

Original Research Article

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Utilisation of Agrowaste Xylan for the Production of Industrially Important Enzyme Xylanase from Aquatic *Streptomyces* sp. and Potential Role of Xylanase in Deinking of Newsprint

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ABSTRACT

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Xylanase is an industrially significant enzyme and its production from pure xylan is expensive. The objective of the current study is to utilise sustainable cost effective substrates -coconut oil cake, corn cob, sugarcane bagasse and water hyacinth, for xylanase production. *Streptomyces* sp. ER1 isolated from the sediments of Cochin estuary was used for xylanase production. The cultural and nutritional conditions for higher xylanase production using the four substrates were optimised using one factor at a time method. Data were analysed by one way ANOVA. The maximum xylanase yield was observed for sugarcane bagasse (10533.33 U/mL), corn cob (7880.9 U/mL) followed by, coconut oil cake (7680 U/mL) and water hyacinth (6930 U/mL) in submerged fermentation. Optimisation studies revealed that optimum fermentation and nutritional factors varied with the substrate. The crude xylanase was vastly effective in deinking of the newspaper at elevated temperature. This study proved that utilising agrowastes provides cost effective and eco-friendly method for xylanase production on large scale. Thus it is an alternative approach to reducing environmental pollution caused due to dumping agro waste. No studies on xylanolytic activity of actinomycetes from Cochin estuary has been done so far.

Introduction

Xylanases (EC 3.2.1.8) are a class of inducible enzymes, liable for the complete hydrolysis of xylan into simpler compounds, consisting mainly of xylose (Gupta and Kar, 2009). Above few years, global market of xylanase is extended swiftly due to its greater potential for industrial use, mainly in the

biotechnological applications in the industry of pulp and paper, baking, textiles, animals feed, biofuels, food and beverages (Ho and Lau, 2014). The marine actinomycetes found in a wide range of aquatic environments, like estuary and mangroves, are well-known to produce chemically diverse compounds with a broad range of biological activities that have commercial applications (Gulve and

Deshmukh, 2011). The marine environment regards with the isolation of indigenous *Streptomyces* as these microbes gained special importance because of their capability to produce novel secondary metabolites or enzymes with a wide range of biological activities (Gulve and Deshmukh, 2011; Solanki *et al.*, 2008). Nevertheless, the expenditure of xylan dependent xylanase production confines its use in industrial applications.

Agricultural by-products containing cellulose, hemicelluloses and lignin could provide as effective and inexpensive sources for xylanase production (Lam, 2006). The accessibility of agricultural waste in India is about 625 million tonnes annually including groundnut cake, rice bran, rice straw, wheat bran, sugarcane bagasse, etc. (Techappun *et al.*, 2003). The pollution problems linked with agro-industrial wastes, like, shortage of places for its disposal, pricey treatment options and enhanced need to save valuable resources have put on to encourage the utilisation and bioconversion of waste into high industrial products (Bhosale *et al.*, 2011). The use of pest plants and cheap agricultural and food-processing by-products is highly favoured so as to develop the commercial viability of bioprocess technology (Sivaramakrishnan and Gangadharan, 2009).

So far, no wide studies have been done in the aquatic actinomycetes and their ability to produce industrial enzymes in Cochin estuary. The estuarine sediment harbours many potent microorganisms, producing xylanase. The mangrove ecosystem associated with Cochin estuary is ideal for growing different microorganisms; due to progressing impact of tides. This, it is crucial that a broad spectrum activity of actinomycetes from hitherto unexplored habitats be considered as sources of xylanase. The current study is an effort to produce xylanase from agrowastes, like

coconut oil cake, corn cob, sugarcane bagasse and water hyacinth (pest plant) using *Streptomyces* sp. ER1 isolated from Cochin estuarine sediment. Numerous reports suggest that apart from the nature of substrate, physical and nutritional parameters also greatly affect the production of xylanase on agricultural waste (Barrios- Gonzalez *et al.*, 1993). Thus during the present study – the effect of physical and nutritional parameters on xylanase production by *Streptomyces* sp. ER1 on different substrates was investigated. The study also focuses on the application of enzyme on newspaper deinking.

Materials and Methods

Microorganism and inoculum preparation

Actinomycete cultures were isolated from sediment samples of Cochin estuary (Rosmine and Saramma, 2016). Isolate ER1 with good xylanase activity was selected and confirmed its identification as *Streptomyces* sp. ER1 by 16S rRNA gene amplification. The sequence was deposited in the Genbank with an accession number KY449279. The selected actinomycete was subcultured in nutrient agar slants containing 1% beech wood xylan (pH 7.0) and incubated at 35°C for five days.

Collection and preparation of substrates

The substrates corn cob, coconut oil cake and sugarcane bagasse were bought from a local market in Ernakulam, Kerala, India, to study about xylanase production using solid state and submerged fermentation. *Eichhornia crassipes* (water hyacinth) was collected from Vembanad Lake. All the substrates were washed with distilled water and then dried out in the oven. Sugarcane bagasse, corn cob and water hyacinth were cut into small pieces (5 mm size) and dried in the hot oven at 80°C for 1 h. Coconut oil cake was then powdered using an electrical grinder and used for xylanase production.

Pre-treatment of substrates

Pre-treatment of substrates was following a modified method of Ali *et al.*, (1991). The prepared substrates were autoclaved for 1 hour with 5% (w/v) NaOH (20mL per gram of substrate) in separate conical flasks for delignification and filtered through muslin cloth. They were then washed with water, neutralized with 1M HCl. and dried at 70°C.

Solid state fermentation (SSF) Vs submerged fermentation (SmF)

The comparative study of the SSF and SmF was carried out using the four substrates as the sole carbon source.

Submerged fermentation

In SmF, the fermentation medium (g/L: KH_2PO_4 1.5, K_2HPO_4 2, $(\text{NH}_4)_2\text{SO}_4$ 4.5, Yeast extract 0.075, Peptone 0.075, Tween 80 0.075, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 140 mg, $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ 160 mg, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 500 mg, $\text{COCl}_2 \cdot 2\text{H}_2\text{O}$ 200 mg, pH.7.0) was used and each of the four substrates were added at 2% (w/v) in separate conical flasks, inoculated and incubated at 35°C for 120 h on an orbital shaker. Each sample was then centrifuged at 10,000 rpm and at 4°C for 20 min, and the clear supernatant was assayed for xylanase activity.

Solid state fermentation

The medium for SSF contained 10 g of each of four substrates and 6 mL of the mineral salt solution: g/L: KH_2PO_4 1.5, K_2HPO_4 2, $(\text{NH}_4)_2\text{SO}_4$ 4.5, Yeast extract 0.075, Peptone 0.075, Tween 80 0.075, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 140 mg, $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ 160 mg, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 500 mg, $\text{COCl}_2 \cdot 2\text{H}_2\text{O}$ 200 mg, Moisture: 6%, pH:7.0). The media was inoculated and incubated at 35°C. After 5 days of incubation, the enzyme was extracted from the SSF media according to the method of Alva *et al.*, (2007).

Xylanase assay

Xylanase activity was determined using beechwood xylan (Sigma, Germany) (Bailey *et al.*, 1992). A 0.2 mL culture supernatant was added to 1 mL xylan solution (1%; pH 7.0; 100 mM sodium phosphate buffer) and incubated at 55°C. After 30 min, 3 mL 3, 5-dinitrosalicylic acid reagent was added to stop the reaction, and the amount of reducing sugars released in the reaction was estimated by measuring the absorbance at 540 nm (Miller, 1959). A control was run concurrently which contained all the reagents but the reaction was terminated prior to the addition of enzyme extract. One unit of xylanase activity was defined as the amount of enzyme catalysing the release of 1 μmol of reducing sugar equivalent to xylose per min under the specified assay conditions. All the experiments were carried out independently in triplicate and the results presented are mean of the three values.

Selection of basal medium

3 different media, A (Techapun *et al.*, 2003), B (M9 medium) (Roy, 2004) and C (Mandels and Sternburg, 1976) were used for comparative studies to find the appropriate basal nutrient medium for the further formulation of the optimal medium.

Optimisation of fermentation conditions

The optimum conditions for enzyme production were studied such as time course of fermentation (1-5days), initial medium pH (6.0–9.0), incubation temperature (30–40°C with 5°C interval), inoculum age (16h, 20 h and 24 h), agitation speed (50,100and 150 rpm), salinity (0 ppt -20 ppt), substrate concentration (0.5-3%) and various nutritional conditions such as additional carbon sources (xylose, glucose, sucrose, cellulose, xylan, starch and glycerol), surfactants (Tween 60,

Tween 80) and other additives (olive oil and polyethylene glycol), nitrogen sources (tryptone, beef extract, yeast extract, peptone, albumin, casein, soya bean meal, urea, ammonium chloride, di ammonium phosphate, ammonium sulphate and potassium nitrate)

Statistical analysis

All experiments were carried out in triplicates, the standard deviation for each experimental result was calculated using Microsoft Excel 2003 and statistically evaluated using ANOVA at a significance level of $p < 0.05$ by using computer based program SPSS (Version 17.0, Chicago, SPSS Inc.).

Application of crude xylanase in deinking of newspaper

Preparation of paper pulp

Old newspapers were pulped by soaking wet in hot water for 2 h and crushed in a domestic mixer with added 0.1 % Tween 80. The pulp was dried at 50°C and stored in sterile container at 4°C until further use (Mohandass and Raghukumar, 2005).

Deinking trials using cell-free bacterial culture supernatants

Streptomyces sp. ER1 was grown in nutrient broth supplemented with Tween 80 and xylan. After 5 days of incubation, the medium was centrifuged and the clear cell-free supernatant was used. The pulp was soaked wet in water for 30 min, prepared at 3-9% consistency and sterilized by autoclaving. It was then incubated with 50 mL of the cell-free supernatant for 5 days. The pulp was washed thoroughly with tap water and filtered over a Buchner funnel under suction to obtain in a form of hand sheets. The hand sheets were

pressed flat using two stainless steel plates and oven-dried at 50°C for 5 days. Newspaper pulp without treatment with actinomycete culture was used as control (Mohandass and Raghukumar, 2005).

Analysis of collected filtrate

The colour removal from the pulp was analysed with a spectrophotometer from λ 200 nm and λ 800 nm. The phenolic and hydrophobic compounds released were measured by measuring the absorbance at λ 237 nm and λ 465 nm, respectively (Patel *et al.*, 1993; Gupta *et al.*, 2000).

Results and Discussion

Comparison of SmF and SSF

The results demonstrated that the used isolate, was able to grow and produce xylanase in SmF even more than SSF (Table 1). Further studies on optimisation of culture conditions and media optimisation were carried out in SmF. Currently, 80-90% of xylanase are produced in submerged culture as the microbial biomass and the substrates are homogeneously distributed in a liquid medium (Hooi Ling, 2014). Most of the studies proved that SSF was a better fermentation technique for xylanase production using agro wastes but the present study reports contrasting results. The decrease in enzymatic activity at 120 h of incubation under SSF may be due to the sporulation of the isolate (Assamoi *et al.*, 2008). Maybe xylanase produced during the first stage of fermentation are degraded or denaturalised after onset of sporulation during SSF (Umsza-Guez *et al.*, 2011).

Selection of substrates for maximum xylanase production

Among all the four substrates, the maximum xylanase yield was observed for corn cob

(7394.4 U/mL) followed by sugarcane bagasse (6965.067 U/mL), water hyacinth (5984 U/mL) and coconut oil cake (4608.133 U/mL) in submerged fermentation suggesting the application of these agro residues for xylanase production. The eminent xylan content in corn cob (40%), the maximum among all agricultural waste, makes it a prospective substrate for xylanase production (Boonchuay *et al.*, 2016). There are many previous reports on the superiority of corn cob as a substrate for xylanase production (Gupta and Kar, 2009; Shanab *et al.*, 2010). Apart from agricultural byproducts, the novel substrate considered in this study is a pest plant- water hyacinth (Perez *et al.*, 2013; Nagar *et al.*, 2010). Its high reproduction rate causes abundant problems like eutrophication, obstruction of rivers, hampers fishing and endangers the existing flora and fauna by preventing the penetration of sunlight. The use of water hyacinth as a suitable substrate is being carefully considered as they do not compete for land, have a insignificant cost and grow rapidly. Sufficient study has not been conducted on water hyacinth, in spite of its higher carbohydrate content (Nagar *et al.*, 2010).

Effect of different media:

Highest xylanase activity was found in the production medium Medium A for both substrates coconut oilcake and water hyacinth while the Medium B (M9 medium) was found optimum for corn cob and sugarcane bagasse (Table 2). The presence of yeast extract and peptone in production medium A might have positively affected the xylanase production using coconut oil cake and water hyacinth. Additionally, the release of ammonium ion from peptone also stimulated the growth of microorganism, thus producing higher xylanase activity (Sanghi *et al.*, 2009). Thus, the optimum medium formulation with essential growth-limiting nutrients is

significant to optimise and increase the xylanase productivity. Lower xylanase activity observed from medium C was most likely owing to the different composition of the medium that was less favourable by *Streptomyces* sp.ER1. ANOVA indicated that the enzyme activity is significant ($p \leq 0.05$).

Effect of incubation period for xylanase production

The production of xylanase from *Streptomyces* sp. ER1 in different time periods (24 to 120 h) exhibited that highest xylanase production was found at 72 h of fermentation and has given the activity of 4608.14 U/mL ($P < 0.01$) with coconut oil cake; 7491.87 U/mL ($P < 0.01$) with corn cob; 6965.07 U/mL ($P < 0.01$) with sugarcane bagasse and 5930 U/mL ($P > 0.05$) with water hyacinth. Similar results were reported by Gupta and Kar (2009) and Ahmad *et al.*, (2012). After 72 h of incubation, the xylanase activities decreased which might be due to both reduction of the nutrients and by the proteolytic enzyme present in the culture medium (Figure 1a). Shorter fermentation time (72 h) is favourable for greater cost-effective industrial xylanase production.

Effect of inoculum age

The production of xylanase from different inoculum age of *Streptomyces* sp. ER1 (16, 20 and 24 h) revealed that maximum xylanase activity was yielded with 5% (v/v) of 20-hour inoculum from sugarcane bagasse (7438 U/mL) ($P < 0.01$), water hyacinth (5948.2 U/mL) ($P < 0.01$), corn cob (7535.2 U/mL) ($P < 0.01$) and coconut oil cake (5333.334 U/mL) ($P < 0.01$). Inoculum of age above 20 h did not support enhanced levels of xylanase production (Figure 1b). However, less xylanase production with 16 h old inoculum, might be because *Streptomyces* sp, ER1 might not have entered into log phase of growth.

The inoculum age of *Streptomyces* sp. is important as it might have caused in the transfer of high quantities of spores if transferred during the stationary phase or death phase and in the long lag phase of the fermentation profile.

Effect of salinity

The effect of salinity on xylanase production was studied by preparing the respective production media with different salinity ranging from 0ppt to 20 ppt. The study shows that 20 ppt salinity was optimum for maximum xylanase production from sugarcane bagasse (7631.6 U/mL) ($P < 0.05$), water hyacinth (5971.667 U/mL) ($P > 0.05$), corn cob (7652.133 U/mL) ($P < 0.05$) and coconut oil cake (5600.54 U/mL) ($P < 0.05$) (Figure 1c). It exhibits the halophilic nature of *Streptomyces* sp. ER1.

Effect of initial pH

The initial pH of the medium is critical for growth and enzyme production as the metabolic activities of microorganisms are very susceptible to pH change (Rekha *et al.*, 2012). *Streptomyces* sp. ER1 showed maximum production in a neutral pH of 7.0 and the production decreased with increase in pH (Figure 1d) with coconut oil cake ($P < 0.01$), corn cob ($P < 0.01$) and sugarcane bagasse ($P < 0.01$) as substrates. However, pH 8 was found optimum for xylanase production with water hyacinth ($P > 0.05$) as the substrate. Similar results were observed by Ahmed *et al.*, (2012) and Rahmani *et al.*, (2014). All the substrates exhibited good activity from pH 6.0 to 9.0 indicating the alkalophilic nature of the xylanase produced and thus could be applied in detergent and textile industries. The inconsistency in optimum pH in different media is dependent on the nature of the substrate and that the enzyme might interact with other media or extract components (Santos *et al.*, 2013).

Effect of incubation temperature

The strain ER1 showed maximum production at 40°C ($P < 0.01$) and the production decreased with increase in temperature (Figure 1e) with coconut oil cake and sugarcane bagasse as substrates. However, 35°C ($P < 0.01$) was found optimum for xylanase production with water hyacinth and corn cob as substrates. Similar results were observed in previous studies (Sivaramakrishnan *et al.*, 2009; Knob *et al.*, 2014). *Streptomyces* sp. ER1, could be qualified as thermotolerant, owing to its inclination towards higher temperature for xylanase production. Thus it might have great role in industrial applications (Immanuel *et al.*, 2006).

Effect of agitation

Enzyme production by *Streptomyces* sp. ER1 with the selected substrates was studied for growth under agitation (50,100 and 150 rpm). In our study, 50 rpm ($P < 0.05$) was found optimum for xylanase production using coconut oil cake (6620.8 U/mL) and sugarcane bagasse (7964.54 U/mL) as substrates while 100 rpm ($P < 0.05$) was optimum for corn cob (7875 U/mL) and water hyacinth (6124.47 U/mL) as substrates (Figure 1f). As agitation speed increased; the higher shear force might have caused lower xylanase production. Similar results were observed by Hooi Ling (2014) and Nasr *et al.*, (2013).

Effect of substrate concentration

With increasing concentrations of substrates, a substantial increase in enzyme production was recorded (Figure 1g). 2% of coconut oil cake, 2.5% of corn cob and water hyacinth; and 3% sugarcane bagasse were found to be optimum for maximum xylanase production ($P < 0.01$). Similar results were observed by

Bhosale *et al.*, (2011) and Sepahy *et al.*, (2011).

Effect of nitrogen sources

Different nitrogen sources were studied for their effect on xylanase production by *Streptomyces* sp. ER1. The results are depicted in Figures 1h and 1i. Among all the organic nitrogen sources tested, peptone, soya bean meal, albumin and urea were found to be the best inducer for xylanase production from coconut oil cake, Corn cob, sugarcane bagasse and water hyacinth respectively and drastically increased xylanase activity (P<0.01). Among the inorganic sources, ammonium chloride produced a maximum xylanase activity from coconut oil cake and corn cob and drastically increased xylanase activity (P<0.05); ammonium sulphate for sugarcane bagasse and potassium nitrate for water hyacinth were found to be optimum for xylanase production and significantly

increased xylanase activity (P< 0.01). Peptone releases NH⁺ 4 ions, which stimulates growth and enzyme yield due to its protease inhibiting nature at low concentration (Bajaj and Abbas, 2011). Soybean meal does not cause catabolite repression and contains approximate all kinds of amino acids (El-Gendy and El-Bondkly, 2014), thus being readily absorbed by *Streptomyces* sp. ER1 mycelium.

Effect of different surfactants

Detergent effects on xylanase production by *Streptomyces* sp. strain ER1 varied with the selected agro waste (Figure 1j). Tween-60, polyethylene glycol and olive oil increased xylanase production in corn cob; coconut oil cake and water hyacinth; and sugarcane bagasse respectively and significantly increased xylanase production (P<0.01). Similar observations were made by El-Gendy and El-Bondkly (2014).

Table.1 Effect of different substrates on xylanase Production under SmF and SSF

Substrate	SmF (U/mL) Xylanase activity	SSF (U/g) Xylanase activity
Coconut oil cake	4608.13±139.47	3069.33± 100.1
Water hyacinth`	5984±149.84	1001.79± 11.89
Sugarcane baggase	6965.067±170.1	1421.33±14.2
Corn cob	7394.4±173.9	479.33±5.0

Table.2 Xylanase production in different production media with different substrates

Production medium	Substrate	Enzyme activity (U/mL)
A	Coconut oil cake	4608.13±139.47
	Corn cob	5255.73±149.84
	Sugarcane baggase	1628.67±170.1
	Water hyacinth	5138.67±173.9
B (M9 medium)	Coconut oil cake	301.86±3.0
	Corn cob	7491.87±174.9
	Sugarcane bagasse	6997.1±179.9
	Water hyacinth	2226.67±121.8
C	Coconut oil cake	3861.47±138.1
	Corn cob	4805.33±138.3
	Sugarcane bagasse	6289.33±172.9
	Water hyacinth	3413.33±134.3

Figure.1a Effect of incubation period for xylanase production using selected substrates

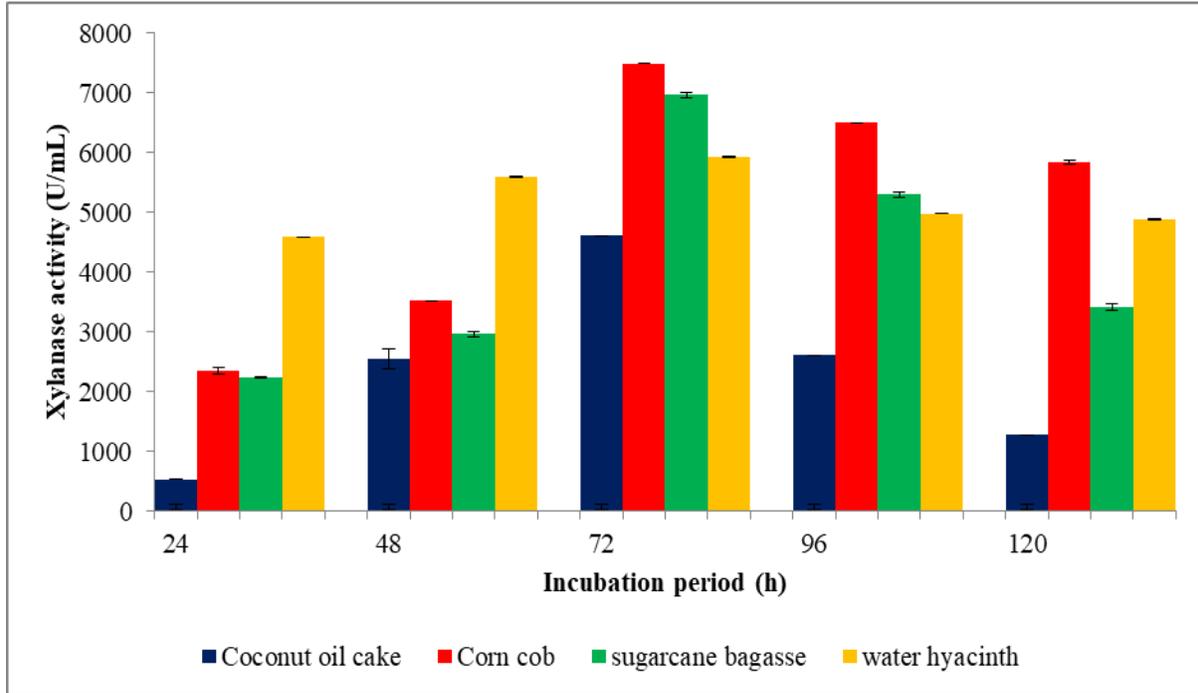


Figure.1b Effect of inoculums age (h) for xylanase production using selected substrates

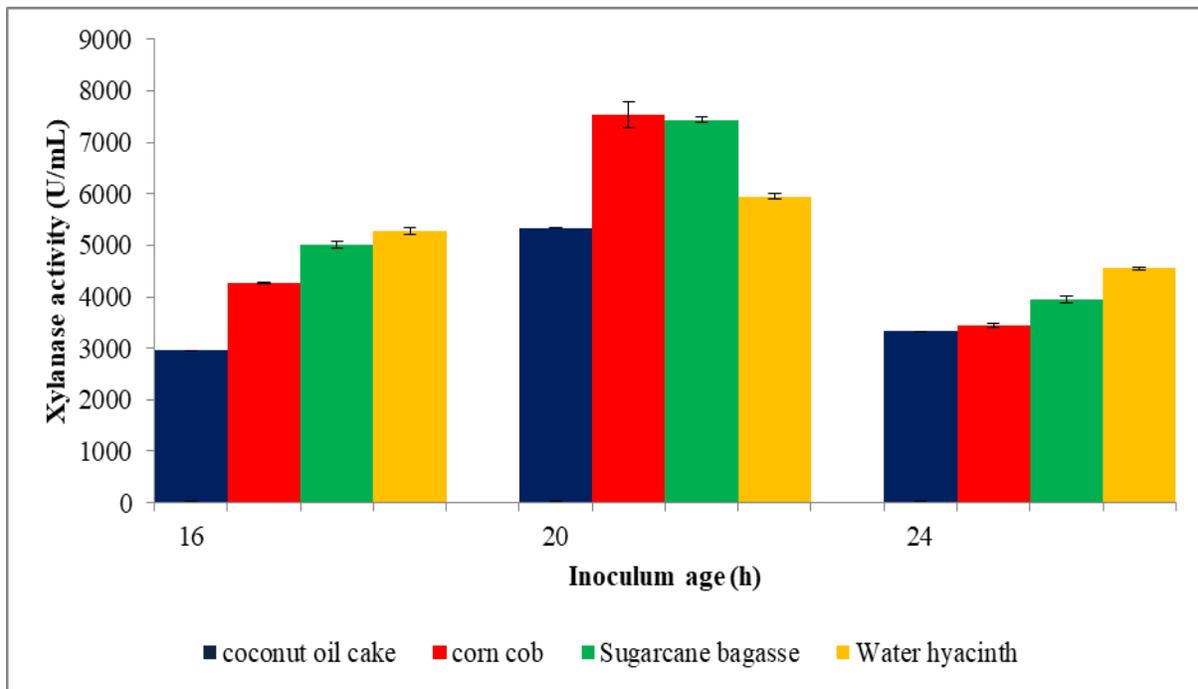


Figure.1c Effect of salinity (ppt) for xylanase production using selected substrates

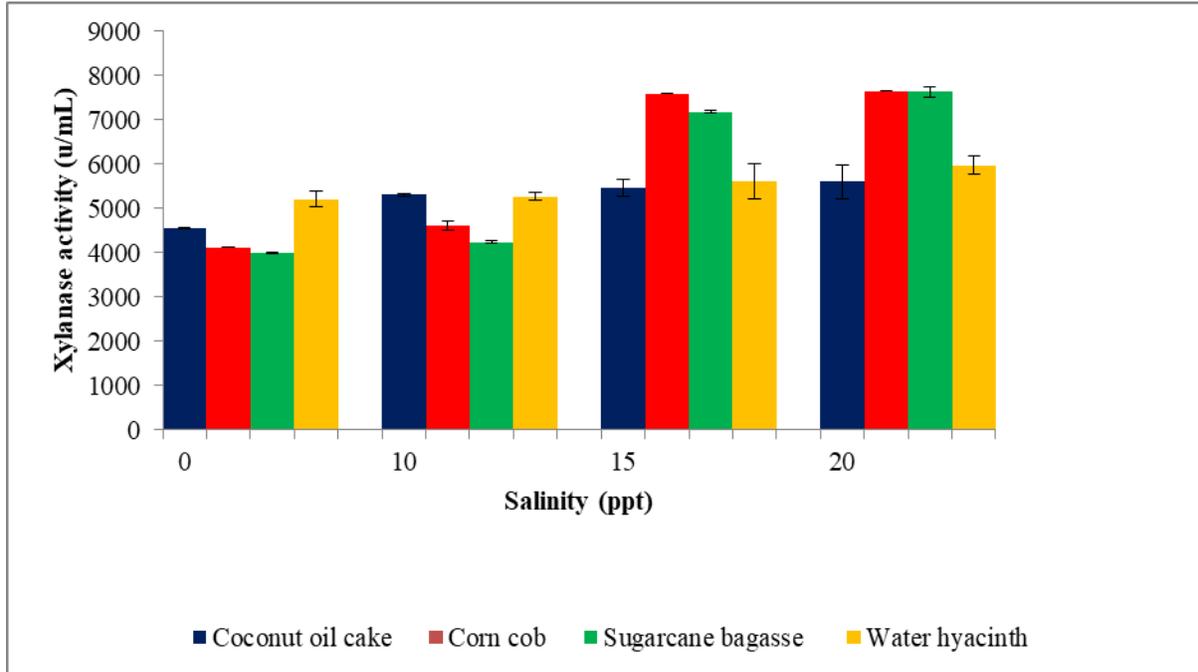


Figure.1d Effect of pH on xylanase production using selected substrates

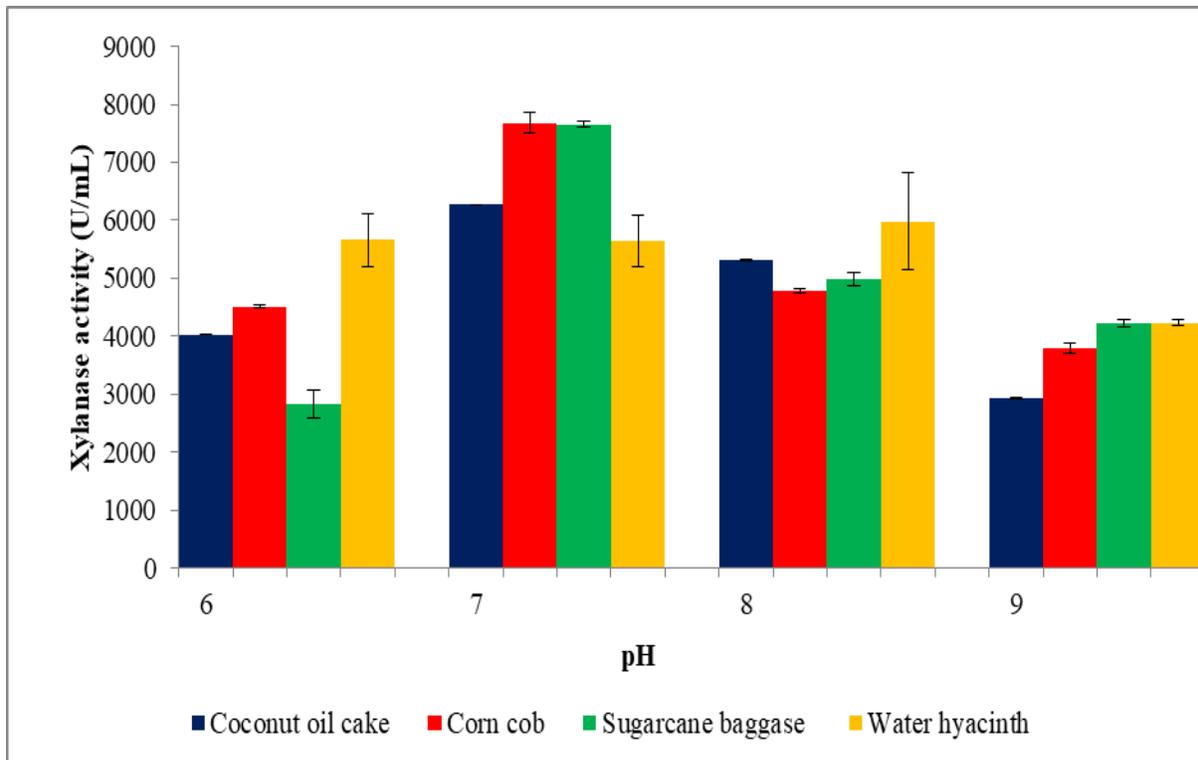


Figure.1e Effect of incubation temperature on xylanase production using selected substrates

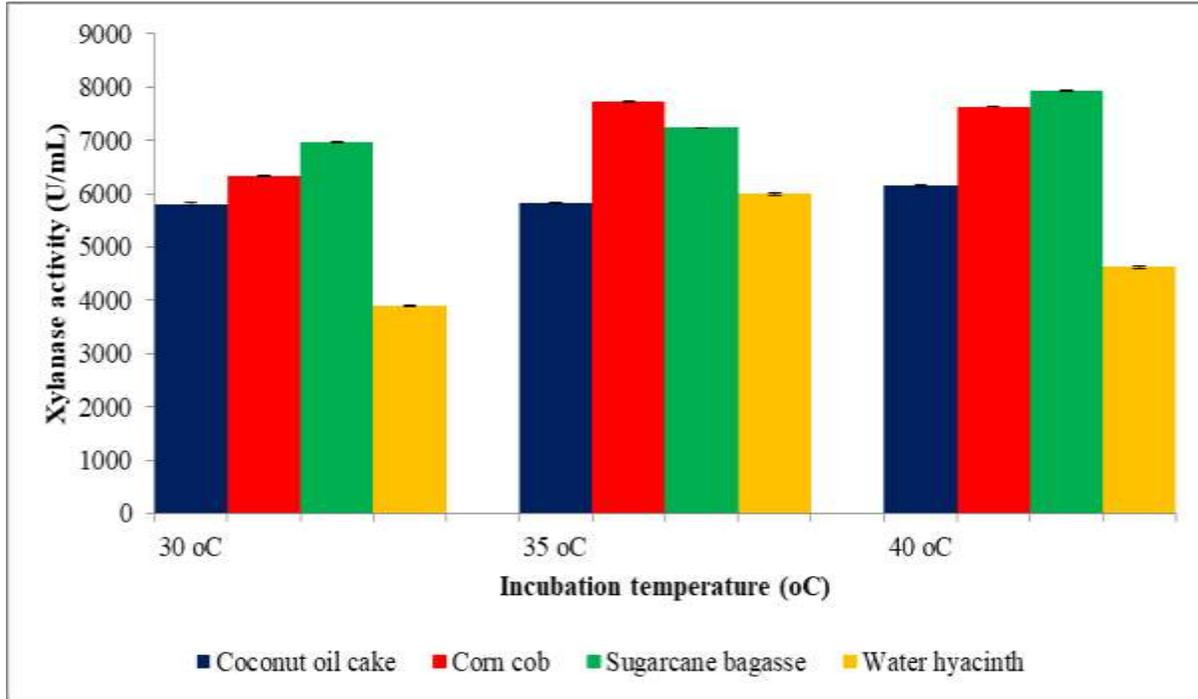


Figure.1f Effect of agitation speed (rpm) for xylanase production using selected substrates

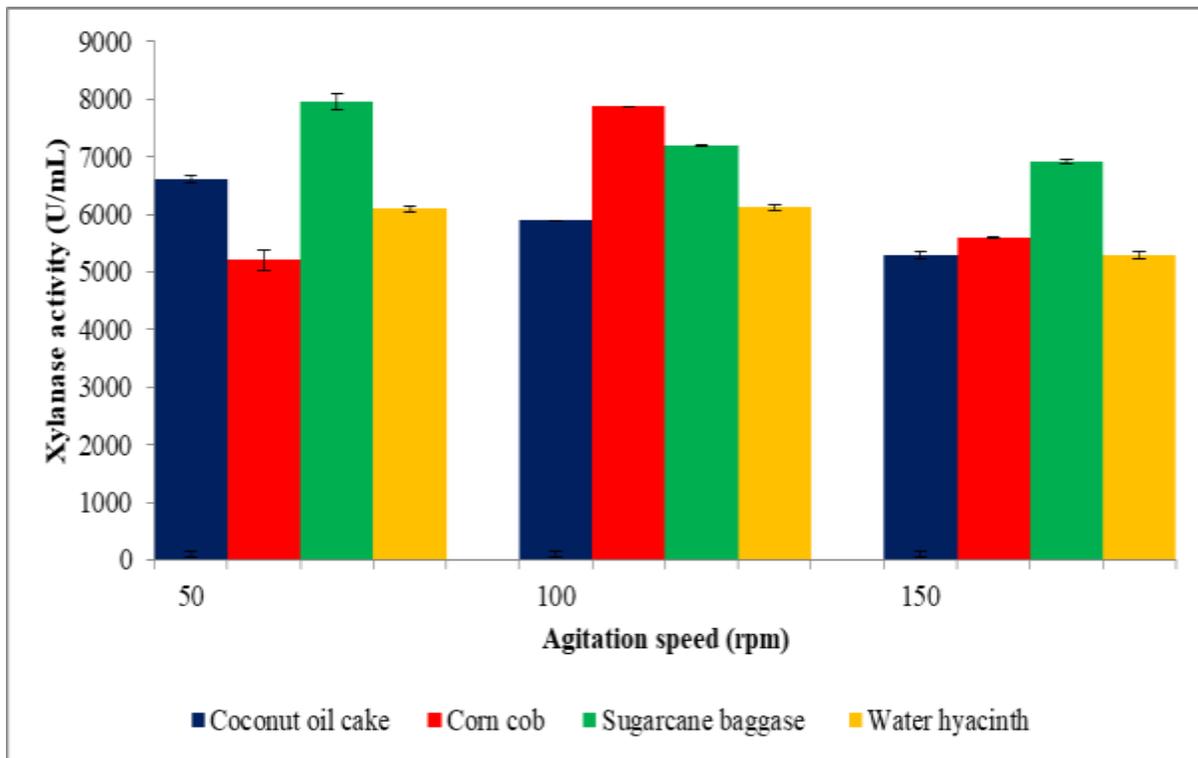


Figure.1g Effect of substrate concentration (%) on xylanase production using selected substrates

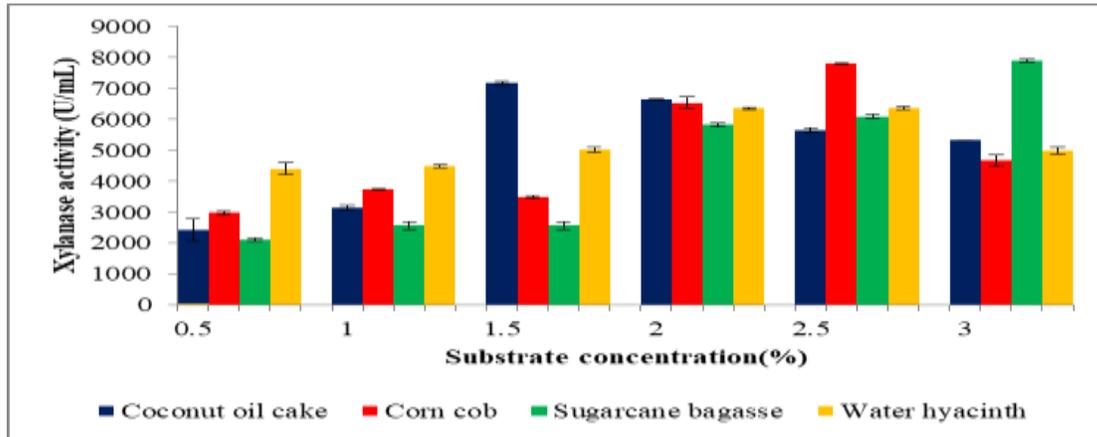


Figure.1h Effect of inorganic nitrogen sources on xylanase production using selected substrates

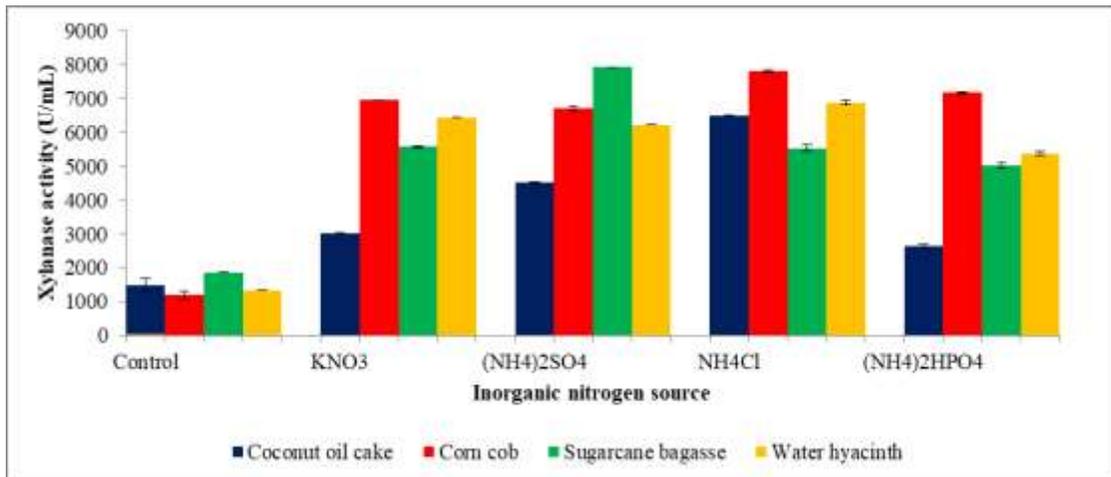


Figure.1i Effect of organic nitrogen sources on xylanase production using selected substrates

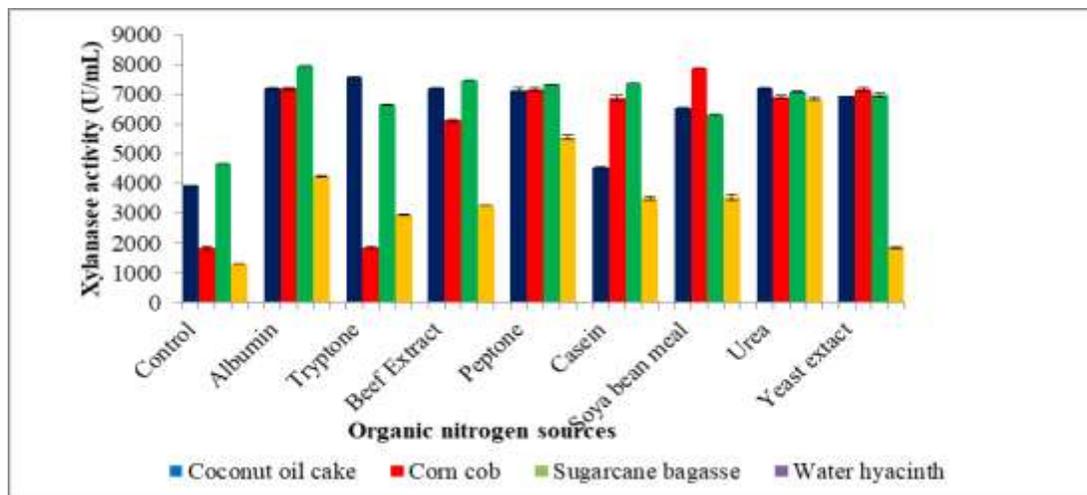


Figure.1j Effect of surfactants on xylanase production using selected substrates

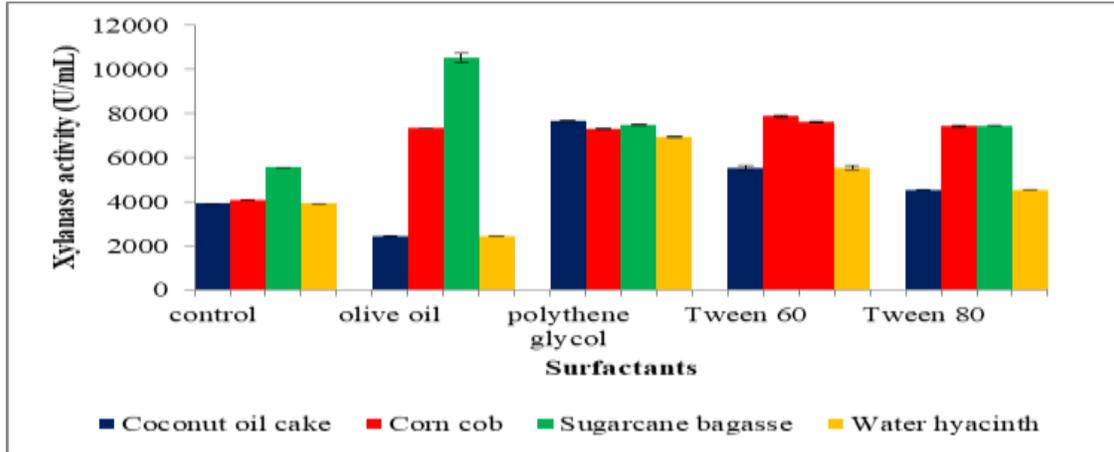
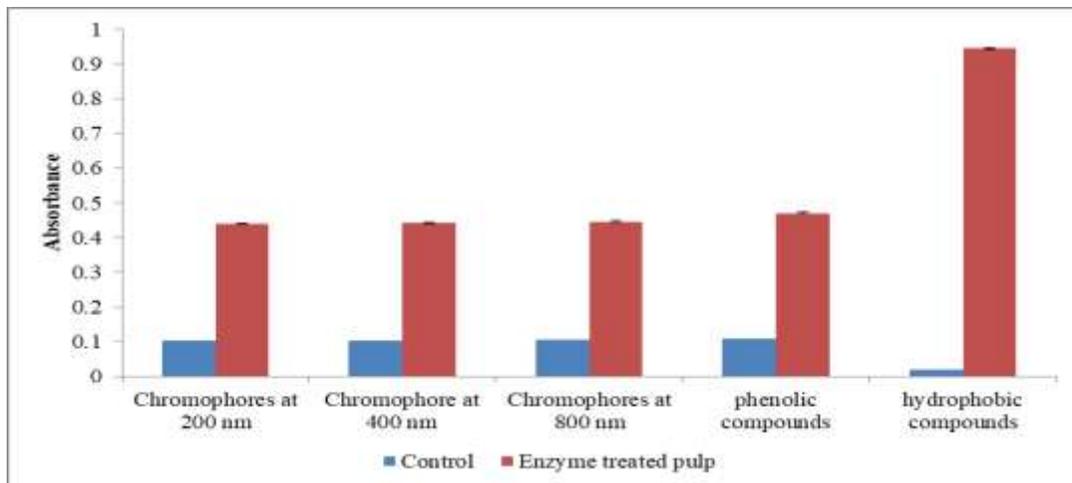


Fig.2 Manually pressed pulp before and after treatment with xylanase



Fig.3 Analysis of phenolic compounds and hydrophobic compounds in effluents released from the paper pulp before and after the enzyme treatment



Stimulatory effect of Tween 60 and olive oil on xylanase production could be due to the effect on cell membrane permeability or by disrupting nonspecific binding of enzymes to substrates. These actions exerts a positive effect on desorption and recycling of xylanase.

Analysis of phenolic compounds and hydrophobic compounds in effluent released before and after xylanase treatment

The results of the current study clearly indicated that the cell-free culture supernatants of *Streptomyces* sp. strain ER1 showed tremendous potential for biological deinking. Treatment with cell-free culture supernatant containing xylanase activity caused several folds of increase in brightness (Figure 2). This might have caused either by decolourization alone or both decolourization and dislodging of ink particles from pulp fibre. On comparing the absorbance of effluents (λ 200 to 800 nm), it was found that xylanase treated pulp effluent showed high absorbance whereas that of control were colourless (Figure 3). Highly purified or concentrated enzymes are being used for deinking purpose (Marques *et al.*, 2003) but, in the current study, the crude culture supernatant alone could bring about deinking of newspaper.

In summary, *Streptomyces* sp. ER1 was identified to be potential xylanase producer but need further studies as xylanase production from *Streptomyces* sp. of Cochin estuary is not well documented. The xylanase enzyme was successfully produced from all agro-industrial wastes tested and sugarcane bagasse was found to be best suited for xylanase production after optimisation. The ability of *Streptomyces* sp. ER1 to produce xylanase on several substrates made it possible to use suitable substrate according to

the seasons, cost effectively and the optimization study in the present work may assist this purpose. The study proves that optimal conditions for xylanase production varied with the substrates and thus it is critical to maintain optimal conditions for maximum enzyme production with each substrate. The crude xylanase produced by the stain ER1 could successfully decolourise the old newspaper samples. Hence, *Streptomyces* sp. strain ER1 can be considered as a promising agent for xylanase production using agricultural wastes which help in converting waste materials in to commercially important valuable products and also its application in deinking used paper.

Conflict of interest statement

We declare that we have no conflict of interest.

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