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Effect of Agro-industrial Residue Composition on the Production of Endo-1, 4-β-Xylanase by *Bacillus pumilus*

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ABSTRACT

Background: *Bacillus pumilus* grows on different substrates and produces Endo-1, 4-β-xylanase (EXase) which degrades the hemicellulose and produces xylooligosaccharides. EXase found applications in paper, pulp and in food industries. The objective of this study is to investigate the compositional effect of lignocellulosic biomass as substrate for the production of EXase by *Bacillus pumilus*.

Methods: The EXase production depends on the composition of substrate (agro-industrial residue), *Bacillus pumilus,* media composition and performance of fermentation method. Thus, we have evaluated the effect of composition on the production of EXase. The higher content of hemicellulose of substrate indulges EXase production. Moreover, ICP-MS, FTIR spectroscopy, XRD and SEM analyses confirmed that significant alternation of substrate structure by *Bacillus pumilus* due to the EXase actions.

Result: Based on the results of the hydrolytic enzyme activation and characterization studies suggested that *Bacillus pumilus* produced EXase highest for sugarcane bagasse 1190 U/mg by degrading the sugarcane bagasse as a low-cost carbon sources and effective media composition (Minimal Salt Yeast Extract Nutrient Medium) for nitrogen source, also with the presence of metal ions such as Cu^{++}, Fe^{++}, Mn^{++} for increased the production of EXase.

Key words: *Bacillus sp*., Endoxylanase, Lignocellulosic biomass, Rice straw, Sugarcane bagasse.

INTRODUCTION

Agro-industrial residues (AIR) are significantly produced by agro-industrial processes and are abundant carbon sources for the production of enzyme (Malik *et al*., 2021). AIR consists of lignin (25-30%), cellulose (35-50%) and hemicellulose (25-30%) (Bhardwaj *et al*., 2019). Production of enzyme using AIR is achieved more interest worldwide. Globally, industrial enzyme production market is 6 billion USD in 2017 and enzyme-based products are used in the field of food and beverages (Karinkanta *et al*., 2018; Ponce *et al*., 2021).

Furthermore, the enzyme production depends on the crystalline nature of cellulose, hemicellulose and lignin (Saroj *et al*., 2018). However, the complex non-porous structure of AIR is limited the hydrolysis effectively and also leading to low degradation of cellulose/hemicellulose for enzyme production (Ayeni *et al*., 2015). To overcome this, utilizing proper methodologies to characterize the degradation process before and after fermentation will help to enhance the enzyme production. The physicochemical characterization of AIR compositions and media composition is mainly aimed to breakdown the hemicellulose/lignin, cellulose and enhance the surface area of residues for its availability to microorganisms for enzyme production (Jantasila, 2012). Therefore, we hypothesize to investigate the compositional effects of media and AIR (sugarcane bagasse (SB), corncob (CC), rice straw (RS) and sawdust (SD)) as substrate for the production of Endo-1, 4-β-xylanase (EXase) by *Bacillus pumilus*.

In this study, we demonstrated the evaluation of compositional effect of AIR and media composition on the production of EXase by *B. pumilus*. Four different AIR substrates such as SB, CC, RS and SD as carbon source have been used with nitrogen rich media composition in the presence of metal ions such as Cu^{++}, Fe^{++}, Mn^{++} for the production of EXase *via* shake flask method at 37°C and neutral pH. The effect of bacterial growth on AIR substrates and its hydrolytic degradation of cellulose/hemicellulose for the production of EXase were determined using different characterization tools such as ICP-MS, FESEM/EDX, FTIR and XRD. The structural and elemental changes (physiochemical changes) of AIR before and after fermentation was investigated and analyzed to understand the mechanism involved to enhance the production of EXase by *Bacillus pumilus*.

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MATERIALS AND METHODS

Microorganism and culture conditions

B. pumilus (MTCC) 10209 was initially grown on nutrient agar medium. The seed culture was produced by inoculating one loop full of *B. pumilus* culture to 50 mL nutrient broth and cultured overnight at 37°C, 120 rpm. *B. pumilus* culture (5.6×10^5 cells per mL) was inoculated to 100 mL MS-YENM containing (g/L): Yeast extract (6.0), $(NH_4)2SO_4$ (0.1), NaCl (0.3), $MgSO_4$ (0.1), $CaC0_3$ (0.02) and 1 mL trace-elements solution consists of $FeSO_4$ (1), $ZnSO_4$ (0.9), $MnSO_4$ (0.2) (50mM sodium phosphate buffer, pH 7) (Adiguzel, 2017). The shake flask studies were adopted at 37°C and 120 rpm for 5 days by using sterile, dried and uniform particle size (mesh size 20) AIR (obtained from Davangere, Karnataka, India *viz.*, SB, CC, RS and SD) of 50 g/L as carbon source for the production of EXase and compared with the standard BWX (Mohlala *et al*., 2016). All experiments were carried out at Siddaganga Institute of Technology, Tumkur, Karnataka, India, during the period from October 2021 to July 2022.

One unit of EXase activity was defined as: the volume of enzyme required to release 1 µmol of reducing sugar (xylose) per minute under the standard assay conditions. The reducing sugar and protein concentration was quantified as described in the previous protocol with few modifications (Adiguzel, 2017). Specific activity was determined according to Javed *et al*., 2017.

Evaluation of Lignocellulosic contents

The AIR *viz*., SB, SD, RS and CC collected were washed with tap water and then with distilled water. This removes the dust particles. The samples were then autoclaved at 121°C, 15 lbs for 15 minutes to prevent microbial contamination. The sterilized agro-industrial waste samples were air dried for 2 h and oven dried for overnight at 60°C. The dried agro-industrial waste samples were milled, screened with 20-mesh to get uniform sized particles and then stored in glass containers which were kept in desiccators to prevent absorption of surrounding moisture for further analysis (Adiguzel, 2017). The composition of AIR (hemicellulose, lignin and cellulose) was determined as described in previous reports (Ayeni *et al*., 2013; Sirisha, 2019) and were as follows.

Hemicellulose

1 g of processed biomass was merged in 450 mL of 0.5 M NaOH and boiled for 3.5 h at 28°C (room temperature). This treatment increases the heating effect and minimizes the lime scales. Next, obtained processed biomass was filtered and washed with distilled water several times to attain neutral pH. Finally, processed biomass was dried in a convection oven at 105°C to attain constant weight. Then, percentage hemicellulose content (%w/w) was evaluated by the difference between weight of AIR before and after this treatment (Ayeni *et al*., 2013).

Lignin

1 g of dried processed biomass was merged in 20 mL of 72% H_2SO_4 and kept at 25°C for 2 h. The biomass samples were shaken at an interval of every 20 minutes to initialize the hydrolysis process. Next, distilled water was added to dilute the solution to 3% H_2SO_4 and subjected for complete hydrolysis by autoclaving at 121°C for 1 h. Finally, slurry was cooled to room temperature and filter the hydrolysates to separate the filtrate and insoluble solids. The insoluble solids were washed with distilled water to remove the traces of sulphuric acid. Finally, the insoluble solids were dried at 105°C to attain constant weight and this indicated the presence of acid insoluble lignin (AIL). The obtained filtrate consists of acid soluble lignin (ASL) and was determined by measuring the absorbance at 280 nm and 215 nm via Klason method. Quantification of lignin was determined by the sum of ASL and AIL content (Sirisha, 2019).

Cellulose

The cellulose content (%w/w) of the processed biomass was determined gravimetrically by adding 15 mL acetic acid and 1.5 mL nitric acid to 3 g of biomass. The sample was refluxed for 20 minutes, then washed with ethanol and finally filtered using whatman filter paper. The obtained biomass solids were dried in over at 105°C overnight to the constant weight. The cellulose content was determined by calculating the difference between initial and final weight (Sirisha, 2019).

Physicochemical measurement of unfermented and fermented AIR

All the samples of AIR before and after fermentation were air-dried and were evaluated with different characterization tools such as ICP-MS, FESEM/EDX, XRD and FTIR for analysis of physicochemical changes (Ameram *et al*., 2019; Evans *et al*., 2019; Gloth, 2013; Hu Chunyan *et al*., 2020; Su *et al*., 2018). For FESEM/EDX, samples were analysed by ultra high-resolution field emission scanning electron microscope (Carl ZEISS, SUPRA ULTRA 55, GEMINI Technology, German) at Center for Nano Science and Engineering (CeNSE), Indian Institute of Science (IISc), Bangalore, India. FTIR analysis was carried out using OPUS 7.5.18 software with frequency range 4000-400 cm^{-1} of FTIR spectroscopy (PerkinElmer, USA) at Chemistry research center, Siddaganga Institute of Technology (Tumkur, India). The obtained spectra were analyzed and quantified for surface functional group changes using reference peaks in the literature. The crystallinity index of all the samples before and after fermentations were analysed by XRD using Ultima IV apparatus (Rigaku Corporation, Japan) at Chemistry research center, Siddaganga Institute of Technology. The dried samples were analyzed with 2θ (scattering angle) between 20° and 30° at a scanning speed of 2°/min. The crystallinity index was calculated using standard formula available in literature. ICP-MS was carried out for all samples prepared by wet acid digestion method(PEQUERUL, 1993)

and analysis using Thermoscientific iCAP RQ(RQ01067) instrument at Chemical Engineering Department, Siddaganga Institute of Technology (Tumkur, India) (Hu *et al*., 2020) compared with standard.

RESULTS AND DISCUSSION

The large-scale production of enzyme in industrial sectors for many product developments is mainly depend on the cost of raw materials and downstream processing. Therefore, cost of the enzyme production can be reduced by appropriate selection of AIR as a carbon source, which can affect the final cost of the products. Furthermore, it is necessary for the investigation of composition and structural changes of AIR as substrate and suitable media composition consumed by *Bacillus pumilus* for the production of EXase. Thus, in this study we have investigated the effect of AIR and media composition on the production of EXase by *Bacillus pumilus* which may reduce the production cost.

Optimize the selection of AIR and media composition for EXase by *Bacillus pumilus*

Previous studies demonstrated that EXase production by microorganisms such as yeasts, fungi and bacteria using low-cost, abundant AIR as carbon source (wheat bran, wheat straw, RS, CC) (Namnuch *et al*., 2021; Sousa *et al*., 2018). Thus, the present study demonstrates the production of EXase by *B. pumilus* using SB, CC, RS and SD. *Bacillus* sp. produce significant amount of their enzymes by degrading hemicellulosic component of AIR. *B. pumilus* (MTCC) 10209 was grown at 37°C for 24 h, maintained in nutrient agar plates and for every fifteen days sub-culturing was done. AIR (CC, SD, SB and RS) was used as carbon source supplemented with MS-YENM at 37°C, 120 rpm for 5 days *via* shake flask method for the production of EXase by *B. pumilus*. The EXase specific activity (Fig 1) was maximum for SB (1190 U/mg) followed by CC (653 U/mg), SD (495 U/mg), RS (502 U/mg) and BWX (461 U/mg) respectively (Fig 1). The hemicellulose of SB consists of branched heteropolymers (xylose and arabinose) and hexoses (mannose, galactose, uronic-acids and glucose) (Kandiyil *et al*., 2018). The SB together with yeast extract (nitrogen source) (Muslim *et al*., 2013) and divalent metal ions such as Cu^{++}, Fe^{++} and Mn^{++} in MS-YENM medium contributes for highest EXase production compared to other carbon sources. Thus, SB may be an alternative for BWX for the large scale-up of EXase production to reduce the cost of production.

Physicochemical characterization of AIR before and after fermentation

The structural, compositional changes and media composition required for the EXase production by *Bacillus pumilus* using AIR were confirmed by FESEM/EDX, FTIR, ICP-MS and XRD analysis. Some of the studies demonstrated the compositional and structural changes for the production of EXase (Namnuch *et al*., 2021). An example demonstrated that 28.49% wheat straw saccharification rate utilized by bacteria *Ochrobactrumoryzae* BMP03 for the xylanase production (Tsegaye *et al*., 2018). Another study showed that 54.38% biomass saccharification rate used by bacteria, *B. subtilis* K18 (Irfan *et al*., 2017). Thus, we investigated the Physicochemical characterization of AIR before and after fermentation.

Utilization of AIR for the production of EXase

The compositional analysis of the AIR were determined by gravimetric analysis [Fig 2 (n = 3)]. The AIR compositions is mainly depending on the type of AIR *i.e* either woody or non-woody, locations of the materials, procedures used for analysis and part of the plant. In this study, we have measured the cellulose (Fig 2A), hemicellulose (Fig 2B) and lignin content (Fig 2C) of AIR utilized by *Bacillus pumilus* for the production of EXase. Even though, the cellulose and hemicellulose content were less in SB compare to CC (Fig 2),

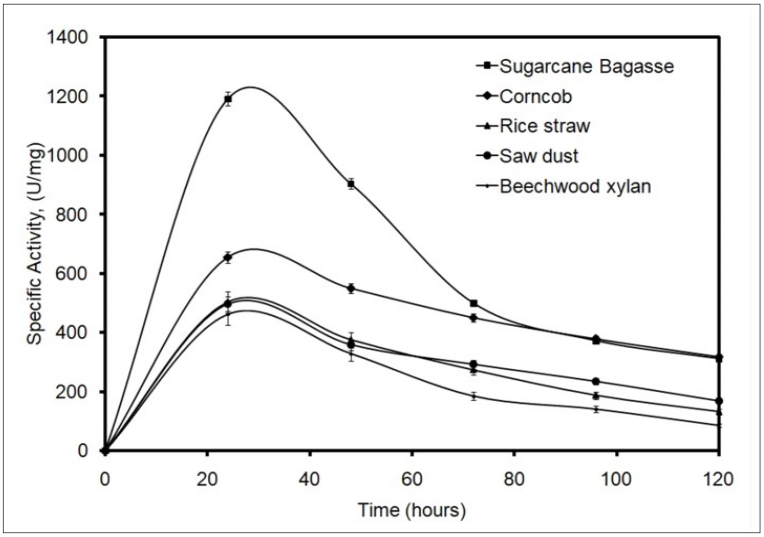
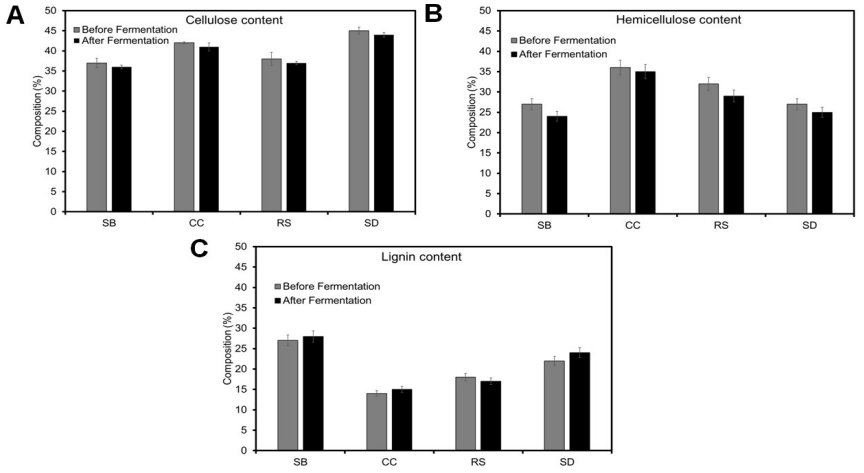


Fig 1: Production of EXase from *B. pumilus* using AIR.

SB has given the highest EXase production. However, lignin value found to be increased as it was not utilized by bacteria. Thus, along with composition of AIR, the structural and compositional change of AIR was investigated using FESEM/EDX, FTIR, ICPMS and XRD diffraction.

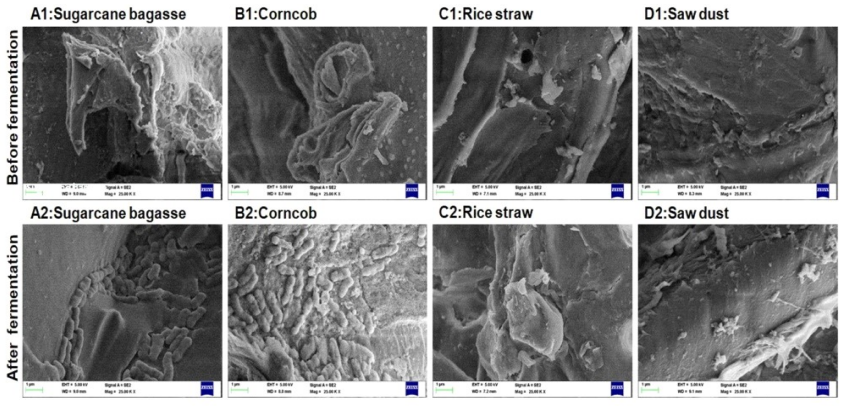
Morphological and elemental analysis of AIR using FESEM/EDX

FESEM was carried out to measure the morphological and elemental changes of all AIR (Before and after fermentation). During this analysis, we observed that bacterial deposition on all of the residues after fermentation with flaky, rigid and distortion of the surfaces (Fig 3A2-3D2) compared to before fermentation (Fig 3A1-3D1). The disruption and disorganized structures of the agro-industrial residue substrate surface indicated that bacteria utilized the substrate for the EXase production. We have observed large pores on the surface of the AIR substrate and this indicated that alternation in the hemicellulose/cellulose/lignin contents. Therefore, elemental changes of all AIR were measured using EDX (Fig 4A-4D). This analysis indicated that changes in the carbon, nitrogen and oxygen contents of all AIR before and after fermentation. Utilization of elements such as carbon, nitrogen and oxygen from AIR for the production of EXase by *Bacillus pumilus* indicated that reduction in the elements after fermentation and hydrolytic activity for the degradation of substrate to enhance the production rate. A study on structural changes of raw wheat straw for the production of xylanase was observed and showed compact, rough and disorganized structures for wheat straw (Tsegaye *et al*., 2018).



A): Cellulose composition of AIR was found highest in SD and low in SB, B): Hemicellulose composition of AIR was found highest in CC and low in SB, C): Lignin composition of AIR was found highest in SB and low in CC.

Fig 2: Fibrous compositional changes of all AIR.



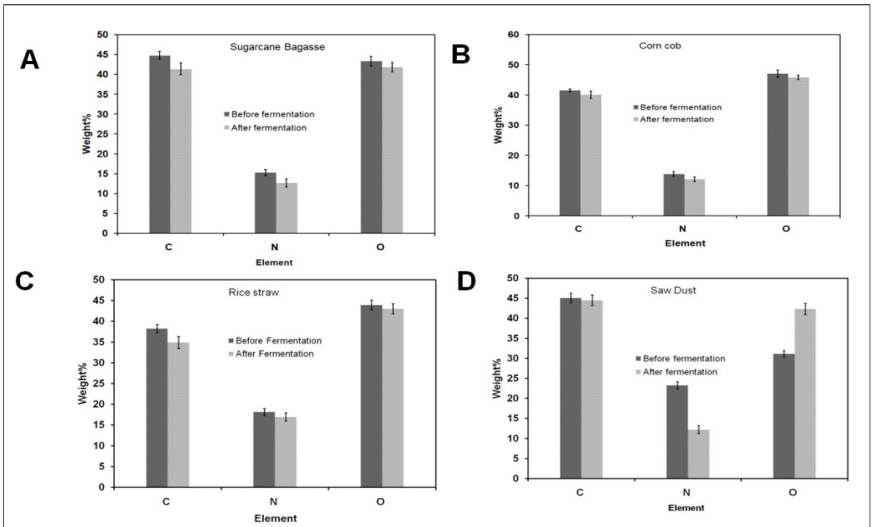
FESEM images of AIR before fermentation (A1: SB, B1: CC, C1: RS, D1: SD) and after fermentation (A2: SB, B2: CC, C2: RS, D2: SD) indicated the change in the surface structure and utilization of AIR for the production of EXase. All the images were imaged at 25 kX magnification.

Fig 3: Structural changes of AIR.

However, this study has not showed the elemental changes before and after treatment. In our study, we have studied both structural and elemental changes before and after fermentation of AIR. Furthermore, we have investigated the elemental, functional group and crystallinity index changes of AIR.

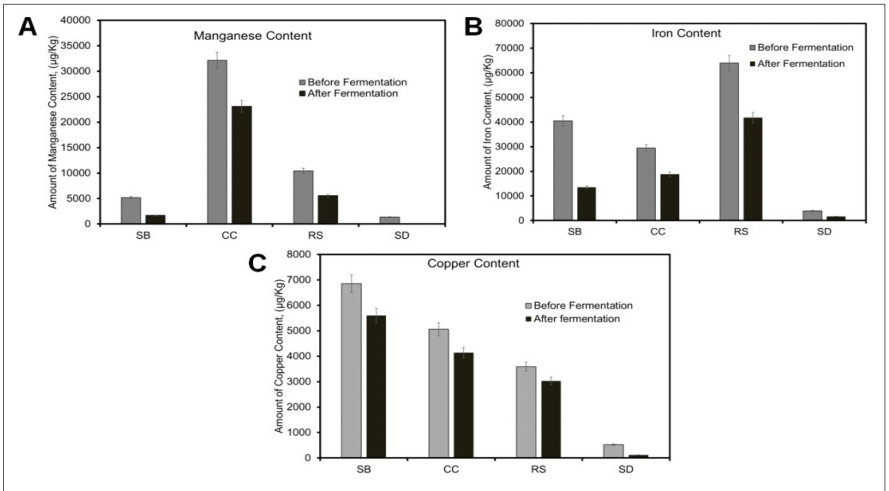
Elemental analysis of AIR using ICP-MS

In present study, we have demonstrated the elemental analysis using ICP-MS for all the AIR to measure the changes in elements before and after fermentation. ICP-MS analysis indicated the changes in divalent metal ions changes (Cu^{++}, Fe^{++} and Mn^{++}) for production of EXase by *Bacillus pumilus* (Fig 5A-5C). This analysis demonstrated that only using AIR not significant for the production of EXase. The media composition is also important for the EXase production. The presence of Cu^{++}, Fe^{++} and Mn^{++}in the Minimal Salt Yeast Extract Nutrient Medium (MS-YENM) as nitrogen source has increased the production of EXase. This investigation showed that enzyme production required divalent metal ions as cofactors in order to protect the enzyme denaturation during production and also maintaining the stability of enzyme to overcome the temperature sensitive. Our studies were correlated with Sinnela *et al*. (2021) studies. Thus, we have confirmed that media composition with divalent metal ions also plays a critical role for the production of EXase using AIR by *Bacillus pumilus*.



The figure shows weight % of carbon, nitrogen and oxygen levels in A): Sugarcane bagasse, B): Corn cob, C): Rice straw, D): Saw dust.

Fig 4: Elemental analysis of AIR by Energy X-ray diffraction (EDX).



Composition analysis of AIR before and after fermentation indicated the changes in the elemental levels of: A): Manganese content, B): Iron content, C): Copper contents on µg/Kg basis.

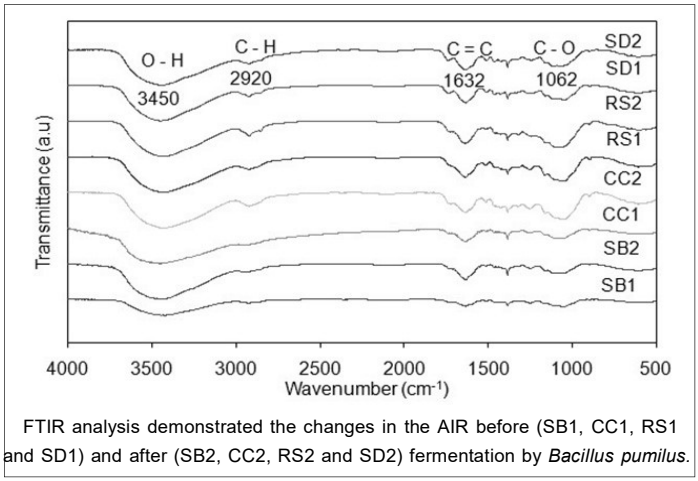
Fig 5: Elemental analysis of AIR using ICP-MS.

Functional group changes of AIR using FTIR analysis

The analysis of chemical and functional group changes using FTIR was carried out for all the AIR before and after fermentation. Fig 6 demonstrated that broad band absorption between 3250 and 3500 cm^{-1}, which exhibited the greatest intensity related to O-H stretching vibration. This indicated the presence of lignin, cellulose and hemicellulose of all AIR. The broadening of the peaks of -OH stretching before and after fermentation indicated the utilization of fibers for the EXase production by *Bacillus pumilus.* Another peak at 2920 and 2910 cm^{-1} depicting the C-H stretching which indicated lignin/ cellulose/ hemicellulose. Similarly, the absorption peaks at 1632 and 1062cm^{-1}indicated the C=C and C-O stretching around the benzene and aromatic ring of cellulose/hemicellulose/lignin, respectively (Table 1) (Ponce *et al*., 2021; Pu *et al*., 2016; Tsegaye *et al*., 2018). However, disappearance or reduction in broadening of the peaks indicated changes in the functional groups and also intra-molecular changes of the cellulose/hemicelluloses.

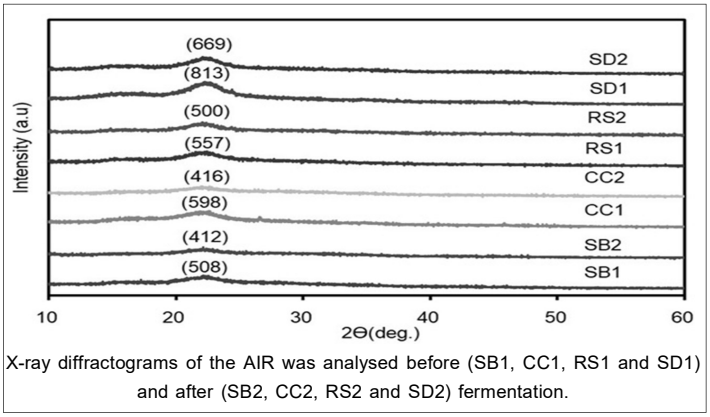
Crystallinity index changes of AIR using X-ray diffraction (XRD) analysis

The changes in the crystallinity index of cellulose/ hemicellulose is an effective factor to investigate the effect of saccharification involved to increase crystallinity percentage by *Bacillus pumilus* for the EXase production using AIR. Therefore, we have measured the crystallinity index changes before and after fermentation of all AIR. Fig 7 indicated the XRD patterns of SB, CC, RS and SD before



FTIR analysis demonstrated the changes in the AIR before (SB1, CC1, RS1 and SD1) and after (SB2, CC2, RS2 and SD2) fermentation by *Bacillus pumilus.*

Fig 6: Functional group changes in AIR.



X-ray diffractograms of the AIR was analysed before (SB1, CC1, RS1 and SD1) and after (SB2, CC2, RS2 and SD2) fermentation.

Fig 7: % Crystallinity index changes in the AIR.

Table 1: Absorbance bands in FTIR spectrum of various AIR.

Wavenumber (cm^{-1})	Assignment	Compounds	Polymers	References
1060	C-O stretching and C-O deformation	Alcohol	Cellulose hemicellulose	(Evans *et al*., 2019; Pu *et al*., 2016; Tsegaye *et al*., 2018)
1632	C=C	Benzene ring	Lignin	
2970-2860	C-H stretching	Alkyl, aliphatic, aromatic	Lignin, cellulose, hemicellulose	
3600-3000	O-H stretching	Acid-Alcohol	Lignin, cellulose, hemicellulose	

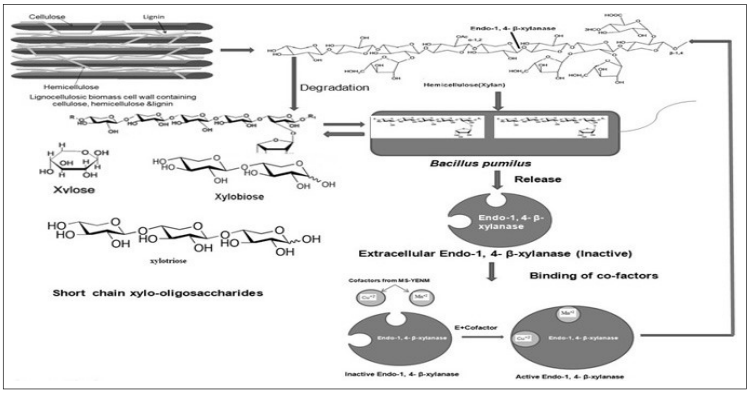


Fig 8: Mechanism of utilization of components as cofactors by EXase of *B. pumilus* to degrade xylan and to produce short chain xylo-oligosaccharides.

Table 2: Intensity change in X-ray diffraction of AIR.

AIR	Highest intensity		Crystallinity index %
	Before fermentation	After fermentation	
SB	508	412	18.88
CC	598	416	30.43
RS	557	500	11.4
SD	813	669	21.52

and after fermentation. After fermentation, the amorphous regions of the AIR were hydrolyzed, leading to the gradual decreased in the peaks after fermentation (SB-18.88%, CC-30.43%, RS-11.4% and SD-21.52%), respectively. These results indicated that depolymerization and degradation of hemicellulose/cellulose contents from AIR for EXase production by *Bacillus pumilus.* The reduction in the intensity of XRD peaks represented the disruption of intra- and inter-chain hydrogen bonds of cellulose/hemicelluloses contents. Our AIR showed that characteristic XRD peaks at 2θ of 20-30° (degree) related to the crystalline region and amorphous regions (Ponce *et al*., 2021). The decrease in peak values and changes in the crystallinity index percentage indicated the deformation of AIR after fermentation for EXase production by *Bacillus pumilus* (Table 2).

Mechanism involved in the EXase production using AIR and media composition

Based on this study, we have observed that maximum production of EXase using SB as substrate by *B. pumilus* MTCC 10209. The EXase production is depend on the both physical parameters such as pH, temperatures and chemical parameters such as composition of AIR, media composition with presence of divalent metal ions as cofactors. These parameters are important to enhance the EXase production using low-cost AIR. The mechanism of degradation of substrate utilized by the bacteria for the production of enzyme has been described in the Fig 8. Usually, divalent metal ions, media composition and AIR composition have been utilized for bacterial growth and produced of EXase.

Conclusion

In the present study, we have investigated the significant production of EXase from *B. pumilus* MTCC 10209 using AIR as carbon source and media composition as nitrogen source with the presence of divalent metal ions. Our study demonstrated the physiochemical characterization of AIR. The structural and compositional changes before and after fermentation analyzed by all the characterization tools showed the hydrolytic degradation of AIR by *B. pumilus* to produce EXase at 37°C, pH-7. It is evident from the structural analysis that *B. pumilus* hydrolyses the substrates to produce enzyme. Even though, composition analysis demonstrated high content of cellulose/hemicellulose in corn cob compared to sugarcane bagasse. Higher EXase production was observed by SB due to the presence of significant divalent metal ions in media composition contributed for the production. Thus, our investigation demonstrated the mechanism involved in the higher production of enzyme depend on the AIR substrate as carbon source, media composition as nitrogen source with presence of divalent metal ions and specific micro-organism bacteria.

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Conflict of interest: None.

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