

Utilization of Egg Shell Waste in Cellulase Production by *Neurospora crassa* under Wheat Bran-Based Solid State Fermentation

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Abstract

A wide variety of waste resources are available on our planet. Egg shell waste is available in huge quantities from the food processing, egg breaking, and the hatching industries. Calcium plays a vital role in *Neurospora crassa* hyphal growth. Batch experiments have been performed using pure calcium salt as well as calcium in the form of pretreated egg shell waste in concentrations of 0.4, 0.8, and 1.2 g/l, under wheat bran-based solid state fermentation by *Neurospora crassa* at 30°C with initial pH 6.0. The present paper describes the utility of wheat bran and egg shell waste in cellulase production. The utilization of economically cheap and abundantly available egg shell waste for cellulase production could be a novel and valuable approach in solid waste management.

Keywords: calcium, cellulase, egg shell waste, *Neurospora crassa*, waste management

Introduction

The bioconversion of waste to usable energy is part of the utilization of waste. Thus the possibility of producing a useful product from wastes will greatly enhance and ensure sustainable economic development as well as waste management problems worldwide [1]. The effective treatment and utilization of biowaste has been emphasized in our society for environmental and economic concerns. Recently, egg shell wastes in the poultry industry have been highlighted because of its reclamation potential [2]. Egg shell waste is available in huge quantities from the food processing, egg breaking, and hatching industries. About 250,000 tons of egg shell waste is produced annually worldwide by the food processing industry only. Fresh egg shell waste is approximately 95% calcium carbonate crystals stabilized by a protein matrix [3]. Uronic acid, sialic

acid, alanine, and glycerol were high in the organic matter of egg shell waste compared to shell membranes [4]. Most good quality egg shell waste shell contains approximately 2.2 g of calcium in the form of calcium carbonate. The average egg shell contains about 95% of calcium carbonate, 0.3% phosphorus, 0.3% magnesium, and traces of sodium, potassium, Zn, Mn, Fe, and Cu. Egg shell waste composed of calcium carbonate, protein membrane, amino acid and collagen [5]. Most of the egg shell waste is commonly disposed of in landfills without any pretreatment because it is traditionally useless and ultimately creates serious environmental problems [3]. Therefore, proper treatment is required to recover valuable calcium ions from egg shell waste.

Calcium, a key signaling molecule in all organisms, is often sequestered in intracellular compartments. In filamentous fungi a calcium gradient has been proposed to play a key role in polar growth. Five genes have been identified in *N. crassa* that may encode organelle calcium transports.

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Calcium is transported into the vacuole by NCA-2 and NCA-3 (P-type ATPase) and CAX (a $\text{Ca}^{++}/\text{H}^{+}$ exchanger), whereas in the golgi it is transported through PMR (a P-type ATPase), thus indicating that the vacuole is a major storage site for calcium [6]. In the tip high cytoplasmic calcium gradient has been identifying as a requirement for hyphal growth in the *Neurospora crassa*. The calcium concentration-dependent growth may relate directly to the biochemical function of calcium in hyphal extension such as vesicle fusion and enzyme activation during cellular expansion. The initiation of tip growth may rely upon random Ca^{++} motion, causing a localized region of elevated calcium [7, 8].

Wheat is one of the cereals used extensively in the many parts of the world for preparation of bread and many bakery products [9]. Industrial wheat bran usually accounts for 14-19% of the grain and comprises the outer coverings, the aleurone layer and the remnants of the starchy endosperm. It consists mainly of starch, arabinoxylans, cellulose, β -glucan, protein and lignin, and has the potential to serve as low-cost feedstock to increase the production of commodity products [10]. Bran is particularly rich in dietary fibers and contains significant quantities of starch, proteins and vitamin and dietary fibers [5]. Wheat bran is a good source of nitrogen due to the presence of protein content; it is also a good source of hemicellulose, as a whole it is a good inducer of the cellulolytic enzyme system [11]. Wheat bran is the outer ~15% of the wheat seed and is composed predominantly of nonstarch carbohydrates (~58%), starch (~19%), and crude protein (~18%). In non-starch carbohydrates ~25% of cellulose present. The high amount of protein present in the wheat bran may also reduce the cellulase biosynthesis but the soluble oligosaccharides, starch, and cellulose present in the wheat bran significantly impact cellulase production [12]. The lignocellulosic biomass, especially agricultural waste, is known to be an excellent carbon source for microbial enzyme production. Cellulase production from lignocellulosic waste materials containing wheat straw: wheat bran (9:1) was investigated by Jecu and reported endoglucanase activity 14.80 IU/ml in 96 hr [13]. Couri et al. investigated the hydrolytic enzymes (polygalacturonase, cellulase, xylanase, and protease) in solid state fermentation using wheat bran as solid substrate [14]. The present paper represents an economical treatment process to recover useful bioproducts from wheat bran and egg shell waste and their utilization in the cellulase production process makes the process cost effective and environmental friendly, and gives a novel avenue for solid waste management.

Experimental Procedures

Pretreatment of Egg Shell Wastes

Egg shells were collected from the local bakeries. To remove impurity and the interference material, egg shells were rinsed several times in deionized water [15]. Chopped and ground egg shell waste (0.4, 0.8, and 1.2 g) with defi-

nite particle size were used for the acid pretreatment. Acid treatments were performed with 5, 10, and 15% HCl (v/v) solution, maintaining the desired solid liquid ratio, soaked at room temperature for two hours. The resulting solution was diluted up to a desired level and made up the resulting solution pH 7.0 with dilute NaOH solution. This solution was further used for the production studies.

Inoculum Development

Neurospora crassa NCIM 1021 strain was procured from the National Chemical Laboratory (NCL) in Pune, India. The procured fungal stock was kept at 4°C in 20% (v/v) glycerol. *Neurospora* culture was grown on M₂ slants at 28°C for 4-5 days. Slants were maintained at 4°C and subcultured about monthly intervals. For the study of growth and production, separate sets of batch experiments were performed. The first set of experiments was carried out (for getting culture solution) in 250 ml Erlenmeyer flasks containing 150 ml of M₂ broth in which 5 loopfull cultures of filamentous mycelia were added and shaken at 180 rpm at 30°C in an incubator shaker for 3-4 days.

Dry Weight Determination

5.0 mL of culture solution was taken from M₂ broth medium. It was then filtered on dried and preweighed Whatman filter paper No. 1. Further the collected solids were washed thoroughly with cold distilled water and with 5.0 mL of 0.9% sterile saline solution. The filter with mycelium was then dried for 24 h at 105°C until attainment of constant weight and weighed. The determination of fungal growth by cell dry weight was expressed as the mean of three independent readings.

Solid State Fermentation

All the chemicals and reagents used to perform experimental work are from Himedia and Sigma aldrich. Solid state fermentation was carried out in 250 ml Erlenmeyer flasks containing sieved wheat bran as the raw material for the growth and production of organisms, impregnated with the following production media in (g/l) Urea, 0.3; $(\text{NH}_4)_2\text{SO}_4$, 1.4; KH_2PO_4 , 2.0; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.3; Peptone, 1.0; Tween 80, 0.2; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.005; $\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.0016; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$; 0.0014; $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.02, with and without CaCl_2 . In the later set of experiments pretreated egg shell waste solution with concentration (0.4, 0.8, and 1.2 g/l) were used in the preparation of basal salt medium in place of pure calcium chloride salt. Throughout the experiment initial pH 6.0 was maintained. Wheat bran bed soaked with treated egg shell waste as well as pure calcium salts containing production media were autoclaved and then inoculated with specific volume (0.56 g/l cell dry weight) of M₂ broth culture solution of *Neurospora crassa*. All the production flasks were placed in an incubator at 25, 30, and 35°C for 8 days.

Table 1. Suitability of acid-treated egg shell waste for *Neurospora crassa* growth [17].

Waste material	Acid treatment dosages	Soaking time (hr)	Neurospora crassa growth measurement
Egg shell waste	Untreated	2	Very light growth
	5% HCl	2	Light growth
	10% HCl	2	Good growth
	15% HCl	2	Very-very light growth

Extraction of Enzyme

Distilled water was added to fermented samples (in a 1:5 proportion in erlenmeyer flasks) and the extraction was done in a shaker at 30°C and 180 rpm. The samples were then filtered and the extract obtained was centrifuged at 6,000 rpm and resulting supernatant was stored and used as enzyme source. All extractions were conducted in duplicate.

Total Cellulase Activity (Filter Paper Activity)

Filter paper activity (FPA) was determined by the method recommended by Ghose [16]. The method is as follows: 0.5 ml of culture supernatant was added to 1 ml of 0.05 M citrate buffer, pH 4.8, and filter paper (50 mg, Whatman filter paper No. 1), mix well and incubated at 50°C for 60 min. The enzymatic reaction was terminated by the addition of 3 ml dinitrosalicylic acid reagent (DNSA). All reducing sugar determinations were performed by the 3,5-dinitrosalicylic (DNS) method at 540 nm. One unit of enzyme activity was defined as the amount of enzyme that released 1 μ mole of reducing sugar equivalent to glucose/min under the assay conditions.

FTIR Spectral Analysis

FTIR spectroscopy is a promising tool for rapid, non-invasive, and multiparameter analysis of the samples. The fourier transform infrared spectroscopy is used for identifying the structure of constituents of lignocellulosic structure. FTIR spectroscopy was performed using a Nicolet 6000 spectrophotometer. Samples were oven dried at 105°C for 4 h, mixed with KBr in the ratio of 1:200 mg (wheat bran: KBr), and pressed under vacuum to form the pellets. Transmittance was measured over a range from 4000-500 cm^{-1} .

XRD Analysis

XRD determined the crystalline content of raw materials. XRD diffraction of samples was recorded on a Bruker AXS D8 Advance diffractometer with a scanning rate of 2 degree/min with Cu K alpha radiation source ($\lambda=1.54060\text{\AA}$) operating at 40 KV and 30 mA. The sample was mounted horizontally while the Geiger counter moved in a vertical arc. The samples were scanned in the range of from 0° to 70° angles.

Results and Discussions

A separate set of batch experiments was performed for the cellulase production study by *N. crassa*. As observed from Table 1, higher and lower dosages of acid concentration were not suitable for the growth of *N. crassa*. Pretreatment of egg shell waste with 10% HCl was found most suitable for growth in comparison to lower and higher acid dosages. At lower acid concentration egg shell waste was not properly dissolved, therefore organisms were unable to take Ca^{++} ions from undissolved (CaCO_3) state, whereas higher acid concentrations were not favorable for growth due to higher acidity.

Treated egg shell wastes are interesting for their ability to support cell growth. As evidence suggested, most quality egg shells contain approximately 2.2 g of calcium in the form of calcium carbonate. Insoluble CaCO_3 is not useful for microbial growth, therefore it is necessary to convert it into soluble form. Insoluble egg shell waste calcium (CaCO_3) becomes soluble (CaCl_2) under treatment with HCl, which provides soluble calcium ions required for microbial growth and the production system.

Good growth has been observed by *Neurospora crassa* on wheat bran-based solid bed impregnated with pure calcium salt containing the production medium [18], as well as calcium salt in the form of acid-treated egg shell waste containing production media-treated wheat bran solid bed as shown by Fig. 1. It has been examined from Table 2 that FPase activity (2.30 IU/ml) of *Neurospora crassa* was found to be the maximum at 30°C on wheat bran solid support. Temperature above or below 30°C was not very suitable for cellulase production by *Neurospora crassa*, which might be due to their physiological nature and protein synthesis capability.

When compared, the cellulase activity in wheat bran bed containing different concentrations of egg shell wastes (0.4, 0.8, and 1.2 g/l) in production media. We have observed from Table 1 and Fig. 2 that 0.8 g/l egg shell waste containing wheat bran solid bed showed better activity (2.11 IU/ml) in comparison to others, which may possibly be due to the fact that at 0.4 g/l of egg shell waste under acid treatment may not provide the desired level of free calcium ions required for proper fungal growth and production, whereas at higher doses many calcium ions and acidity may inhibit microbial growth as observed from Fig. 1.

On the other hand, wheat bran was found as a suitable raw material for cellulase production by *Neurospora crassa* under solid state fermentation, which might be due to the

Table 2. Cellulase activity by *Neurospora crassa* in pure calcium salts as well as acid-treated egg shell waste (varying concentrations) containing wheat bran solid bed under optimized acid treatment dosages.

Calcium salt used	Conc. (g/L)	Optimized acid treatment dosages	Volume of acid used (mL)	Temp (°C)	FPase (IU/mL)
Pure	0.4	-	-	25	1.95
				30	2.30
				35	1.78
ESW	0.4	10% HCl	25	25	1.71
				30	1.89
				35	1.57
ESW	0.8	10% HCl	50	25	1.85
				30	2.11
				35	1.72
ESW	1.2	10% HCl	75	25	1.78
				30	1.96
				35	1.65

presence of soluble oligosaccharides, starch, and easily available cellulose, which significantly induces the cellulase production. Oligosaccharides present in wheat bran may also be converted into a strong inducer for cellulase production such as sophrose and gentiobiose by transglucosylation. As the literature reported that wheat bran is a good source of nitrogen due to the presence of protein content, it is also a good source of hemicellulose. As a whole it is a good inducer for the cellulolytic enzyme system [12]. Although the cellulose percentage in wheat bran are low [11], but it is easily utilizable by microbes, this can be proved by the XRD pattern of wheat bran. A lesser number of peaks with smaller peak height in the XRD pattern of wheat bran shown by Fig. 3 revealed that the cellulose present are easily available for microbial hydrolysis.

A lower percentage of lignin may also provide fruitful conditions for the easier uptake of cellulose and other inducers required for cellulase production. This situation may also be confirmed by FTIR spectral analysis.

FTIR Spectra of Wheat Bran Raw Material

The composition of wheat bran could be identified from the peak presence between 1650 cm^{-1} and 1000 cm^{-1} . FTIR spectra of wheat bran showed several peaks (1641 cm^{-1} , 1552 cm^{-1} , 1413 cm^{-1} , 1321 cm^{-1} , 1256 cm^{-1} , 1152 cm^{-1} , and 1039 cm^{-1}) in this region. The spectra of wheat bran shows a band at 3444 cm^{-1} related to the $-\text{OH}$ band of either hydrogen-bonded or hydroxyl groups in the phenolic and aliphatic compounds. The band near 1321 cm^{-1} may be ascribed to

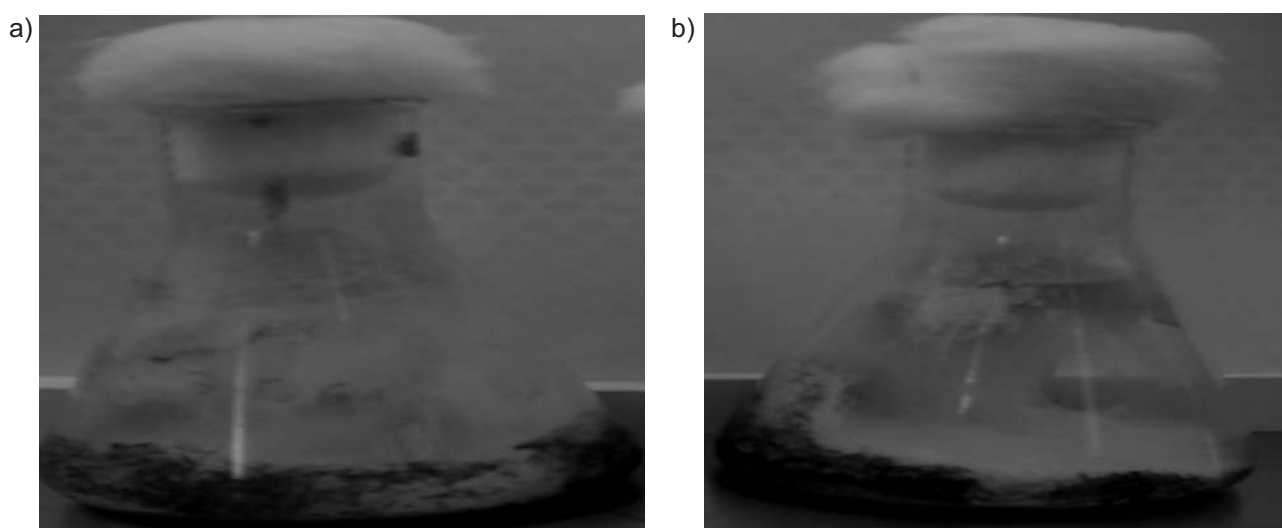


Fig. 1. Growth of *N. crassa* on wheat bran-based solid bed impregnated with pure calcium salt/calcium salt in the form of acid treated egg shell waste containing production media at 30°C and $\text{pH } 6.0$ (1a and 1b, respectively).

Table 3. Peak positions of untreated lignocellulosics in FTIR spectra [19-24].

Wave number (cm ⁻¹)	Predictable groups
3600-3200	O-H stretching and hydrogen bonds
3000-2750	C-H stretch band of methyl group and CH ₂ stretching in cellulose and hemicelluloses,(-OCH ₃) methoxy group present in lignin
1375	C-H in plane deformation (symmetric) for cellulose and hemicellulose
1328	Syringyl ring breaking C-O stretching of phenol
1235-1270	C-O stretching in the acetyl and phenolic groups
1168	C-O-C antisymmetric bridge stretching vibration in cellulose and hemicelluloses, arabinoxylan structure
1044	C-O stretching in cellulose and hemicelluloses
898	C-H deformation of cellulose, β-glucosidic linkage between sugars

syringyl ring breaking C-O stretching of phenol, whereas the band at 1256 cm⁻¹ may possibly be due to C-O stretching in the acetyl and phenolic groups.

The absorption peaks at 1,152 cm⁻¹ and 1,039 cm⁻¹ showed the existence of C-N stretching (amines) and -CO stretching of -COOH in raw wheat bran [25]. The presence of a vibrational peak at 895 cm⁻¹ may be consigned to C-H

deformation of cellulose as well as β-glucosidic linkage between sugars. The FTIR spectra showed that peak height at 3,444 cm⁻¹, 1,321 cm⁻¹, and 1,256 cm⁻¹ bands are very weak with lower absorbance percentages suggesting that the lignin and phenolic compounds are present in lesser amounts, which provides a condition for effortless uptake of cellulose by fungal system.

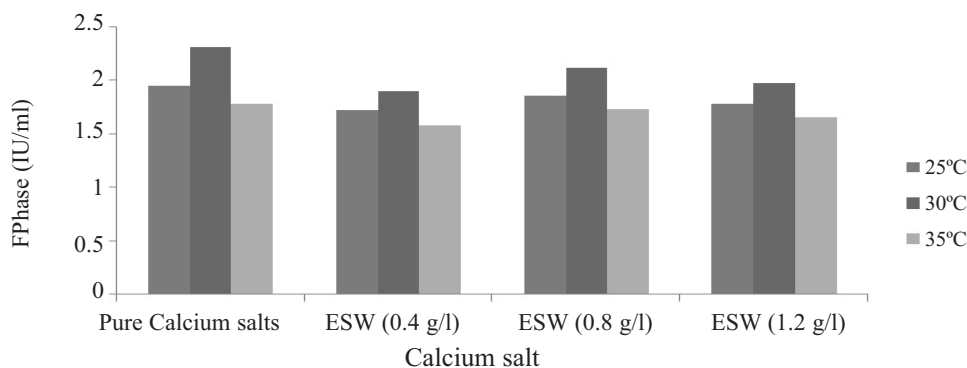


Fig. 2. Bar diagram represents cellulase activities of *Neurospora crassa* using pure calcium salt as well as calcium in treated egg shell wastes (ESW) form, in production medium under wheat bran-based solid state cultivation at 25°C, 30°C, and 35°C.

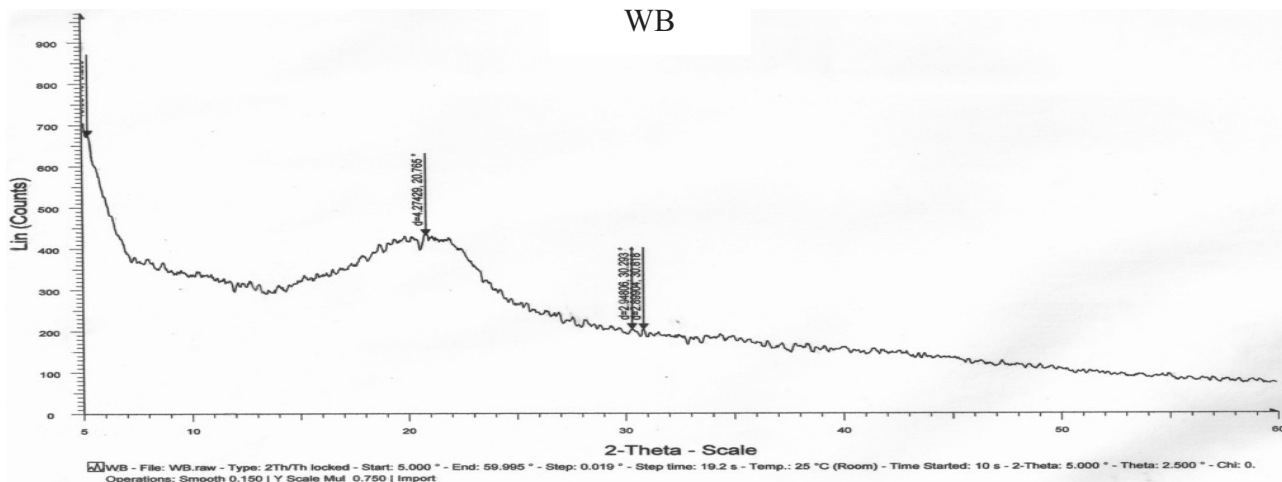


Fig. 3. XRD pattern of wheat bran.

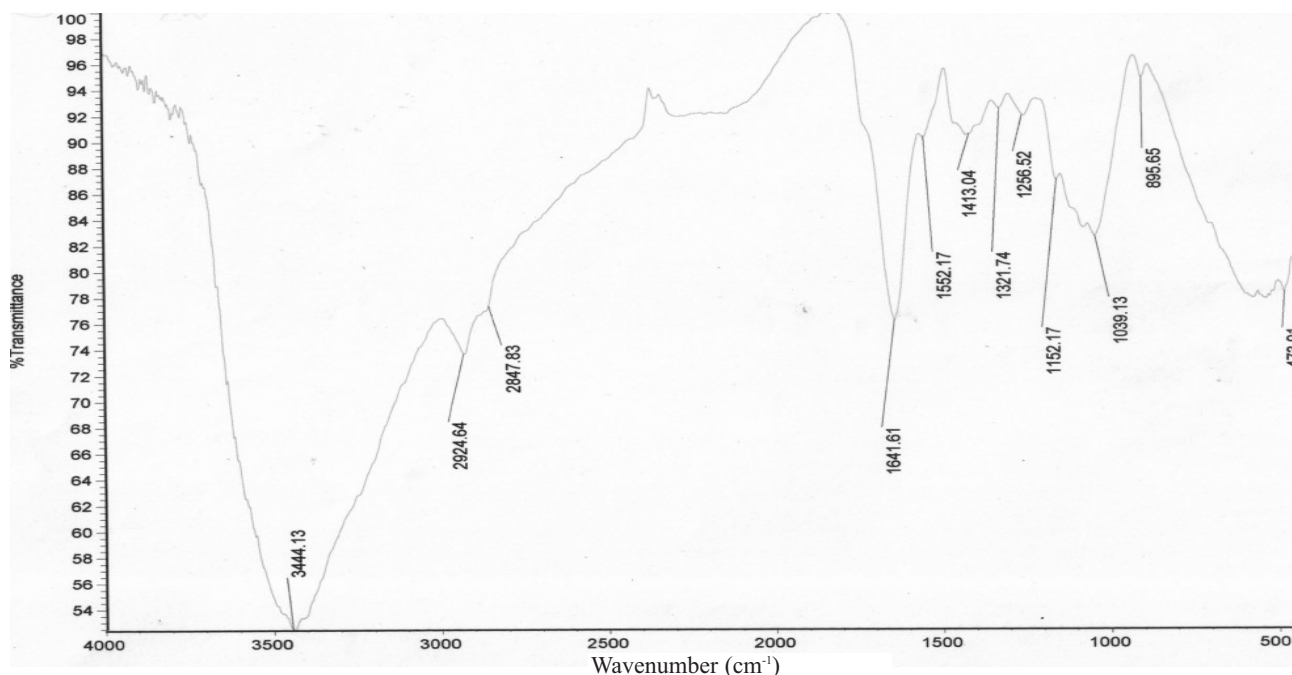


Fig. 4. FTIR spectral diagram of untreated wheat bran.

Conclusion

Waste utilization is another approach in the waste management practice. Keeping in view the importance of calcium in microbial growth, it is important to utilize the calcium present in waste form. Therefore, recovery and utilization of calcium from egg shell waste in cellulase production would be a novel approach. On the other hand, consumption of wheat bran in enzyme production also makes for cost-effective cellulase production technology. On an industrial point of view, solid state fermentation using larger bioreactors such as rotary drum type tray type may be more effective for utilization of egg shell waste as well as wheat bran on mass scale to minimize pollution load more efficiently. Hence the utilization of both waste material, not only provides a cost-effective and environmentally friendly technology, but also is helpful to some extent in the solid waste management process.

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