cellulase-free xylanase from *Streptomyces* sp. Ab106 and a significant amounts of xylanase were produced by *Aspergillus fumigatus* on a variety of agro-wastes but wheat bran supported higher xylanase production followed by rice bran, rice straw and corn cobs as the sole carbon source (8,450; 5,500; 4,600 and 4,500 U/L, respectively) [2]. It was previously reported that in xylanase production corncobs act as efficient inducer due to its high content of xylan, [36] sugarcane bagasse verified due to its high water retention capacity [10] but wheat bran as a rather complete nutrient (18% protein, 5% fat and 62% carbohydrate) enhance growth and xylanase production [2]. Thus when corncob mixed with sugarcane bagasse and wheat bran at a ratio of 0.5: 1.0: 1.0 in SSF, the highest xylanase production (2364 Ugds^{-1}) was achieved (Figure 4). This study highlighted that developing a xylanase production process based upon mixture of CC, SCB and WB as a technical substrate is very attractive as they are cheap and readily available sources of carbon.

Incubation temperature

As shown in Table 6, maximum xylanase activity (3112.19 Ugds^{-1}) was attained at 30-40°C. Streptomycetes generally are mesophilic in nature with a growth temperature range of 15-45°C, then very low temperature may not trigger the metabolism of the organism while very high temperature (over 45°C) results in the denaturation of the metabolic enzymes. This optimal temperature is similar to those described for *S. chartreusis* L1105 (40°C) [36] but quite different from *Streptomyces* sp. Ab106 xylanase (55°C) [37].

Initial pH: The effect of initial pH values on xylanase production is shown in Table 6. When initial pH was equal to 3.0, no xylanolytic activity was detected. Xylanase productivity gradually increased with increasing pH reaching an optimum level at pH 7.0 (3112.00 Ugds^{-1}) and thereafter decreased at higher values reaching 1005.45 Ugds^{-1} at pH 10.0. Our findings agree well with earlier studies that showed that xylanase production is markedly dependent on pH due to it influences the enzymatic systems and its transport across the cell membrane [36]. Previous studies indicated neutral pH values between 6.0 and 7.0 for optimal xylanase production by other *Streptomyces* strains as *Streptomyces* sp. 594 and *S. chartreusis* L1105 [36].

Inoculum level

The amount of *Streptomyces* sp. ESRAA-301097 inoculum added to the fermentation medium has significant effect on xylanase production under SSF. Optimum enzyme productivity (3370.52 Ugds^{-1}) was obtained with an inoculum level of 1x10^{7} spore gds^{-1}. Higher or lower inoculum level decreased xylanase production to 701.40 and 1750.19 Ugds^{-1} at inoculums concentration 10^{3} and 10^{10} spore gds^{-1}, respectively (Table 6). Lower inoculum density than optimum level may not be sufficient for producing the require biomass while higher inoculum can cause fierce competition for nutrients [8]. Our data are similar to those obtained by Alberton et al. [10] for maximum xylanase production by *Streptomyces* viridosporus T7.

Initial moisture content (IMC)

Initial moisture level of the substrate acts as a fundamental controlling parameter for enzyme production in SSF. The highest

Agro industrial residues	Xylanase production (Uml^{-1}) during different fermentation period (day)						
	1	2	3	4	5	6	7
Wheat bran (WB)	10.16	15.94	23.70	28.72	33.19	20.12	16.00
Rice bran (RB)	8.90	13.32	18.05	19.78	20.31	20.00	18.64
Sugarcane bagasse (SCB)	16.42	29.00	30.93	31.33	40.30	47.30	40.30
Corncob (CC)	9.06	17.16	24.20	32.16	46.12	40.53	40.51
Rice straw (RS)	2.80	7.52	11.43	15.80	18.02	15.65	14.00
Wheat straw (WS)	3.17	9.00	11.68	16.02	19.35	19.35	19.00
Barely bran (BB)	5.30	6.91	12.26	16.00	16.00	14.50	11.62
Banana stalk (BS)	1.00	1.84	4.00	7.39	8.50	11.14	9.54
Sorghum stalk (SS)	2.25	3.90	5.16	8.06	10.04	13.90	11.00
Maize stalk (MS)	4.10	8.30	13.99	20.31	22.18	21.40	21.00

Table 5: Impact of different agro-industrial residues under SMF on ESRAA-301097 xylanase production (Uml^{-1}) over different incubation periods.

Process parameter	Xylanase production ($Ugds^{-1}$)	Process parameter	Xylanase production ($Ugds^{-1}$)
Incubation temperature (°C)		Carbon supplements (0.5 %)	
25	2000.41	Control	3819.00
28	2365.00	Glucose	600.16
30	3112.19	Fructose	1032.82
35	3112.19	Mannose	2160.27
40	3112.19	Maltose	1019.50
45	2600.54	Lactose	1618.04
Initial pH		Sucrose	1204.35
3.0	0.0	Arabinose	1032.82
3.5	100.28	Galactose	1322.50
4.0	304.76	Starch	2002.25
4.5	700.11	Nitrogen supplements (0.2 %)	
5.0	1280.24	Control	3819.00
5.5	1900.04	Yeast extract	3422.00
6.0	2212.65	Peptone	3068.00
6.5	2609.31	Soybean meal	4500.39
7.0	3112.00	Corn steep solid	4385.40
7.5	2819.30	Casein	3270.10
8.0	2750.74	Urea	2477.18
9.0	1907.00	Phenylalanine	2301.00
10.0	1005.45	Arginine	3290.75
Spore concentration (spores gds^{-1})		Glutamine	2000.08
$1X10^3$	701.40	Tryptophan	4029.40
$1X\ 10^4$	1100.17	NH_4NO_3	2989.26
$1X\ 10^5$	1930.29	$NaNO_3$	3999.07
$1X\ 10^6$	3112.18	$(NH_4)_2SO_4$	2149.37
$1X\ 10^7$	3370.52	NH_4Cl	2594.22
$1X\ 10^8$	2915.84	Soybean meal + Corn steep solid	4734.03
$1X\ 10^9$	2180.16	Metal supplements (0.1%)	
$1X\ 10^{10}$	1750.19	Control	4734.03
Initial moisture level (%)		$CaCl_2$	4604.40
40	1041.79	NaCl	4175.62
50	1960.00	KCl	4182.02
60	2803.17	$MgCl_2$	4214.00
70	3372.58	K_2HPO_4	4673.55
75	3410.41	Detergents additives (1%)	
80	3650.50	Control	4734.03
85	3650.50	Triton X-100	5150.18
90	3407.19	Tween-20	5709.20
Particle size (µm)		Tween-80	5294.00
200	1209.20	Sodium dodecyl sulphate (SDS)	750.29
400	2300.08	Polyethylene glycol	5000.10
600	3650.29	Sodium tetraborate	1804.70
800	3819.00		
1000	3043.59		
2000	2218.13		

Table 6: Optimization of SSF physical and nutritional process parameters of xylanase production by *Streptomyces* sp. ESRAA-301097.

enzyme production (3650.50 $Ugds^{-1}$) was obtained at 80-85% initial moisture content and then xylanase activity was decreased with higher or lower initial moisture content (Table 6). Whereas increase in SSF moisture content reduce the porosity of solid particles thus limiting oxygen transfer, a decrease in moisture content reduce the solubility and swelling of solid substrate with higher water tension [8]. Ideal moisture content for xylanase production from *S. viridosporus* T7A and *S. chartreusis* L1115 was over 90% but 75% elucidated xylanase yield from *Streptomyces* sp. QG-11-3 to 2360 $Ugds^{-1}$ [10,36,38].

Substrate particle size

Particle size of solid substrate and therefore the specific surface area was found to be one among the crucial factors affecting xylanase production by endophytic *Streptomyces* sp. ESRAA-301097 (Table 6). Maximum xylanase productivity (3819.00 $Ugds^{-1}$) was obtained from 800 µm sized particles and less enzyme detected with bigger or smaller particles. These results are in line with that obtained for particle size of sorghum straw for xylanase production by Thermomyces lanuginosus under SSF [35]. Lesser enzyme titer obtained on sized particles below

800 µm may be attributed to increasing mycelia thickness around the substrate particles with decreasing porosity of the substrate bed and then *Streptomyces* sp. ESRAA-301097 mycelium could not penetrate deep into the particles but with larger particle sizes, the saturated surface area for growth is less and productivity correspondingly less [8]. Mixture of substrate support with 800 µm particle size possibly provided sufficient surface area and aeration to actinobacteria for growth resulting in increased xylanase production.

Carbon supplementations

In the present study, the supported mixture (CC+SCB+WB) was able to function only as nutrient and inducer for xylanase production by *Streptomyces* sp. ESRAA-301097 without needing any carbon supplementation (Table 6). In comparison with the control (3819.00 $Ugds^{-1}$), the highest reduction (600.16 $Ugds^{-1}$) was obtained with glucose. This decrease may be attributed to xylanase synthesis repressed when easily metabolizable carbon sources present, suggesting that enzyme synthesis is controlled by a transitory regulatory status and catabolic repression [10]. Conversely, the highest level of *S. lividans* xylanase production was detected in wheat straw supplemented with 2% glucose [39].

Nitrogen supplementations

The mechanisms that govern the formation of enzymes are influenced by the availability and type of nitrogenous precursors for protein synthesis. Adding of soybean meal, corn steep solid and $NaNO_3$ enhanced xylanase productivity to 17.842, 14.831 and 4.715 %, respectively (Table 6). Moreover, using a mixture of soybean and corn steep solid as nitrogen source resulted in 23.96% increase in xylanase production by *Streptomyces* sp. ESRAA-301097. Conversely, with the exception of tryptophan (4029.40 $Ugds^{-1}$) there was significant decrease in enzyme yield with amino acids and ammonium salts supplementations. Nitrogen source can significantly affect the pH of the medium during the course of fermentation which in turn may influence enzyme activity and stability. Soybean meal is complex and conditioned nitrogen source that does not cause catabolite repression and probably contains approximately all kinds of amino acids [2] which can be readily absorbed by *Streptomyces* sp. ESRAA-301097 mycelium. Similar to our results, soybean meal was observed to be the best nitrogen source for xylanase production by alkalophilic *Streptomyces* species CD3 [40] and *Aspergillus* fumigates [2].

Metal supplementations

No enhancement in xylanase production was occurred when *Streptomyces* sp. ESRAA-301097 was grown on metal salts source (Table 6), thus the salt requirements for the production of this particular enzyme was apparently provided by the solid substrates (CC, SCB, WB, SB and CSS) used in SSF. These finding are important in terms of the cost of xylanase production process [8]. In contrast, Abd El-Nasser et al. [39] reported that, some divalent metal salts supplemented to wheat straw as agriculture byproduct stimulated xylanase enzyme formation by *Streptomyces lividans.*

Detergents additives

Detergent effects on xylanase production by *Streptomyces* sp. ESRAA-301097 were varied. Tween-20; Tween-80; Triton X-100 and polyethylene glycol increased xylanolytic productivity to 5709.20; 5294.00; 5150.18 and 5000.10 $Ugds^{-1}$, respectively but the addition of the ionic surfactants sodium dodecyl sulphate (SDS) to the fermentation medium resulted in a severe reduction in enzyme yield

Leaching parameter	Xylanase activity ($Ugds^{-1}$)	Leaching parameter	Xylanase activity ($Ugds^{-1}$)
Leaching agents (1:10, w/v)		Soaking time (min)	
H_2O	2670.81	60	3305.21
Methanol	2150.16	90	4890.40
Acetone	2218.43	120	6290.10
Butanol	3500.80	150	5970.12
Glycerol	3902.24	180	4600.59
NaCl (1.0%)	5000.29	pH of extraction process	
Citrate buffer (pH 4.0, 0.1 M)	5708.51	3	5734.40
Citrate buffer (pH 6.0, 0.1 M)	5295.90	4	6290.10
Phosphate buffer (pH 7.0, 0.1 M)	3366.70	5	5585.90
Glycine-NaOH buffer (pH 10.0, 0.1 M)	560.84	6	4431.90
Tween 80 (0.1%)	5720.00	7	1600.30
Tween 80 (0.2%)	5812.04	8	1170.28
Tween 80 (0.3%)	5769.28	9	1054.22
Citrate buffer (pH 4.0, 0.1 M) + Tween 80 (0.2%)	6290.10	10	754.22
Leaching agent: fermented substrate ratio (w /v)		Temperature of extraction process (°C)	
1:2	1002.33	30	2216.65
1:4	1930.16	40	4372.10
1:6	4893.10	45	6290.10
1:8	6290.10	50	6312.45
1:10	6290.10	55	5396.00
1:12	6034.24	60	4153.07
1:20	5481.00	70	3305.19
Soaking time		Agitation mode (150 rpm)	6312.45
30	2196. 74	Static mode	3103.72

Table 7: Optimization of extraction process parameters of ESRAA-301097 xylanase ($Ugds^{-1}$).

Purification step	Total protein (mg)	Xylanase activity (U)	Specific activity (U/mg)	Yield (%)	Purification fold
Crude extract	1986.50	21975.00	11.06	100	1
$(NH_4)2SO_4$ fractionation (60-80%)	671.00	17395. 42	25.93	79.16	2. 344
DEAE- cellulose chromatography	151.11	11736.86	77.67	53.41	7.021
Sephadex G-200 chromatography	23.62	9088.65	384.79	41.36	34.784
Sephadex G-100 chromatography	14.48	7145.53	493.48	32.52	44.610

Table 8: Purification profile of xylanase isolated from *Streptomyces* sp. ESRAA -301097 after growing in SSF.

to 750.29 Ugds^{-1} (Table 6). Whereas stimulatory effect of Tween 20 on xylanase production could be attributed to its effect on cell membrane permeability or by disrupting nonspecific binding of enzymes to substrates and thus exertion a positive effect on desorption and recycling of xylanase, the severe reduction in enzyme yield by SDS might be due to conformational changes in the tertiary secondary structure of the protein, binding of surfactants to the active site of the enzyme or by changing the substrate nature through decreasing the availability of reaction sites. Previously xylanase production by alkalophilic *Streptomyces* species and *Streptomyces chartreusis* L1105 was greatly enhanced when the medium supplemented with Tween 80 [36,40,41].

Optimization of leaching process parameters for *Streptomyces* sp. ESRAA-301097 xylanase

Recovery of the enzyme from the fermented matter is an important factor that affects the cost-effectiveness of the overall process. Among various leaching agents, the highest enzyme yield (6290.10 Ugds^{-1}) was leached from the fermented matter by citrate buffer (0.1 M, pH 4.0) containing 0.2% Tween 80 (Table 7). By increasing the ratio of leaching agent from 1:2 to 1:8 - 1:10 (w/v), the efficiency of leaching process was increased 6.276-fold (Table 7). Furthermore, the yield of leached enzyme was increased 2.863-fold when contact time was extended from 30 to 120 min. Moreover, data in Table 7 indicated that the quantum of xylanase recovery from the fermented mixture (CC+SCB+WB+SB+CSS) at leaching pH 4.0, leaching temperature of 50°C and under agitation mode (150 rpm) increased to 6312.45 Ugds^{-1}.

Purification of ESRAA-301097 xylanase

The elution profile of ESRAA-301097 Xylanase on DEAE-cellulose chromatography showed only one pool of xylanase activity indicating absence of multiple forms (Figure 5). The summary of purification procedures is presented in Table 8. The overall level of recovery was 32.52 % while 44.61-fold purification of xylanase was achieved with specific activity of 493.48 Umg^{-1}. The purified enzyme showed a single protein band on SDS-PAGE indicating its monomeric nature with molecular weight ~31.5 kDa (Figure 6). The molecular weight of xylanase of the other xylanolytic microorganisms such as *Streptomyces chartreusis* L1105, *Streptomyces* sp. QG-11-3, *Streptomyces* sp B-12-2, *S. thermoviolaceus* OPC-520 and *S. viridisporus* T7A were 31.6, 20.5, 23.8-40.5, 33-54, 59 and 15-36 kDa [36,38,42- 44].

Characterization of the purified xylanase

Incubation temperature, pH, substrate concentration and substrate specificity: In this study a classical pattern of temperature–activity relationship with optimum reaction temperature at 55-70°C was observed (Figure 7). ESRAA-301097 Xylanase was stable at temperature lower than 85°C and retained more than 50% of its activity after heating at 100°C for 1 h. Many investigators reported optimum reaction temperature of 55 to 75°C for xylanase activity and stability from other *Actinomycetes* such as *Streptomyces* sp. S38, *S. chartreusis* L1105, *Streptomyces* sp. Ab106; *Streptomyces* sp. B-12-2; *S. thermoviolaceus* OPC-520; *S. viridisporus* T7A and *Streptomyces* sp. QG-11-3 [1,36-38,42- 44]. On the other hand, the purified xylanase of *Streptomyces* sp. ESRAA-301097 exhibited maximum activity at pH 6.0 – 8.0 with pH stability over a wide range of pH (pH 4.0 - 9.0, Figure 8). The optimum pHs for xylanolytic activity and stability of *S. chartreusis*, *Streptomyces* sp. B-12-2, *S. thermoviolaceus* OPC-520, *S. viridisporus* T7A and *Streptomyces* sp. QG-11-3 were 6.7 - 7.7; 6.0 – 7.0; 7.0 -8.0;

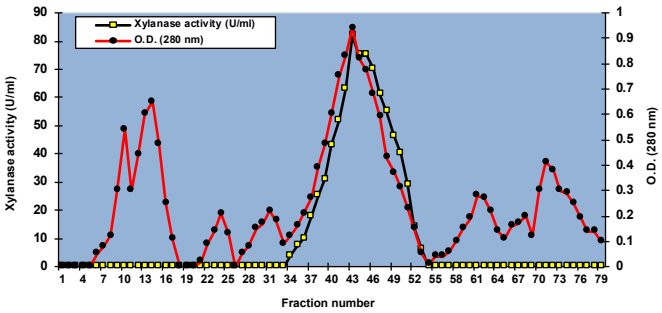

Figure 5: Elution profile of ESRAA-301097 Xylanase on DEAE-cellulose chromatography.

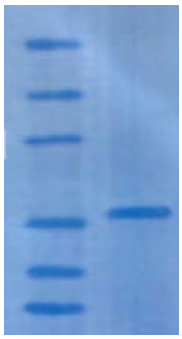

Figure 6: SDS-PAGE analysis of the purified xylanase from *Streptomyces* sp. ESRAA-301097. Lift lane, standard marker protein (97.4, 66.2, 45.0, 29.0, 20.1 and 14.4 kDa); right lane, xylanase after Sephadex G-100 column chromatography, which showed a single band.

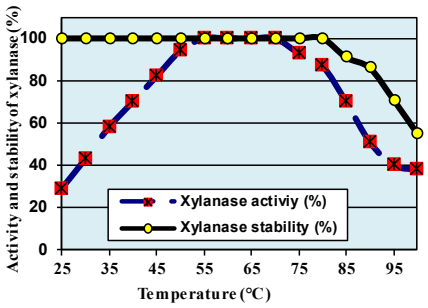

Figure 7: Effect of temperature on xylanase activity and stability of endophytic *Streptomyces* sp. ESRAA-301097.

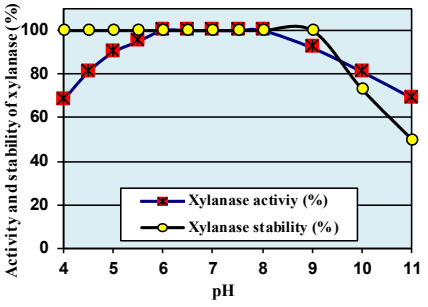

Figure 8: Effect of pH on xylanase activity and stability of endophytic *Streptomyces* sp. ESRAA-301097.

8.6 and 6.8 - 7.8, respectively [36,38,42-44] compared to pH 9.0 for xylanases from *S. actuosus* A-151, *S. olivaceoviridis* A1 and *Streptomyces* sp. Ab106 [37]. Furthermore, xylanase activity was increased with increasing substrate concentration up to 3% and then abrupt decrease was observed (Table 9) due to the saturation of active sites of the enzyme [44]. The relative activity of xylanase towards different substrates in Table 9 showed higher activity for the highly substituted xylan such as oat spelt xylan (OSX) than less branched birchwood xylan (BWX). These data are corroborated well with the results recorded for xylanases from *Streptomyces matenis* [14]. ESRAA-301097 xylanase exhibited no specificity towards carboxymethyl cellulose (CMC) and filter paper, which showed that ESRAA-301097 xylanase could be described as cellulose free xylanases. Cellulase-free xylanases is of industrial importance in paper and biobleaching of pulp industries to avoid cellulose degradation as previously reported for cellulase-free xylanases produced by other *Streptomyces* species [37,38,42].

Evaluation of chemical additives as activators or inhibitors

Relative to control (no additive), several multivalent metal ions (Co^{2+}, Mn^{2+}, Cu^{2+}, Mg^{2+}, Zn^{2+}, Fe^{3+}, Ba^{2+} and Ca^{2+}) as well as Na^{+} enhanced xylanase activity produced by *Streptomyces* sp. ESRAA-301097 to 145, 152, 169, 125, 138, 186, 107, 111 and 110 %, respectively but Hg^{2+}, Cd^{2+}, Pb^{2+}, Ni^{+} and Li^{+} reduced it to 38, 87, 90, 79 and 81%, respectively (Table 10). In contrast, xylanases of *Streptomyces* sp AMT-3 were strongly inhibited by Cu^{2+}, Mg^{2+}, and Fe^{3+} [45]. It has been suggested that the effect of metal ions as activators or inhibitors could be attributed to a change in the solubility, the behavior of the ionized nutrients at interfaces and changes in the catalytic properties of the enzyme itself [46]. Table 10 demonstrates that xylanase activity was greatly reduced in 1% of sodium tripolyphosphate, SDS and sodium tetraborate to 29, 41 and 50%, respectively but it was enhanced in the presence of Tween 20, Tween 80 and Triton X-100 to 119, 114 and 103%, respectively after 1 h storage. Whereas 10 and 50 mM of serine protease inhibitors (PMSF) had strong reducing xylanase activity to 21 and 0%, respectively; the inhibitors of cysteine protease (1,10-phenanthroline and Dithiothreitol), thiol protease (iodoacetamide and p-chloromercuribenzoate) and metalloprotease (EDTA and EGTA) at a concentration of 10 and 50 mM had no to minor inhibitory effect on xylanase activity; the enzyme retained (97 and 89%); (99 and 95%), (100, 98%); (100, 100%); (100, 100%) and (100 and 96%) of its activity, respectively. These data revealed that serine not cysteine residues are involved in the catalytic mechanisms of enzyme and it is not metaloproteins or thioproteins but it could be considered as a serine protease [46]. Moreover, effect of such solvent on xylanase activity was varied depending on its polarity. The detected activity of xylanase with 1-Propanol, propyleacetate, benzene, toluene, n-Hexane, decanol, isooctane, tetradecane, n-Hexadecane and ethyl acetate were found to be 49, 70, 111, 90, 109, 94, 100, 102, 108 and 60%, respectively of the control (Table 10). Whereas the increase in activity with non-polar solvents is due to their hydrophobicity properties, decreasing of activity with propyle acetate, propanol and ethyl acetate is attributed to the high polarity of these solvents that stripped the water layer surrounding the enzyme causing enzyme inactivation [47]. Consequently our study clearly indicated that the properties of *Streptomyces* sp. ESRAA-301097 xylanase make this enzyme potentially very effective and economical for industrial applications. For instance, alcohol -tolerant xylanase is required for biofuel production, solvent

Substrate	Relative activity (%)
concentration % (Birchwood xylan)	
1	100
2	117
3	121
4	121
5	115
6	106
Substrate specificity	
Birchwood xylan	100
Xylan- Oat Spelt	116
CM-cellulose	0
Filter paper	0

Table 9: Impact of substrate concentration and specificity on purified ESRAA-301097 xylanase activity.

Parameter	Relative activity (%)*
Control (without additives)	100
Metal additives (10 mM)	
Co^{2+}	145
Mn^{2+}	152
Cu^{2+}	169
Mg^{2+}	125
Zn^{2+}	138
Fe^{3+}	186
Na^{+}	110
Ba^{2+}	107
Hg^{2+}	38
Cd^{2+}	87
Pb^{2+}	90
Ca^{2+}	111
Ni^{+}	79
Li^{+}	81
Detergent additives (1%)	
Tween 20	119
Tween 80	114
Triton X-100	103
Sodium dodecyl sulphate (SDS)	41
Sodium tripolyphosphate	29
Sodium tetraborate	50
Protease inhibitors (mM)	
Paramethyl sulfonyl fluoride (PMSF)	
10	21
50	0
p-Chloromercuribenzoate (PCMB)	
10	100
50	100
Iodoacetamide	
10	100
50	98
1,10- Phenanthroline	
10	97
50	89
Dithiothreitol (DTT)	
10	99
50	95
EDTA	
10	100
50	100
EGTA	
10	100
50	96
Organic solvents (50%)	
1-Propanol	49
Propyleacetate	70
Benzene	111
Toluene	90
n-Hexane	109
Decanol	94
Isooctane	100
Tetradecane	102
n-Hexadecane	108
Ethyl acetate	60

*One hundred percent (%) was assigned to the activity in the absence of these chemical additives

Table 10: Some factors affecting xylanase activity produced by *Streptomyces* sp. ESRAA-301097.

and salt tolerant xylanases are applied for bioremediation of solvent contaminated industrial wastewaters, solvent and surfactant tolerant xylanases are used in deinking of recycled paper and solvent tolerance facilitates the selective precipitation, recovery and reuse of enzymes [47].

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