Nova Biotechnol Chim (2022) 21(2): e1226
DOI: 10.36547/nbc.1226

Nova Biotechnologica *et* Chimica

Optimization of procedural factors for advanced xylanase synthesis by *Lysinibacillus fusiformis* using Kolanut husk as substrate

Suliat Olatidayo Omisore✉, Daniel Juwon Arotupin, Michael Tosin Bayode

Department of Microbiology, Federal University of Technology, P.M.B. 704, Akure, Nigeria

✉ *Corresponding author: omisore34@gmail.com*

Article info

Article history:
Received: 13th October 2021
Accepted: 1st December 2022

Keywords:
Kolanut husk
Lysinibacillus fusiformis
Optimization
Substrate
Xylan
Xylanase

Abstract

Xylan is a complex hetero-polysaccharide consisting of different monosaccharides held together by glycosidic and ester bonds. Extracellular xylanase fashioned by numerous microbes principally from bacterial species such as *Bacillus* species are responsible for cleaving the glycosidic linkages. Microbial xylanases exhibit different substrate specificities and biochemical peculiarities. This study was carried out for optimization of cultivation conditions for xylanase production using the bacterium *Lysinibacillus fusiformis* and Kolanut husk as a component of cultivation medium. The bacterium was isolated from Kolanut plantation waste soil and screened for the production of xylanase qualitatively on xylan nutrient agar and quantitatively under submerged fermentation. The different conditions optimized included substrate concentration, additional sugars, incubation period, temperature, initial pH, nitrogen supplementation and inoculum mass through one factor at a time approach. Maximum xylanase production was obtained at substrate concentration of (1 % xylan and 1.5 % Kolanut husk), nitrogen source (yeast extract plus peptone), carbon source (sucrose), incubation period (24 h), pH (5.0), temperature (35 ºC) and inoculum size (1 %). *Lysinibacillus fusiformis* has been proven to be a promising bacterium for xylanase production using Kolanut husk as substrate. The use of Kolanut husk as foremost carbon source is predominantly precious as being an agricultural waste, affordable, and locally available compared to expensive commercially sold xylan.

Introduction

Hemicellulose serves as the second most ample source of polysaccharides in nature, exhibiting 25 % 30 % of the dry weight of lignocellulose biomass (Coman *et al.* 2011; Walia *et al.* 2015). It is the major amount of lignocellulose, comprising of varied 5- and 6-carbon sugars such as arabinose, galactose, glucose, mannose, and xylose (Girisha 2018). Xylan is a polymer of xylose molecules which plays the pivotal role in holding the plant cell walls together (Irfan *et al.* 2016; Girisha 2018). Considerable xylanolytic enzymes comprising α-arabino-furanosidase, act collectively for total disintegration of xylan. The most important endo β-1, 4-xylanase plays an imperative part to catalyze the breakdown of xylan into diminutive oligosaccharides (Irfan *et al.* 2012; Khusro *et al.* 2016).

Udey et al. (2016) reported the latent applications of xylanase in numerous fields such as saccharification of lignocellulosic biomass for ethanol and xylo-oligosaccharides generation in the biofuel industry. Its use as a fading agent and

lighting up of pulp to reduce chlorine in paper and pulp production operations was also reported (Raghukumar *et al.* 2004; Sridevi *et al.* 2016; Patel and Dudhagara 2020a). It helps in the smooth relocation of the water core in bread dough ensuring a softer dough formation (Beg *et al.* 2001; Garg *et al.* 2010). Furthermore, xylanolytic enzymes have been reported by Garg et al. (2010) for its application in the animal industry for the conversion of lignocellulose into feedstocks and fuels.

Xylanases are produced by a diverse number of microbes such as bacteria, fungi and actinomycetes (Battan *et al.* 2006; Chakdar *et al.* 2016; Ramanjaneyulu *et al.* 2017). However, xylanases from bacteria are the most-preferred for industrial applications due to thermo-stability and ability to withstand alkali-substrates (Nagar *et al.* 2013; Aarti *et al.* 2015; Mandal *et al.* 2015). Xylanolytic bacteria are the generally striking producers of high-level extracellular xylanase as compared to fungi and actinomycetes, which have time-consuming generation time alongside the production of extremely glutinous polymers and deprived oxygen transport. There is limited exploitation of xylanase commercially due to its high cost of production. In this view, the search for cost-effective means of production, such as the use of low-cost agro agricultural residues has captivated the attention of scientists (Patel and Dudhagara 2020b). Gautério *et al.* (2020) and Ratnadewi *et al.* (2020) also buttressed the utility of agriculture waste derivatives like wheat bran, corn cobs, rice straw for exploitation as a low-grade substrate for xylanase production.

Kolanut husk alongside its testa has been proven as a budding substrate to produce the microbial enzyme particularly xylanase (Fabunmi *et al.* 2018). Therefore, the study was carried out to determine the optimal process parameters needed to improve the yield of xylanase produced from simple inexpensive Kolanut husk using *Lysinibacillus fusiformis*. In biotechnological process, the best results are achieved when the individual and combined effects of parameters involved are considered (Tandon *et al.* 2016). The current study aims at optimizing the cultural conditions such as carbon source, nitrogen supplementation, temperature, incubation time and inoculum sizes for xylanase production by *Lysinibacillus fusiformis*, an isolate of Kolanut plantation waste soil using a medium with Kolanut husk as substrate in other to know the best conditions for highest xylanase.

Experimental

Kolanut plantation soil and Kolanut husk collection

Kolanut husks and Kolanut plantation soil from the Kolanut husk dumpsite were obtained from a Kolanut farm at Ile Ife City, Osun State, Nigeria (7.4905° N, 4.5521° E). Sample transported to the laboratory of Microbiology Department, Federal University of Technology Akure, Ondo State in a ziploc bag. The soil samples were preserved at -20 ºC before use.

Isolation and identification of bacteria isolates

One gram of the soil sample was suspended in 100 mL of distilled water then incubated in a control incubator shaker (Gallenkamp, Cambridge, UK) at 200 rpm for 30 min. Mixtures were allowed to settle, and serial dilutions of up to 10^4 diluents were prepared using sterile distilled water. Bacterial isolation was conducted by aseptically plating
100 µL from the diluent on xylan nutrient agar (nutrient agar supplemented with 1 % xylan) and incubated aerobically for 37 ºC for 24 h. Purification of bacteria was done by repeated streaking and sub-culturing for purity and preserved at 4 ºC until use (Kartik *et al.* 2016).

Authentication of the xylanolytic ability of isolates by well plate diffusion assay

The organism was however authenticated qualitatively for positive xylanase production by growing isolates on nutrient agar medium containing 1 % birchwood xylan. The media was sterilized, poured, and allowed to solidify. Upon solidification, wells were made with the aid of sterile cork-borer of 10 mm diameter. To each well bored in the solidified media, 100 µL of the overnight pure bacterial culture was poured and incubated for 24 – 36 h. The plate was flooded with

0.25 % Congo red solution for 15 min after which it was decolorized with 0.5 % acetic acid for 15 min and finally washed twice by 1 M NaCl for de-staining as described (Chan *et al.* 2016). Clear zones around the well further authenticated xylanolytic activity.

Submerged fermentation for xylanase production

Submerged fermentation was conducted using Kolanut husk as the substrate in addition to minimal salt medium containing ($g.L^{-1}$): xylan 10.0, K_2HPO_4 0.1, $CaCl_2$ 0.5, $MgSO_4.7\ H_2O$ 0.5, peptone 2.0, $FeSO_4 \cdot 7\ H_2O$ in trace amount, pH 6.8, sterilized at 121 ºC for 15 min. 50 mL of the sterile minimal salt medium in 250 mL flasks were inoculated with 1 mL of bacterial culture in broth and incubated at 37 ºC for 24 h in a rotary shaker (HunterLab Digital Colorimeter, Reston, USA) with agitation speed of 150 rpm. After the incubation period, the enzyme production medium was subjected to cold centrifugation at 10,000 rpm for 10 min to eliminate the bacterial cells and superfluous elements. The supernatant considered as source of the crude enzyme was gently decanted into sterile vials (Sari *et al.* 2016).

Xylanase assay

Xylanase activity in the supernatant was assayed by determining the concentration of reducing sugar released by the activity of the crude enzyme on its substrate xylan by 3,5-dinitrosalicylic acid (DNSA) reagent (Miller 1959). Reaction mixture containing 0.5 mL of culture enzyme with 0.5 mL 1 % of Birchwood xylan solution primed in phosphate buffer pH 6.8 was incubated for 20 min at 45 ºC. The control experiment was set up to authenticate the results from the actual testing; with the control tube comprising a similar volume of a substrate with enzyme solution being replaced with sterile distilled water. After incubation, the reaction was terminated by adding 1 mL of DNSA reagent as described by Miller (1959). The tubes enclosing the resultant blend were excited in the boiling water bath for 10 min, observed for brick red colour development and cooled swiftly in a water bath at room temperature. The optical density of the cooled mixture was measured with a spectrophotometer (Lab-Tech Digital Colorimeter) at 540 nm against a blank and articulated as xylose equivalent. One unit of endo-1,4-β-xylanase was defined as the amount of enzyme requisite to discharge 1 µmol of xylose per minute in model assay conditions as depicted by Sharma and Bhajaj (2005); Bhalla *et al.* (2015).

Protein estimation

The amount of protein released in the supernatant was estimated as described Bradford (1976) using Bovine Serum Albumin (BSA) (Sigma-Aldrich, Darmstadt, Germany) as standard. 1mL of the enzyme extract was taken in a glass tube and added to it 3 mL of Bradford's reagent, the mixture was incubated at room temperature for 7 – 10 min. The absorbance was measured at 595 nm with a spectrophotometer (HunterLab Digital Colorimeter, Virginia, USA) and compared with the Bovine Serum Albumin standard curve.

Optimization of cultural and nutritional conditions

The optimization of xylanase production by *Lysinibacillus fusiformis* was carried out using "one factor at a time technique" (varying one factor while keeping the rest constant) in order to estimate xylanase activity for each varied parameter. Parameters such as the effect of Kolanut husk as carbon source at concentration (0.5 – 2.5 %), Kolanut husk induction with xylan (0.5 – 1.5 %), additional carbon source at 0.5 % (glucose, fructose, sucrose, galactose and lactose), nitrogen supplementation at 0.25 % (yeast extract, urea, gelatin, tryptone, casein, ammonium chloride, ammonium nitrate, ammonium sulfate, potassium nitrate), incubation period (0 – 42 h), pH (2 – 10), temperature (25 – 45 ºC) and inoculum size (0.5 – 2.5 %) were optimized for enhanced xylanase production by *Lysinibacillus fusiformis.* The fermentation broth was separated from the bacterial cells by centrifugation, the supernatant served as a source of a crude enzyme tested for xylanase activity. The entire experimentations were done in triplicates with each mean shown in the result section. Each experiment had its control with the control tube not having the tested process parameters.

Statistical analysis

The data obtained after the optimization studies were statistically evaluated using the one-way analysis of variance (ANOVA) at significance level of $P < 0.05$ using the statistical package IBM SPSS Statistics v. 23.0 (Chicago, USA).

Results

Screening and authentication of obtained xylanolytic bacteria

Isolate appeared cream-coloured, regularly shaped, round edges with 1 – 2 mm diameter on nutrient agar plate. It was Gram-positive and rod-shaped and conforms to its earlier identification by Fabunmi *et al.* (2018) as *L. fusiformis.* Organisms showed zones of hydrolysis on xylan agar plates after incubation, indicating its ability to degrade xylan in the media alongside its growth during the incubation period with an average of 29 mm diameter zone of hydrolysis on xylan agar medium.

Optimization of culture and nutritional conditions

The effect of different substrate (Kolanut husk) concentration (0.5 – 2.5 %) as sole source of xylan in submerged fermentation by *L. fusiformis* is in Table 1. Result indicated that the highest enzyme activity was at Kolanut husk concentration of 2.0 % with value of 419.28 $\mu mol.mL^{-1}.min^{-1}$ while the least at 0.5 % with a value of 201.52 $\mu mol.mL^{-1}.min^{-1}$.

Table 1. Upshot of substrate concentration (Kolanut husk) on xylanase production by *L. fusiformis* under SmF.

Kolanut husk [%]	Substrate optimization			
	Absorbance	Enzyme activity [$\mu mol.min^{-1}.mL^{-1}$]	Protein concentration	Specific enzyme activity [$\mu mol.min^{-1}.mg^{-1}$]
0.5	0.54 ± 0.00^{a}	206.52 ± 2.02^{a}	1.96 ± 0.06^{a}	100.18 ± 0.16^{b}
1.0	0.70 ± 0.00^{b}	266.83 ± 2.04^{b}	2.17 ± 0.01^{b}	124.28 ± 0.11^{e}
1.5	0.92 ± 0.00^{c}	341.43 ± 0.29^{c}	3.89 ± 0.02^{d}	88.34 ± 0.08^{a}
2.0	1.11 ± 0.00^{e}	419.28 ± 0.36^{e}	3.91 ± 0.01^{d}	106.83 ± 0.12^{c}
2.5	1.01 ± 0.00^{d}	381.36 ± 0.31^{d}	3.11 ± 0.06^{c}	121.65 ± 0.03^{d}

Values are obtainable as Mean ± S.E (n = 3). Values with different superscripts are statistically significantly different ($P < 0.05$). SmF – Submerged fermentation.

Effect of xylan supplementation with Kolanut husk on xylan production by L. fusiformis

The effect of different concentrations of a husk (0.5, 1.0, 1.5, 2.0, and 2.5 %) in combination with a commercial birchwood xylan was supplemented to the fermentation medium for enhanced production of xylanase as shown in Table 2. Xylan supplementation as inducer showed maximum activity at 1.5 % Kolanut husk supplemented with 1 % commercial xylan with xylanase activity of 422.63 $\mu mol.mL^{-1}.min^{-1}$ and lowest at 1 % Kolanut husk with 0.5 % xylan with activity of 303.06 $\mu mol.mL^{-1}.min^{-1}$.

Effect of carbon source on xylanase production by Lysinibacillus fusiformis

Effect of sugar optimization revealed sucrose to having the highest activity effect amongst others with enzyme activity of 549.75 $\mu mol.mL^{-1}.min^{-1}$ exceeding other sugars as shown in Table 3. The monosaccharide galactose gave the least activity of 494.5 $\mu mol.mL^{-1}.min^{-1}$, the remaining sugars gave moderate improvement on enzyme production but not comparable to sucrose.

Table 2. Effect of Xylan (X) supplementation as inducer with varied Kolanut husk concentration on xylanase production by *L. fusiformis* under SmF.

Kolanut husk + Xylan [%]	Substrate optimization			
	Absorbance	**Enzyme activity [µmol.min^{-1}.mL^{-1}]**	**Protein concentration**	**Specific enzyme activity [µmol.min^{-1}.mg^{-1}]**
K1.0 + X0.5	0.80 ± 0.00^{a}	303.06 ± 0.54^{a}	1.87 ± 0.01^{a}	162.84 ± 0.07^{d}
KI.0+ X1.0	1.01 ± 0.00^{d}	384.13 ± 0.59^{e}	2.09 ± 0.05^{b}	190.69 ± 0.85^{f}
K0.5+X1.0	0.97 ± 0.01^{c}	367.12 ± 1.16^{c}	2.38 ± 0.01^{c}	153.37 ± 0.49^{b}
K1.5+X0.5	0.79 ± 0.00^{a}	374.65 ± 0.87^{d}	2.38 ± 0.07^{c}	166.72 ± 0.43^{d}
K1.5+X1.0	1.12 ± 0.00^{e}	422.54 ± 1.44^{f}	2.39 ± 0.01^{c}	178.55 ± 0.23^{e}
2.0K.H	0.92 ± 0.00^{b}	348.36 ± 0.31^{b}	2.36 ± 0.02^{c}	147.21 ± 1.17^{a}

Values are obtainable as Mean ± S.E (n = 3). Values with different superscript are statistically significantly different (P < 0.05). SmF – Submerged fermentation.

Table 3. Effect of sugar supplementation (carbon source) on xylanase production by *Lysinibacillus fusiformis* under SmF.

Carbon sources [%]	Carbon sources optimization			
	Absorbance	**Enzyme activity [µmol.min^{-1}.mL^{-1}]**	**Protein concentration**	**Specific enzyme activity [µmol.min^{-1}.mg^{-1}]**
Control (peptone water)	0.86± 0.02^{a}	320.66 ± 0.88^{a}	1.79 ± 0.05^{d}	177.61 ± 0.87^{a}
Glucose	1.39 ± 0.01^{d}	521.74 ± 0.80^{e}	2.06 ± 0.02^{e}	250.47 ± 0.86^{b}
Fructose	1.38 ± 0.01^{d}	518.02 ± 1.13^{d}	1.21 ± 0.04^{c}	452.10 ± 0.58^{d}
Sucrose	1.45 ± 0.02^{e}	550.58 ± 0.71^{f}	1.08 ± 0.02^{b}	500.57 ± 0.86^{e}
Galactose	1.32 ± 0.01^{c}	495.16 ± 0.44^{c}	0.88 ± 0.01^{a}	566.38 ± 2.34^{f}
Lactose	1.25 ± 0.02^{b}	476.16 ± 1.48^{b}	1.76 ± 0.02^{d}	264.52 ± 0.86^{c}

Values are obtainable as Mean ± S.E (n = 3). Values with different superscript are statistically significantly different (P < 0.05). SmF – Submerged fermentation.

Effect of organic nitrogen on xylanase production by Lysinibacillus fusiformis

Nitrogen sources revealed yeast extract giving the highest xylanase titre (437.75 µmol.mL^{-1}.min^{-1}) and gelatin (339.38 µmol.mL^{-1}.min^{-1}) displayed the lowest enzyme titre (Table 4).

Table 4. Effect of organic nitrogen source on xylanase production by *Lysinibacillus fusiformis* under SmF.

Inoculum [%]	Organic sources optimization			
	Absorbance	**Enzyme activity [µmol.min^{-1}.mL^{-1}]**	**Protein concentration**	**Specific enzyme [µmol.min^{-1}.mg^{-1}]**
Control (peptone water)	1.05 ± 0.00^{b}	401.06 ± 0.23^{b}	2.76 ± 0.07^{b}	149.06 ± 0.38^{b}
Gelatin	0.86 ± 0.01^{a}	340.12 ± 0.47^{a}	2.79 ± 0.06^{b}	116.61 ± 0.45^{a}
Casein	1.12 ± 0.00^{c}	426.90 ± 0.31^{c}	2.72 ± 0.01^{b}	155.44 ± 0.29^{c}
Urea	1.13 ± 0.00^{c}	430.72 ± 0.14^{d}	2.39 ± 0.01^{a}	181.06 ± 0.52^{e}
Tryptone	1.15 ± 0.00^{d}	437.98 ± 0.89^{e}	2.36 ± 0.01^{a}	184.73 ± 0.63^{f}
Yeast extract	1.15 ± 0.00^{d}	438.55 ± 0.47^{e}	2.70 ± 0.01^{b}	162.89 ± 0.58^{d}

Values are obtainable as Mean ± S.E (n = 3). Values with different superscript are statistically significantly different (P < 0.05). SmF – Submerged fermentation.

Effect of inorganic nitrogen on xylanase production by Lysinibacillus fusiformis

Table 5 showed the effect of inorganic nitrogen with KNO_3 resulting in the highest xylanase activity (464.25 µmol.mL^{-1}.min^{-1}) and NH_4CO_3 with the least (325.70 µmol.mL^{-1}.min^{-1}).

Table 5. Outcome of inorganic nitrogen source on xylanase production by *Lysinibacillus fusiformis* under SmF.

Inoculum [%]	Inorganic sources optimization			
	Absorbance	Enzyme activity [µmol.min^{-1}.mL^{-1}]	Protein concentration	Specific enzyme [µmol.min^{-1}.mg^{-1}]
Control (peptone water)	1.05 ± 0.00^{b}	400.56 ± 0.86^{b}	2.68 ± 0.02^{a}	148.96 ± 1.15^{e}
KNO_3	1.22 ± 0.01^{d}	463.74 ± 0.90^{d}	3.83 ± 0.01^{c}	121.27 ± 0.36^{d}
$(NH4)_2SO_4$	1.05 ± 0.00^{c}	398.01 ± 0.57^{b}	3.52 ± 0.02^{b}	113.49 ± 0.86^{c}
$NaNO_3$	1.07 ± 0.01^{c}	405.86 ± 0.59^{c}	4.30 ± 0.05^{d}	96.44 ± 0.28^{b}
NH_4CO_3	0.85 ± 0.00	325.70 ± 1.19^{a}	4.50 ± 0.01^{e}	92.45 ± 0.27^{a}

Values are obtainable as Mean ± S.E (n = 3). Values with different superscript are statistically significantly different (P < 0.05). SmF – Submerged fermentation.

Effect of pH on xylanase synthesis by Lysinibacillus fusiformis

Media pH is an extra significant dynamic considered in Table 6, enzyme activity was highest value of 537.79 µmol.mL^{-1}.min^{-1} at pH of 5.0 and the lowest enzyme activity at pH of 2.0 (368.20 µmol.mL^{-1}.min^{-1}). A supplementary boost in pH above 5.0 resulted in a significant drop-off in the activity recorded.

Table 6. Upshot of pH on xylanase synthesis by *Lysinibacillus fusiformis* under SmF.

pH	pH optimization			
	Absorbance	Enzyme activity [µmol.min^{-1}.mL^{-1}]	Protein concentration	Specific enzyme [µmol.min^{-1}.mg^{-1}]
2	0.88 ± 0.08^{a}	368.20 ± 0.90^{a}	3.55 ± 0.02^{d}	102.69 ± 0.11^{a}
3	1.11 ± 0.01^{bc}	424.08 ± 0.46^{b}	3.52 ± 0.03^{cd}	121.53 ± 0.03^{b}
4	1.20 ± 0.00^{cd}	456.88 ± 1.55^{c}	3.05 ± 0.02^{b}	148.83 ± 0.06^{d}
5	1.41 ± 0.00^{g}	537.79 ± 1.10^{h}	3.73 ± 0.12^{e}	141.29 ± 0.12^{c}
6	1.39 ± 0.00^{fg}	528.31 ± 0.84^{g}	3.35 ± 0.07^{c}	161.22 ± 0.14^{g}
7	1.31 ± 0.00^{ef}	497.30 ± 0.84^{f}	3.14 ± 0.03^{b}	159.14 ± 0.13^{f}
8	1.28 ± 0.00^{de}	486.26 ± 1.86^{d}	3.10 ± 0.05^{b}	161.25 ± 0.14^{g}
9	1.20 ± 0.00^{cd}	458.18 ± 0.92c^{c}	2.99 ± 0.05^{b}	152.71 ± 0.11^{e}
10	1.09 ± 0.00^{b}	490.56 ± 0.72^{e}	2.75 ± 0.02^{a}	141.44 ± 0.02^{c}

Values are obtainable as Mean ± S.E (n = 3). Values with different superscript are statistically significantly different (P < 0.05). SmF – Submerged fermentation.

Effect of temperature on xylanase production by Lysinibacillus fusiformis

Xylanase production was at its peak at 35 ^{o}C (572.83 µmol.mL^{-1}.min^{-1}) and least at 45 ^{o}C (347.33 µmol.mL^{-1}.min^{-1}) (Table 7).

Table 7. Effect of temperature on xylanase synthesis by *Lysinibacillus fusiformis* under SmF.

Temp [^{o}C]	Temperature optimization			
	Absorbance	Enzyme activity [µmol.min^{-1}.mL^{-1}]	Protein concentration	Specific enzyme [µmol.min^{-1}.mg^{-1}]
25	1.20 ± 0.00^{c}	457.64 ± 1.30^{c}	3.07 ± 0.06^{a}	151.82 ± 0.03^{c}
30	1.39 ± 0.00^{d}	531.41 ± 0.33^{d}	3.11 ± 0.11^{a}	169.65 ± 0.17^{d}
35	1.51 ± 0.00^{e}	572.77 ± 1.29^{e}	3.50 ± 0.05^{b}	167.62 ± 1.44^{d}
40	1.10 ± 0.00^{b}	419.86 ± 0.32^{b}	3.39 ± 0.05^{b}	124.51 ± 0.86^{b}
45	0.91 ± 0.00^{a}	346.11 ± 0.67^{a}	2.94 ± 0.02^{a}	119.35 ± 0.87^{a}

Data are presented as Mean ± S.E (n = 3). Values with different superscript are statistically significantly different (P < 0.05). SmF – Submerged fermentation.

Effect of incubation time on xylanase production by Lysinibacillus fusiformis

Incubation period optimization results showed that 24-h cultivation (624.29 µmol.mL^{-1}.min^{-1}) gave the best production as seen in Fig. 1 and further increase revealed a corresponding reduction in enzyme activity.

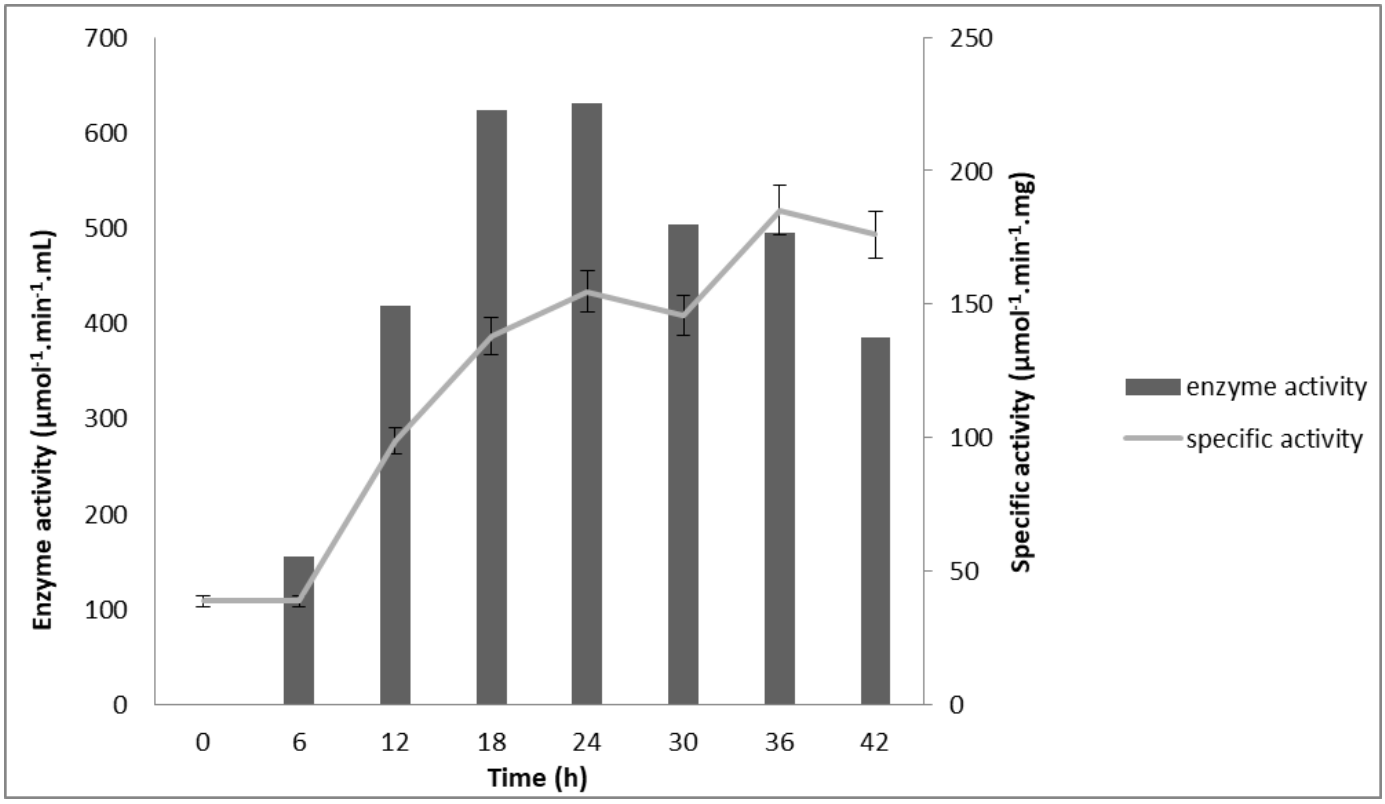

Fig.1. Effect of time on xylanase production by *Lysinibacillus fusiformis* under submerged fermentation.

Effect of inoculum size on xylanase production by Lysinibacillus fusiformis

Inoculum size is a vital dynamic in enzyme synthesis and maximum enzyme activity was recorded at 1.5 % with a value of 647.63 µmol.mL^{-1}.min^{-1} and the least observed at 2.5 % with 616.07 µmol.mL^{-1}.min^{-1} (Table 8).

Table 8. Effect of inoculum size on xylanase production by *Lysinibacillus fusiformis* under SmF.

Inoculum [%]	Inoculum optimization			
	Absorbance	Enzyme activity [µmol.min^{-1}.mL^{-1}]	Protein concentration	Specific enzyme [µmol.min^{-1}.mg^{-1}]
0.5	1.62 ± 0.00^{a}	615.27 ± 0.36^{a}	0.89 ± 0.01^{b}	697.68 ± 0.71^{d}
1.0	1.67 ± 0.00^{b}	624.07 ± 0.51^{b}	0.96 ± 0.01^{c}	653.53 ± 0.74^{b}
1.5	1.70 ± 0.00^{d}	647.63 ± 0.32^{d}	1.25 ± 0.01^{d}	518.78 ± 0.32^{a}
2.0	1.69 ± 0.00^{c}	643.35 ± 0.86^{c}	0.97 ± 0.01^{c}	671.07 ± 0.52^{c}
2.5	1.62 ± 0.00^{a}	616.07 ± 0.51^{a}	0.84 ± 0.00^{a}	732.31 ± 0.56^{e}

Data are presented as Mean ± S.E (n = 3). Values with different superscript are statistically significantly different ($P < 0.05$). SmF – Submerged fermentation.

Discussion

Kolanut husk in nature is composed of hemicellulose (40.41 %), lignin (21.29 %), cellulose (38.72 %) as reported by Adeyi (2010) and Patel and Dudhagara (2020a), making it an appropriate substrate for xylanase production. They are mostly disposed of as agricultural waste at production site of Kolanut forming a landfill and environmental problems. Nevertheless, as a substrate for enzyme production could help to solve environmental pollution problems as well as reduction in production cost of xylanase. Kolanut husk as an enzyme source was optimized as a component of the microbial fermentation medium. Among the concentration of Kolanut husk tested 2.0 % gave the maximum xylanase production. Concentration above 2.0 % demonstrated a declined xylanase production but not as low as when lower concentrations of 0.5 % and 1.0 % was used (Table 1). This indicated that adding further substrate does not show any noteworthy change in xylanase synthesis. A general boost in substrate absorption could lead to swell in enzyme output velocity at certain point till it reaches substrate saturation where no enzyme activity could be recorded (Heskia *et al.* 2018). Furthermore, the fermentation medium was induced with commercial birchwood xylan alongside Kolanut husk. This was intended to boost xylanase production in the medium. Optimum xylanase production was attained at a lesser substrate concentration in contrast to medium with solely Kolanut husk. Different xylan concentrations have also been reported to give maximum xylanase activity of 0.5 % (Annamalai *et al.* 2009; Heskia *et al.* 2018). Thus, indicating that xylanase stimulation is an intricate trend in which the intensity of reaction varies with altered concentration combinations. Decreases in enzyme activity at higher substrate concentration might be owed to the formation of thick suspension in the presence of higher substrate concentration which could have resulted in improper mixing of the substrate or non-uniform circulation of a nutrient under agitation condition (Karim *et al.* 2015). Karim *et al.* (2015) has revealed that substrate concentration used is a decisive element for utmost xylanase optimization and that there are different concentrations for various ligno-cellulose substrates.

Supplementation of medium with additional carbon sources involving both monosaccharide and disaccharide improved the enzyme activity produced. Sucrose had the highest enzyme activity and closely followed by other sugars (Table 2 and Table 3). The results obtained agree with Singh *et al.* (2011) who stated that amylase synthesis is favored by galactose, linking to the fact growth and enzyme productions of any organism are greatly influenced by both environmental conditions as well as the nutrient present in the growth medium. However, these findings contradict reports that wholesome sugars only have good growth but poor xylanase synthesis (Shah and Madamwar 2005) and that of Seyis and Aksoz (2005) that sucrose and glucose gave inhibitory effect on enzyme activity. Their reports were attributed to catabolite repression of xylanase genetic material which is guarded at two levels as reported by De Graaff *et al.* (1994), repressed gene transcription and ultimately by repressed transcriptional activator.

In addition to carbon sources, nitrogen sources have a significant end product on the synthesis of xylanolytic enzymes produced by bacteria. Overall, crude sources gave a higher xylanase production than inorganic sources (Table 4 and Table 5). This agrees with the report of Bajaj *et al.* (2010), who stated that peptone combined with yeast extract as the largely reasonable and accustomed nitrogen sources in terms of elevated output of xylanase from diverse bacteria. Nagar *et al.* (2010) reported maximum xylanase production by *B. pumilus* SV-85S using peptone followed by yeast extract as nitrogen source. However, it is contrary with the report of Sepahy *et al.* (2011) where tryptone in combination with yeast extract gave the maximum xylanase titre. Peptone and yeast extract are multifaceted nitrogen sources rich in diverse growth factors such as minerals and vitamins and hence improve the growth of bacteria and enzyme synthesis (Bibi *et al.* 2014) as few among the inorganic KNO_3 resulted in higher titre among others.

Sepahy *et al.* (2011) stated the pH of the medium has a strong influence on enzymatic processes as it affects the growth and metabolic activities of

individual microorganisms. Influences of pH ranging from acidic to alkaline were tested for xylanase production as *B. megaterium* had the highest production at pH 5.0, indicating that acidic favours this particular organism when acting on Kolanut husk as substrate. The alkalinity or acidity of the medium affect bacteria growth coupled with other enzymatic reactions through transport of ions, metabolites, and enzyme structure (Liang *et al.* 2010). Our results revealed that as the pH of the medium increases from extremely acidic (pH 2.0) to slightly acidic (pH 5.0), so does the enzyme activity increased but tending towards neutral pH, there was a drop in the value and the least value recorded at alkaline pH (Table 6). This report is in contrary with the observations of Sepahy *et al.* (2011); Irfan *et al.* (2016) who reported the highest xylanase synthesis at pH 8.0 using *B. mojanvensis* and *B. subtilis* under submerged fermentation. This could possibly be attributed to the alkaline medium possessing an inhibitory influence on *Lysinibacillus fusiformis* and moreover each microorganism has its pH for optimum growth and enzyme production.
Temperature is another key dynamic that influences the success of optimization system. The best medium temperature was 35 ^{o}C which is close to the optimal temperature for utmost xylanase titre in submerged fermentation (SmF) (Table 7) from other *Bacillus* species reported as 37 °C (Qureshi *et al.* 2002; Battan *et al.* 2007; Geetha and Gunasekaran 2010; Nagar *et al.* 2012) but contradicts the report of 50 °C temperature (Sa-Pereira *et al.* 2002; Azeri *et al.* 2010; Sepahy *et al.* 2011), 50 – 55 °C (Anuradha *et al.* 2007; Sharma *et al.* 2011) and 55 °C (Annamalai *et al.* 2009). This indicates that productions of xylanase are favored at ambient temperature as compared to extremophilic temperature. This could be due to the fact that mesophilic temperature favors the growth of organisms and hence their ability to produce the desired end product. Though, thermophilic temperature is best preferred as it diminishes the risk of mesophilic microbial defects (Yeoman *et al.* 2010). Generally, *Bacillus* sp. shows ability to grow and produce enzymes at 37 ^{o}C under suitable fermentation conditions (Sanghi *et al.* 2008; Nagar *et al.* 2010). The finest temperature for microbial enzyme synthesis may fluctuate as it is linked to their proliferation.
The organism used in this study showed relatively high xylanase production within a short period of incubation which could be attractive for large-scale xylanase production. Xylanase activity was found to decrease with prolonged incubation which could be linked to depletion in the available nutrient as time progresses and accumulation of end-products (Fig. 1). Production of toxic metabolites inhibits enzyme synthesis also (Irfan et al. 2016). Apparently, the increased incubation period affected any xylanase yield but reduced enzyme activity. This appears to be similar to Simphiwe *et al.* (2011) who reported 24-h as an utmost incubation period using digested bran as substrate. In another report (Anuradha *et al.* 2007), xylanase synthesis by *Bacillus* species was growth-related with highest production at 24-h incubation period. However, Prakash *et al.* 2011 reported contrarily with maximum production after 48-h of fermentation with *B. habdurans*. The decline in xylanase production during the later stage of fermentation could be attributed to the liberation of a limited volume of protease from the mature cells penetrating into autolysis or shortage of impenetrable xylan particles in the medium. Limited fermentation time presented better and gainful production which is a favourable condition in large-scale industrial production.
Optimal inoculum size is crucial for preserving balance between growth sizes and obtainable nutrients in order to obtain the utmost yield. The outcome of this study as illustrated in Table 8 is parallel to Irfan *et al.* (2012) where 1.5 % inoculum size as peak of yield using *B. megaterium* on corn cob but in variance with that of Nagar *et al.* (2010) who tested higher inoculum size (1.0 – 5.0 %) for xylanase production. The reduction might be related to the depletion of available nutrients from the fermentation medium as inoculum level increases which could have resulted to decline in enzyme synthesis.

Conclusion

The search for agricultural waste such as Kolanut husk as potential source of enzyme production rather than environmental nuisance is desirable as it reduces cost of production and increases bioconversion of waste to value-added compounds

by economically feasible techniques. The results obtained therein showed that *L. fusiformis* can produce xylanase via Kolanut husk as a substrate under submerged fermentation. Results indicated that cultural and nutritional parameters are paramount to enzyme production. The optimization process is an important pre-requisite to be considered for a cost-effective production and since Kolanut husk is an agro-waste, it could be an alternative to the expensive commercial xylan as substrate especially at large scale.

Acknowledgements

Authors cherish the efforts of the technical staff of the Department of Microbiology, Federal University of Technology, Akure, (FUTA) Nigeria for their technical support in this study.

Conflict of Interest

The authors declare that there is no conflict of interest.

References

Aarti C, Arasu MV, Agastian P (2015) Lignin degradation: A microbial approach. South Indian J. Biol. Sci. 1: 119-127.

Adeyi O (2010) Proximate composition of some agricultural wastes in Nigeria and their potential use in activated carbon. J. Appl.Sci. Environ. Manag. 14: 1.

Annamalai N, Thavasi, R, Jayalakshmi S, Balasubramanian T (2009) Thermostable and alkaline tolerant xylanase production by *Bacillus subtilis* isolated from marine environment. Indian J. Biotechnol. 8: 291-297.

Anuradha P, Vijayalaxmi K, Prasanna ND, Sridevi K (2007) Production and properties of alkaline xylanases from *Bacillus* sp. isolated from sugarcane field. Curr. Sci. 90: 1283-1286.

Azeri C, Tamer AU, Oskay M. (2010) Thermo-active cellulase-free xylanase production from alkaliphilic *Bacillus* strains using various agro-residues and their potential in bio bleaching of kraft pulp. Afr. J. Biotechnol. 9: 063-072.

Bajaj BK, Singh NP (2010) Production of xylanase from an alkalitolerant *Streptomyces* sp. 7b under solid state fermentation, its purification and characterization. Appl. Biochem. Biotechnol. 162: 1804-1818.

Battan B, Sharma J, Dhiman SS, Kuhad RC (2007) Enhanced production of cellulase-free thermostable xylanase by *Bacillus pumilus* ASH and its potential application in paper industry. Enzyme Microb. Technol. 41: 733-739.

Battan B, Sharma J, Kuhad RC (2006) High level xylanase production by alkaliphilic *Bacillus pumilus* ASH under solid state fermentation. World J. Microb. Biotechnol. 22: 1281-1287.

Beg QK, Kapoor M, Mahajan L, Hoondal GS (2001) Microbiology xylanases and their industrial applications: a review. Appl. Microbiol. Biotechnol. 56: 326-338.

Bhalla A, Bischoff KM, Sani RK (2015) Highly thermostable xylanase production from a thermophilic *Geobacillus* sp. Strain WSU utilizing lignocellulosic biomass. Front. Bioeng. Biotechnol. 3: 84.

Bhardwaj N, Kumar B, Verma P (2019) A detailed overview of xylanases: an emerging biomolecule for current and future prospective. Bioresour. Bioprocess. 6: 23-37.

Bibi Z, Asma A, Raheela RZ, Shah AUQ, Afsheen A (2014) Production of xylan degrading endo-1,4-β-xylanase from thermophilic *Geobacillus stearothermophilus* KIBGE-IB29. J. Radiat. Res. Appl. Sci. 8: 1-8.

Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye binding. Anal. Biochem. 72: 248-254.

Chakdar HM, Kumar M, Pandiyan K, Singh A, Nanjappan K, Kashyap PL, Srivastava AK (2016) Bacterial xylanases: biology to biotechnology. 3 Biotechnol. 6: 150.

Chan XY, Hong KW, Yin WF (2016) Microbiome and biocatalytic bacteria in monkey cup (*Nepenthes* Pitcher) digestive fluid. Sci. Rep. 6: 2016.

Coman G, Bahrim G (2011) Optimization of xylanase production by *Streptomyces* sp. P12-137 using response surface methodology and central composite design. Ann. Microbiol. 61: 773-779.

De Graaff LH, Van den Broeck HC, Van Ooijen AJJ, Visser J (1994) Regulation of the xylanase encoding xlnA gene of *Aspergillus tubigensis*; Mol. Microbiol. 21: 1273-1281.

Fabunmi TB, Arotupin DJ, Adegunloye DV (2018) Study of bacterial associated with Kolanut soil plantation and waste looking at their benefit to man and his environment. Niger. J. Technol. 37: 1128-1136.

Garg P, Mahatman KK, Kumar A (2010) Xylanse: applications and biotechnological aspects. Saarbruckeri: Lambert Academic Publishing AG and CO, 60 p.

Gautério GV, da Silva LGG, Hübner T, da Rosa Ribeiro T, Kalil SJ (2020) Maximization of xylanase production by *Aureobasidium pullulans* using a by-product of rice grain milling as xylan source. Biocat. Agric. Biotechnol. 23: 101511.

Geetha K, Gunasekaran P (2010) Optimization of nutrient medium containing agricultural waste for xylanase production by *Bacillus pumilus* B20. Biotechnol. Bioprocess. Eng. 15: 882-889.

Girisha M, Shilpa SC (2018) Production and applications of xylanase-an overview. Biotechnologia 99: 59-72.

Heskia K, Mubarik NP, Palar R, Pugoh SY (2018) Optimization of process parameters and scale up of xylanase production using corn cob raw biomass by marine

bacteria *Bacillus subtilus* LBF M8 in stirred tank bioreactor. Pakistan J. Biotechnol. 15:707-714.

Irfan M, Nadeem M, Nelofer R, Syed Q (2016) Optimization of process parameters for xylanase production by *Bacillus* sp. In submerged fermentation. J. Radiat. Res. Appl. Sci. 9: 139-147.

Irfan M, Nadeem M, Syed Q, Baig S (2012) Effect of medium composition on xylanase production of *Bacilllus subtilis* using various agricultural wastes. Am-Eurasian J. Agric. Environ. Sci. 12: 561-565.

Karim A, Nawaz MA, Aman A, Qader SA (2015) Hyper production of cellulose degrading endo (1,4) β-d-glucanase from *Bacillus licheniformis* KIBGE-IB2. J. Radiat. Appl. Sci. 8: 160-165.

Kartik VP, Jinal HN, Amaresan N (2016) Characterization of cadmium-resistant bacteria for its potential in promoting plant growth and cadmium accumulation in *Sesbania bispinosa* root. Int. J. Phytoremed. 2016: 1-17.

Khusro A, Kaliyan BK, Al-Dhabi NA, Arasu MV, Agastian P (2016) Statistical optimization of thermo-alkali stable xylanase production from *Bacillus tequilensis* strain. Electron. J. Biotechnol. 22: 16-25.

Liang Y, Feng Z, Yesuf J, Blackburn JW (2010) Optimization of growth medium and enzyme assay conditions for crude cellulases produced by a novel thermophilic and cellulolytic bacterium, *Anoxybacillus* sp. 527. Appl. Biochem. Biotechnol. 160: 184-185.

Mandal, A (2015) Review on microbial xylanases and their applications. Int. J. Life Sci. 4: 178-187.

Miller GL (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar. J. Chem. Anal. 31: 538-542.

Nagar S, Gupta VK, Kumar L, Kuhad RC (2010) Production and optimization of cellulase-free, alkali-stable xylanase by *Bacillus pumilus* SV-85S in submerged fermentation. J. Indian Microbiol. Biotechnol. 37: 71-83.

Nagar S, Jain RK, Thakur VV, Gupta VK (2013) Biotechnological application of cellulase poor and alkali stable xylanase by *Bacillus pumilus* SV-85S in bio-bleaching. 3 Biotechnol. 3: 277-285.

Nagar S, Mittal A, Gupta VK (2012) Enzymatic clarification of fruit juices (apple, pineapple and tomato) using purified *Bacillus pumilus* SV-85S xylanase. Biotechnol. Bioprocess. Eng. 17: 1165-1175.

Patel K, Dudhagara P (2020a) Compatibility testing and enhancing the pulp bleaching process by hydrolases of the newly isolated thermophilic *Isoptericola variabilis* strain UD-6. Biocatal. Biotransf. 38: 144-160.

Patel K, Dudhagara P (2020b) Optimization of xylanase production by *Bacillus tequilensis* strain UD-3 using economical agricultural substrate and its application in rice straw pulp bleaching. Biocatal. Agric. Biotechnol. 30: 101846.

Prakash S, Veeranagouda Y, Kyoung L, Sreeramulu K (2011) Xylanase production using inexpensive agricultural wastes and its partial characterization from a halophilic *Chromohalobacter* sp. TPSV 101. World J. Microbiol. Biotechnol. 25: 197-204.

Qureshy AF, Khan LA, Khanna S (2002). Cloning, regulation and purification of cellulose free xylanase from *Bacillus circulans* Teri-42. Indian J. Microbiol. 42: 35-41.

Raghukumar C, Muraleedharan U, Gaud VR, Mishra R (2004) Xylanase of marine fungi of potential use for bio-bleaching of paper-pulp. J. Indian Microbiol. Biotechnol. 31: 433-441.

Ramanjaneyulu G, Ramya A, Shanthi KB, Dileep KK (2015) Isolation and screening of xylanase producing fungi from forest soils. Int. J. Curr. Microbiol. Appl. Sci. 4: 586-591.

Ratnadewi AAI, Zain MHA, Kusuma ANN, Handayani W, Nugraha AS, Siswoyo TA (2020) *Lactobacillus casei* fermentation towards xylooligosaccharide (XOS) obtained from coffee peel enzymatic hydrolysate. Biocatal. Agric. Biotechnol. 23: 101446.

Sanghi A, Garg N, Sharma J, Kumar K, Kumar RC, Gupta VK (2008) Optimization of xylanase production using inexpensive agro-residues by alkalophilic *Bacillus subtilis* ASH in solid state fermentation. World J. Microbiol. Biotechnol. 24: 633-640.

Sa-Pereira P, Mesquita A, Duarte JC, Barros MRA, Costa-Ferreira M (2002) Rapid production of thermostable cellulase free xylanase by a strain of *Bacillus subtilis* and its properties. Enzyme Microbiol. Technol. 30: 924-933.

Sari SA, Pangastuti A, Susilowati A, Purwoko T, Mahajoeno E, Hidayat W, Panuntun FD, Kurniawati D, Anitasari R. (2016) Cellulolytic and hemicellulolytic bacteria from the gut of *Oryctsrhinoceros* larvae. Biodiversity 17: 78-83.

Sepahy AA, Ghazi S, Sepahy MA (2011) Cost-effective production and optimization of alkaline xylanase by indigenous *Bacillus mojavensis* AG 137 fermented on agricultural waste. Enzyme Res. 2011: 593624.

Seyis I, Akzoz N (2005) Effect of carbon and nitrogen sources on xylanase production by *Trichoderma harzianum* 1073 D3. Int. Biodeter. Biodegr. 55: 115-119.

Shah AR, Mandamwar D (2005) Xylanase production by a newly isolated *Aspergillus foetidus* strain and its characterization. Process Biochem. 40: 1763-1771.

Sharma P, Bajaj BK (2005) Production and partial characterization of alkali-tolerant xylanase from an alkalophilic *Streptomyces* sp. CD3. J. Sci. Ind. Res.64: 688-697.

Sharma SCD, Shovon MS, Asaduzzaman AKM, Sarowar-Jahan MG, Yeasmin T, Roy N (2011) Optimization of alkali-thermostable and cellulase-free xylanase production from *Bacillus* sp. J. Bio-Sci. 19: 7-14.

Simphiwe P, Ademola B, Olaniran O, Pillay B (2011) Sawdust and digestive bran as cheap alternate substrate for xylanase production. Afr. J. Microbiol. Res. 5: 742-752.

Singh S, Dutt D, Tyagi CH, Upadhyaya JS (2011) Bio-conventional bleaching of wheat straw soda AQ pulp with crude xylanases from SH- 1 NTCC-1163 and SH-2 NTCC-1164 strains of *Copimellus dissenminatus* to mitigate AOX generation. New Biotechnol. 21: 233-243.

Sridevi A, Sandhya A, Ramanjaneyulu G, Narasimha G, Devi PS (2016) Bio-catalytical activity of *Aspergillus* niger-xylanase in paper pulp bio-bleaching. 3 Biotech. 6: 1-7.

Tandon P, James MT, Abraldes JG, Karvellas CJ, Ye F, Pannu N (2016) Relevance of new definitions to incidence

and prognosis of acute kidney injury in hospitalized patients with cirrhosis: A retrospective population-based cohort study. PLoS One. 11: e0160394.

Uday USP, Choudhury P, Bandyopadhyay TK, Bhunia B (2016) Classification, mode of action and production strategy of xylanase and its application for biofuel production from water hyacinth. Int. J. Biol. Macromol. 82: 1041-1054.

Walia A, Mehta P, Guleria S, Shirkot CK (2015) Improvement for enhanced xylanase production by *Cellulosimicrobium cellulans* CKMXL using central composite design of response surface. Biotechnol. 5: 1053-66.

Yeoman, CJ Han, Y Dodd, D Schroeder, CM Mackie, RI, Cann IK (2010) Thermostable enzymes as biocatalyst in the biofuel industry. Adv. Appl.Microbiol. 70:1-55.