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 comcob (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 10⁷ 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 Actinomyces 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. NRCS 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...
Isolation of plant-derived endophytic Actinomycetes

Each organ (roots, stems and leaves) of the collected Egyptian medicinal plants Cymopogon proximus (halafbar), Anethum graveolens (dill), Artemisia judaica (shih balady) and Corchorus olitorius (malukiyah) were surface sterilized and sectioned into small fragments as previously described [5]. These surface sterilized tissue segments were plated onto three different isolation media, 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 dianimonipelic 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 DNeasy 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 was 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 KP877333.
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 (1×10⁶, 1×10⁷, 1×10⁸, 1×10⁹, 1×10⁹, 1×10¹⁰, 1×10¹¹, 1×10¹², 1×10¹³, 1×10¹⁴ and 1×10¹⁵ 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 (NH₄NO₃, NaNO₃, (NH₄)₂SO₄ and NH₄Cl ), 0.1% of metal source (CaCl₂, NaCl, KCl, MgCl₂ and K₂HPO₄) 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 leaching 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 sulfate, 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⁺² and Li⁺²), 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-phenanthroline, dithiothreitol 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, decanal, isoctane, 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, Cymophobogon proximus (baflab) which used In Egyptian folk medicine as an effective renal anti-microbial, diuretic and antiinflammatory 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, antiparasitic and antihypercholesterolaenic 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.

### Table 1: Medicinal plants selected for endophytic Actinomycetes isolation using different cultivation media.

| 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) (DYM) (XA) | 8 3 7 |
| | | | Stems | (AIA) (DYM) (XA) | 6 1 4 |
| | | | Roots | (AIA) (DYM) (XA) | 10 4 10 |
| *Anethum graveolens* | Dill | Antimicrobial, antihyperlipidaemic, antispasmodic, antihypercholesterolaemic agent and for some gastrointestinal ailments such as flatulence, indigestion, stomachache and colic | Leaves | (AIA) (DYM) (XA) | 0 0 0 |
| | | | Stems | (AIA) (DYM) (XA) | 0 0 0 |
| | | | Roots | (AIA) (DYM) (XA) | 0 0 0 |
| *Artemisia judaica* | Shih balady | Used as antiseptic agent or tinctures applied for the relief of rheumatic pains | Leaves | (AIA) (DYM) (XA) | 0 0 0 |
| | | | Stems | (AIA) (DYM) (XA) | 0 0 0 |
| | | | Roots | (AIA) (DYM) (XA) | 0 0 0 |
| *Corchorus olitorius* | Malukhiyah | Exhibits several antifertility, anti-convulsive, antioxidants, anti-inflammatory, anti-proliferative, antimicrobial and antitumor activities with gastroprotective effects | Leaves | (AIA) (DYM) (XA) | 11 4 14 |
| | | | Stems | (AIA) (DYM) (XA) | 16 14 16 |
| | | | Roots | (AIA) (DYM) (XA) | 16 6 21 |

*Actinomycetes isolation agar (AIA), dextrose yeast extract malt extract agar (DYM) and xylan agar (XA) media*
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 well and ranged in color from reddish olive to dark gray but the spore chains with spiny spore surface (Figure 1). It was developed against clindamycin, furazolidone, amikacin, penicillin G, kanamycin, acid, malonic acid, pectin, malic acid, starch, cellulose, inulin, xylan has degradable activity towards chitin, aesculin, citric acid, succinic acid, 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 (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, ascelin, 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

| isolate | Primary screening (diameter of xylan digestion zone, mm) | Secondary screening (U/ml) |
|---------|----------------------------------------------------------|-----------------------------|
| 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 | 38.00 |
| 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 (U/ml) of hyper xylanolytic endophytic actinobacteria after primary and secondary screening at 30°C for 5 days in selective xylan medium.
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 Ugd-1 (with banana stalk and corncob, respectively). The production of xylanase, xylan containing substrate such as corncob was significant with the enzyme activity measured up to 18 % being recorded after 370 time:

| Table 5: | Chemotypic characteristics of the hyper xylanolytic producer strain ESRAA 301097 |
|--------------------------|------------------------------------------------------------------|
| Spore surface            | Spiny                                                                 |
| Spore chain morphology   | straight                                                             |
| Spores / chain           | >10                                                                 |
| Melanin production       | +                                                                  |
| H2S production           | +                                                                  |
| Soluble pigment on       | +                                                                  |
| ISP 2                    | +                                                                  |
| ISP 3                    | +                                                                  |
| ISP 4                    | +                                                                  |
| ISP 5                    | +                                                                  |
| Milk coagulation         | +                                                                  |
| Milk peptization         | +                                                                  |
| NaNO2 reduction          | +                                                                  |
| Cell wall amino acids    | L, L-diaminopimelic acid, lysine, glutamic acid, glycine         |
| Whole cell sugars        | Glucose, mannose, galactose, xylose                                |
| Major fatty acids (%)    |                                                                    |
| 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                                                               |
| C18:2                    | 0.21                                                               |
| Characteristic phospholipids | PE, DPG, PG, PIMS and PI                                   |
| Major menaquinones       |                                                                    |
| MK-8(H8)                 | 2 %                                                                |
| MK-9(H6)                 | 31 %                                                               |
| MK-9(H8)                 | 24 %                                                               |
| MK-9(H10)                | 8 %                                                                |
| MK-10(H4)                | 2 %                                                                |
| MK-10(H6)                | 22 %                                                               |
| MK-10(H6)                | 10 %                                                               |
| MK-10(H10)               | 1 %                                                                |
| Hydrolysis activity (0.1 %) |                                                                 |
| Chitin                   | +                                                                  |
| Aesculin                 | +                                                                  |
| Citric acid              | +                                                                  |
| Succinic acid            | +                                                                  |
| Malonic acid             | -                                                                  |
| Pecdin                   | +                                                                  |
| Fumaric acid             | -                                                                  |
| Malic acid               | +                                                                  |
| Starch                   | +                                                                  |
| Cellulose                | +                                                                  |
| Inulin                   | +                                                                  |
| Xylan                    | +                                                                  |
| Gelatin                  | +                                                                  |
| Carbon utilization (%)   |                                                                    |
| D- Glucose               | +                                                                  |
| D-Ribose                 | +                                                                  |
| L-Arabinose              | +                                                                  |
| Fucose                   | +                                                                  |
| D- xylose                | +                                                                  |
| Rhamnose                 | +                                                                  |
| D-Mannitol               | -                                                                  |
| Adonitol                 | +                                                                  |
| Lactose                  | +                                                                  |
| Sucrose                  | +                                                                  |
| Maltose                  | +                                                                  |
| Raffineose               | +                                                                  |
| L-Insolitol              | -                                                                  |
| Nitrogen source utilization |                                                                 |
| Casein                   | +                                                                  |
| L-Histidine              | +                                                                  |
| L-phenylalanine          | +                                                                  |
| DI-Methionine            | +                                                                  |
| L-Serine                 | +                                                                  |
| L-Valine                 | +                                                                  |
| Urea                     | +                                                                  |
| Response to antibiotics  |                                                                    |
| Clindamycin (2 mg/disc)  | R                                                                  |
| Furazolidone (15 mg/disc)| R                                                                  |
| Gentamycin (10 mg/disc)  | S                                                                  |
| Amikacin (30 mg/disc)    | R                                                                  |
| Penicillin G (10 mg/disc)| R                                                                  |
| Kanamycin (30 mg/disc)   | R                                                                  |
| Furazolidone (15 mg/disc)| R                                                                  |
| Tetracycline (30 mg/disc)| S                                                                  |
| Lincomycin (2 mg/disc)   | S                                                                  |
| Streptomycin (10 mg/disc)| R                                                                  |
| Vancomycin (30 mg/disc)  | S                                                                  |
| Chloromycycin (30 mg/disc)| S                                                                  |
| Erythromycin (15 mg/disc)| R                                                                  |
| Ceclazolin (30 mg/disc)  | R                                                                  |
| Rifampicin (5 mg/disc)   | S                                                                  |
| Tobramycin (10 mg/disc)  | S                                                                  |
| pH range for growth      | 4 - 11                                                             |
| Temp. range for growth (ºC) | 15 - 45               |
| NaCl tolerance (%)       | Up to 18 %                                                         |
| DNA G+C content (mol %)  | 70.4                                                               |

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

**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
aspen (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 corncob 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 cyaneus SN32, Techapun et al. [37] supported cane bagasse as inducer for cellulase-free xylanase from Streptomyces sp. Ab106 and a significant amounts of xylan 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; 5,400 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 1x10⁶ spore gds⁻¹. Higher or lower inoculum level decreased xylanase production to 701.40 and 1750.19 Ugds⁻¹ at inoculums concentration 10³ and 10⁷ spore gds⁻¹, respectively (Table 6). Lower inoculum density than optimum level may not be sufficient for producing the required 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 viridosphorus T7.

Initial moisture content (IMC)

Initial moisture level of the substrate acts as a fundamental controlling parameter for enzyme production in SSF. The highest
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 or lower initial moisture content (Table 6). Whereas increase in SSF moisture content and then xylanase activity was decreased 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 sized particles below 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

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

| Agro industrial residues | Xylanase production (Umg⁻¹) during different fermentation period (day) |
|--------------------------|---------------------------------------------------------------------|
| Kyphosus edulis (KE)     | 105.00                                                              |
| Ceratocystis fimbriata (CF) | 104.00                                                             |
| Picea mariana (PM)       | 103.00                                                              |
| Pinus radiata (PR)       | 102.00                                                              |

#### Table 6: Optimization of solid state fermentation and leaching process parameters for improvement of xylanase production by endophytic Streptomyces sp. ESRAA-301097.

| Process parameter | Xylanase production (Uml⁻¹) | Process parameter | Xylanase production (Uml⁻¹) |
|-------------------|------------------------------|-------------------|------------------------------|
| Incubation temper. | Carbon supplements (0.5 %)   | Control           | 3819.00                      |
| pH                | Glucose                      | 600.16            |                             |
| pH                | Fructose                     | 1032.82           |                             |
| pH                | Maltose                      | 1019.50           |                             |
| pH                | Lactose                      | 1618.04           |                             |
| Spore conc.       | Control                      | 3819.00           |                             |
| Spore conc.       | Yeast extract                | 3422.00           |                             |
| Spore conc.       | Peptone                      | 3068.00           |                             |
| Spore conc.       | Soybean meal                 | 4500.39           |                             |
| Spore conc.       | Corn steep solid             | 4385.40           |                             |
| Spore conc.       | Casein                       | 3270.10           |                             |
| Spore conc.       | Urea                         | 2477.18           |                             |
| Spore conc.       | Phenylalanine                | 2301.00           |                             |
| Spore conc.       | Arginine                     | 3290.75           |                             |
| Spore conc.       | Glutamine                    | 2000.08           |                             |
| Spore conc.       | Tryptophan                   | 4029.40           |                             |
| Spore conc.       | NH₄NO₃                      | 2989.26           |                             |
| Spore conc.       | (NH₄)₂SO₄                    | 2149.37           |                             |
| Spore conc.       | NH₄Cl                        | 2594.22           |                             |
| Spore conc.       | Soybean meal + Corn steep solid | 4734.03       |                             |
| Spore conc.       | Metal supplements (0.1%)     | 4734.03           |                             |
| Spore conc.       | Control                      | 4734.03           |                             |
| Initial moisture level (%) | CaCl₂                      | 4604.40           |                             |
| Initial moisture level (%) | NaCl                        | 4175.62           |                             |
| Initial moisture level (%) | KCl                         | 4182.02           |                             |
| Initial moisture level (%) | MgCl₂                      | 4214.00           |                             |
| Initial moisture level (%) | K₂HPO₄                      | 4673.55           |                             |
| Initial moisture level (%) | Detergents additives (1%)   | 4734.03           |                             |
| Initial moisture level (%) | Control                     | 4734.03           |                             |
| Particle size (µm) | Sodium dodecyl sulphate (SDS) | 750.29           |                             |
| Particle size (µm) | Polyethylene glycol          | 5000.10           |                             |
| Particle size (µm) | Sodium tetaborate            | 1804.70           |                             |

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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 supplements

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 Ugd s⁻¹), the highest reduction (600.16 Ugd s⁻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 catabolite repression [10]. Conversely, xylanase production by corn steep solid as nitrogen source resulted in 23.96% increase in %, respectively (Table 6). Moreover, using a mixture of soybean and corn steep solid as nitrogen source resulted in 23.96% increase in %, respectively (Table 6). Moreover, using a mixture of soybean and corn steep solid as nitrogen source resulted in 23.96% increase in %, respectively (Table 6). Moreover, using a mixture of soybean and corn steep solid as nitrogen source resulted in 23.96% increase in %, respectively (Table 6). Moreover, using a mixture of soybean and corn steep solid as nitrogen source resulted in 23.96% increase in %, respectively (Table 6).

### Nitrogen supplements

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 NaNO₃ 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 Ugd s⁻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; 5150.18 and 5000.10 Ugds⁻¹, respectively but the addition of detergents additives. 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].

### Table 7: Optimization of extraction process parameters of ESRAA-301097 xylanase (Ugd⁻¹).

| Leaching parameter | Xylanase activity (Ugd⁻¹) | Leaching parameter | Xylanase activity (Ugd⁻¹) |
|--------------------|--------------------------|--------------------|--------------------------|
| Leaching agents (1:10, w/v) | 2670.81 | Soaking time (min) | 60 |
| H₂O | 671.00 | | 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 | | |
| Citrate buffer (pH 4.0, 0.1 M) | 5708.51 | pH of extraction process | 3 |
| Citrate buffer (pH 6.0, 0.1 M) | 5295.90 | 5 | 5585.90 |
| Phosphate buffer (pH 7.0, 0.1 M) | 3636.70 | 6 | 4431.90 |
| Glycine-NaOH buffer (pH 10.0, 0.1 M) | 560.84 | 7 | 1690.30 |
| Tween 80 (0.1%) | 5720.00 | 8 | 1170.28 |
| Tween 80 (0.2%) | 5812.04 | 9 | 1054.22 |
| Tween 80 (0.3%) | 5769.28 | | |
| Citrate buffer (pH 4.0, 0.1 M) + Tween 80 (0.2%) | 6290.10 | Temperature of extraction process (°C) | 30 |
| Leaching agent: fermented substrate ratio (w/v) | 10 | 2216.65 |
| 1:2 | 1002.33 | 40 | 4372.10 |
| 1:4 | 1930.16 | 45 | 6290.10 |
| 1:6 | 4893.10 | 50 | 6312.45 |
| 1:8 | 6290.10 | 55 | 5396.00 |
| 1:10 | 6290.10 | 60 | 4153.07 |
| 1:12 | 6034.24 | 70 | 3305.19 |
| 1:20 | 4818.00 | 70 | 6312.45 |
| Soaking time | 2216.65 | Agitation mode (150 rpm) | 3103.72 |
| 30 | 2196.74 | Static mode | |

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

| Purification step | Total protein (mg) | Xylanase activity (U) | Specific activity (U/mg) | Yield (%) | Purification fold |
|-------------------|-------------------|----------------------|--------------------------|----------|------------------|
| Crude extract     | 1986.50           | 21975.00             | 11.06                    | 100      | 1.00             |
| DEAE- cellulose chromatography | 151.11 | 17395.42 | 25.93 | 79.16 | 2.344 |
| Sephadex G-200 chromatography | 23.62 | 11736.86 | 77.67 | 53.41 | 7.021 |
| Sephadex G-100 chromatography | 14.48 | 9088.65 | 384.79 | 41.36 | 34.784 |
| | | 7145.53 | 493.48 | 32.52 | 44.610 |
to 750.29 Ugd⁻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 Ugd⁻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 Ugd⁻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 chromatography, which showed a single band.

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; and *S. viridisporus* T7A were 31.6, 20.5, 23.8-40.5, 33-54, 59 and 15-36 kDa [36,38,42-44].

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; 6.7 - 7.7; 6.0 – 7.0; 7.0 -8.0; 6.0 – 7.0; 7.0- 8.0; 7.0-8.0; respectively (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 also 6.7 – 7.7; 6.0 – 7.0; 7.0 - 8.0; 6.7 - 7.7; 6.0 – 7.0; 7.0 -8.0; 6.0 – 7.0; 7.0- 8.0; 7.0-8.0; respectively (Figure 8).

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8.6 and 6.8 - 7.8, respectively [36,38,42-44] compared to pH 9.0 for xylanases from S. actuaus 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 (BXW). 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 cellulase 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 (Co²⁺, Mn²⁺, Cu²⁺, Mg²⁺, Zn²⁺, Fe³⁺, Ba²⁺ and Ca²⁺) as well as Na⁺ enhanced xylanase activity produced by Streptomyces sp. ESRAA-301097 to 145, 152, 169, 125, 138, 186, 111 and 110 %, respectively but Hg²⁺, Cd²⁺, Pb²⁺, 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 Cu²⁺, Mg²⁺, and Fe³⁺ [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, 114 and 110%, respectively after 1 h storage. Whereas 10 and 50 mM of serine protease inhibitors (PMSF) had strong reducing activity to 21 and 95%, 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 (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 metalloproteins or thiopeptides 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, isocetane, tetradeoline, n-Hexadeconyl 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

| Parameter | Relative activity (%)* |
|-----------|------------------------|
| Control (without additives) | 100 |
| Metal additives (10 mM) | |
| Co²⁺ | 145 |
| Mn²⁺ | 152 |
| Cu²⁺ | 169 |
| Mg²⁺ | 125 |
| Zn²⁺ | 138 |
| Fe³⁺ | 186 |
| Na⁺ | 110 |
| Ba²⁺ | 107 |
| Cd²⁺ | 38 |
| Ca²⁺ | 87 |
| Ni⁺ | 90 |
| Li⁺ | 111 |
| 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-Phenantherline | |
| 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 |
| Isocetane | 100 |
| Tetradeconale | 102 |
| n-Hexadeconyl | 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.

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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.
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