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PURIFICATION, FRAGMENTATION AND CHARACTERIZATION OF GUM FROM *Cyamopsis tetragonolobus* TO ENHANCE ITS NUTRACEUTICAL ATTRIBUTES

Shazia Anwer Bukhari[1], Muhammad Shahid[2'*], Fozia Anjum[1] Ammara Rasheed[1]and Hira Munir[2]

[1]Department of Chemistry, Government College University, Faisalabad. 3800, Pakistan; [2]Department of Biochemistry, University of Agriculture, Faisalabad-38040, Pakistan.
[*]Corresponding author's e.mail: mshahiduaf@yahoo.com

Guar gum was purified to remove impurities like proteins, fibers and fats and hydrolyze by different chemical and enzymatic means to decrease its roughness and increase thermal stability etc. Swelling ratio of crude guar gum was 2.73 g that was higher than purified (2.57g), base (2.49g), acid (2.23 g). and enzyme (2.31) hydrolyzed samples. Fourier Transform Infra Red analysis of crude guar gum showed a broad peak at 3298 $cm^{-1,}$ 2916 cm^{-1} and 1217 cm^{-1} due to stretching modes of OH, CH and aliphatic amines respectively whereas in the purified and acid hydrolyzed gum (PGG), slight modifications were observed and peaks for OH and CH were noted at 3257 cm^{-1} and 2923 cm^{-1} and, 2920 cm^{-1} and 3290 cm^{-1} and peaks of aliphatic amines were absent. No major functional group transformations during enzymatic hydrolysis of guar gum was detected. X-Ray Diffraction Spectroscopic analysis of enzymatically hydrolyzed guar gum has confirmed the increase in crystallinity as well as fragmentation. Granular form of guar gum was changed into fibrillar structure after enzymatic hydrolysis revealed by Scanning Electron Microscopy whereas Electron Dispersive X-Ray Spectroscopy demonstrated higher percentage of carbon 59.03% and oxygen 40.97% in guar gum. Thermo Gravimetric Analysis for crude and hydrolyzed guar gum depicted thermal sustainability by demonstrating curve at 80°C and 100°C. Presence of mannose and arabinose were detected by High Performance Liquid Chromatography. There are many drawbacks to use the native gums while hydrolysis and fragmentation has exhibited the potential to overcome these pitfalls and applicable further.
Keywords: Guar gum, Purification, Characterization, Hydrolysis, HPLC, SEM/EDX and XRD

INTRODUCTION

Gum is a resinous material associated with certain species of plant kingdom. The polysaccharide material is typically of high molecular weight and most often highly hydrophilic (Schroder and Monika, 2003, Shahid *et al.,* 2013). Guar gum is a carbohydrate having high molecular mass, obtained from the natural seed of *Cyamopsis tetragonolobus* (guar plant) (Pal, 2008). Guar gum is a polysaccharide composed of a mannose backbone and a galactose side chain with an average ratio of 1:2. It contains polysaccharides having high molecular mass of galactomannan which are straight chains of (1→4) β-D-mannopyranosyl units linked with (1→6) α-D-galactopyranosyl residue as side chain. This mannose and galactose group constitutes the galactomannan section of seed endosperm (Mudgil *et al.,* 2012). Guar gum (galactomannan) has a polymeric constitution of hydroxyl groups, which are used to manufacture a variety of derivatives in pharmaceutical, cosmetic, textile, oil recovery and food industries. However, modification of guar galactomannan is appreciated because of its diversified applications. Hydrolyzed gum having low molecular weight depolymerized is a source of soluble dietry fiber used in development of nutraceuticals and functional foods (Mudgil *et al.,* 2012). The hydrolyzed guar gum powder is almost fragrance-free and is off white to yellowish in color (Soumya *et al.,* 2009).

Guar gum is usually used as a stabilizer and economical gelling agent in food and pharmaceutical applications (Pal, 2008; Hussain *et al.,* 2014). Apart from being the commercial stabilizer, it provides water binding and texture improvement properties. The most significant property of guar gum is its ability to hydrate quickly in cold water to achieve regular and high viscosity at comparatively low concentration (Schroder and Monika, 2003). Guar gum and its derivatives (anionic and cationic) are generally used in a variety of industries such as pharmaceuticals, textile, cosmetics, food, explosives, mining, paper and oil drilling (Mudgil *et al.,* 2012). Present study was planned to achieve purified and hydrolyzed guar gum to increase crystalinity, thermal stability and characterize them as a functional soluble dietary fiber for food.

MATERIALS AND METHODS

***Materials*:** Guar gum was purchased from local market of Faisalabad, Pakistan and identified from the Department of

Agronomy, University of Agriculture, Faisalabad, Pakistan. All chemicals of analytical grade used in this study were purchased from either E. Merck (Darmstadt. Germany) or Sigma-Aldrich unless otherwise noted.

***Purification*:** Natural polysaccharides contain small amount of other bio macromolecules like proteins, fats and fiber materials as impurities. To remove these impurities, purification of guar gum was done by different methods as reported by Vinod *et al* (2010), Sandolo *et al* (2007), Kwakye *et al* (2010) and Dodi *et al* (2011).

***Hydrolysis of guar gum*:** Acidic as well as basic hydrolysis of purified gum was done by method as reported by Grobl *et al.* (2005) and Olga *et al.* (2008). For the reduction in viscosity of guar gum, enzymatic hydrolysis was carried out with the help of cellulase enzyme using method reported by Mudgil *et al.* (2012).

***Swelling behavior of guar gum*:** Extent of swelling (Swelling ratio and percentage) of guar gum was calculated using following equations.

$$\text{Swelling ratio} = W_{water} / W_{gel}$$

Where W *water* is the sample weight after soaking, W *gel* is the sample weight after freeze drying (Dodi *et al.,* 2011).

***Determination of monosaccharide composition*:** Monosaccharide contents were analyzed according to the method described by Jahanbin *et al.* (2012). Pure lyophilized gum sample (10 mg) was hydrolyzed by 1 mL of 2M trifluoroacetic acid (TFA) for 3 h at 120°C in a capped tube. The samples were heated at 40°C in a water bath and flash evaporation was done to remove excess acid. The sample (1.0mL) was distilled up to 4mL with water and $NaBH_4$ (50 mg) was added to the resulting product to reduce it. After filtration the sample was injected to the HPLC (Sykam GmbH, Kleinostheim, Germany), where the column (Razex RCM-Monosaccharide Ca^{+2} Phenomenex), mobile phase (DDH_2O), and flow rate was 0.6ml/min at 80^{o}C using refractive index as detector.

***Haemolytic activity*:** Human blood (3 mm) was mixed thoroughly and then poured into a 15 mL screw capped tube to centrifuge for 5 min according to the method of Tabasum *et al.* (2013). The supernatant was collected after centrifugation and the viscous pallets were washed again for three times with phosphate buffer saline (PBS) (5 mL) solution. The washed cells were suspended in a final volume of 20 mL chilled, sterile PBS and the cells counted on a haemacytometer. Ice was used to maintain the blood cell suspension and diluted with sterile PBS. Peptide aliquotes (20 mL) were placed into 20 mL micro fuge tubes. Diluted blood cell aliquots (180 mL) suspension were aseptically placed into each 2 mL tube and smoothly mixed three times with a wide mouth pipette tip. At 37°C tubes were incubated with agitation for 35 min. Immediately the tubes were placed for 5 min on ice and centrifuged for 5 min. Aliquots of 100 mL of supernatant were carefully collected, placed into a sterile 1.5 mL microfuge tube, and diluted with 900 mL chilled, sterile PBS. All tubes were maintained on wet ice after dilution. Three replicates were run for each. Absorbance was measured at 576 nm.

***Scanning Electron Microscopy-X-Ray Dispersive Spectroscopy (SEM-EDX)*:** Scanning electron microscope (Hitachi S-2380, Japan) was used for analyzing the surface morphology of crude, purified and hydrolyzed gum samples in powder form. To determine the elements present at the surface of the particular area of the gum sample, Electron Dispersive X-ray Spectroscopic analysis (SEM-EDX, Hitachi S-2380, Japan) was carried out by following the method as described by Zia *et al.,* (2013).

***Structure elucidation of purified and hydrolyzed guar gum by FTIR*:** Information regarding the presence of different functional groups as well as molecular structure can be obtained from infrared spectroscopy. Crude, purified and partially hydrolyzed guar gum samples was analyzed to confirm their functional groups by FTIR Nicolet (FT-IR Bruker, Impact 400 IR spectrophotometer) and infrared spectra that was recorded in a range from 4000 to 500 cm^{-1} (Mudgil *et al.,* 2012).

***X-Ray Diffraction Analysis (XRD)*:** X-ray diffractometry analysis of lyophilized samples of crude, purified and partially hydrolyzed guar gum (in powder form) was analyzed by using X-ray diffractometer (JDX 3532, JEOL, Japan). Measurements were carried out with a diffraction angle range of 5–60^o at room temperature with a scan step of 0.01 (Mudgil *et al.,* 2012).

***Thermogravimetric analysis (TGA)*:** Thermo-gravimetric (TGA) analysis was carried out with the help of a TGA instrument. Thermo-Gravimetric and Differential Thermal Analyzer (TG/DTA) (TG/DTA Perkin Elmer, USA) was used to evaluate the effect of rise in temperature with respect to time in crude, purified and hydrolyzed samples of guar gum. Analysis was carried out in a temperature range from 30°C to 1200°C with a uniform heating rate of 10°C/min in nitrogen inert atmosphere (Zuber *et al.,* 2014).

RESULTS AND DISCUSSION

***Purification of guar gum*:** Purification and hydrolysis have resulted in the removal of impurities as well as insoluble fractions. Purification has been done by following the different methods named as GG1, GG2, GG3 and GG4. Sample was best purified by the method GG4 (78.5%) followed by GG1 (75.0%), GG3 (72.2%) and GG2 (33.8%) (Fig. 1).The yield is the most economical vital feature of polysaccharide purification and purification yield was dependent on the method applied. The reason for the highest yield (78.5%) of GG4 was probably the long time period used for dissolving the crude sample or might be due to the recovery of almost all the precipitated polysaccharide by using ethanol. For economical reasons method 4 was used for purification.

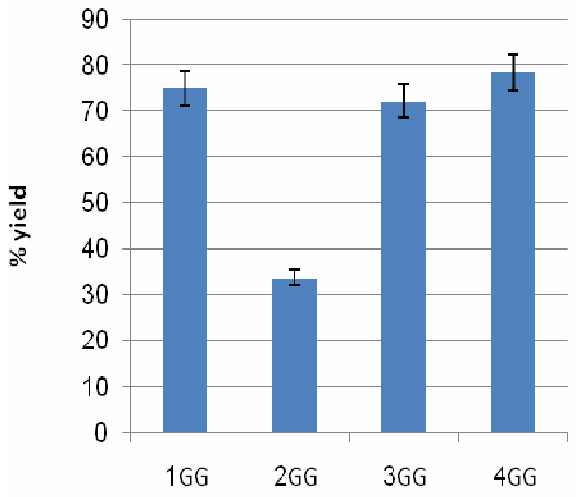

Figure 1. Purification yield of guar gum by different purification procedures

***Hydrolysis of guar gum*:** Guar gum has positive physiological benefits; its high viscosity makes it difficult to incorporate into food products and solutions. Native guar can be modified by both chemical and enzymatic methods to extend its range of applications. Partially hydrolyzed guar gum (PHGG) was produced to provide a dietary fiber source that could be added easily to the diet. Partially hydrolyzed guar gum was produced by three different methods like acidic hydrolysis using TFA (Grobl *et al.,* 2005), basic hydrolysis using $Ba(OH)_2$ (Olga *et al.,* 2008) enzymatic hydrolysis using cellulase (Mudgil *et al.,* 2012). PHGG has low molecular weight and less viscosity and could be added easily to the diet and would be acceptable to consumers (Salvin and Greenberg, 2003). The low viscosity, the small amount of remained glutaraldehyde, and the thermal stability indicates that the guar hydrogel has potential to be applied as biomaterial with specific rheological requirements (Cunha *et al.,* 2005).

***Swelling behavior of guar gum*:** In Table 1, crude guar gum displayed a higher swelling ratio relatively to purified and hydrolyzed guar gum. More swelling ratio, more uptake of moisture contents and less will be the potential for commercial applicability (Cunha *et al.,* 2005; Dodi *et al.,* 2011). Nep and Conway (2010) and Dodi *et al.* (2011) also detected higher swelling ratio of crude guar gum as in present research work. An important functional property of polysaccharide gums is their ability to hydrate and swell to form highly viscous solutions or dispersions. The hydration rate and degree of swelling is therefore critical to their successful application with a wide range of uses in clinical nutrition (Salvin and Greenberg, 2003) and is dependent on a number of factors such as pH and temperature.

Table 1. Swelling ratio of hydrolyzed and unhydrolyzed guar gum samples

Sr. No.	Guar sample	Swelling ratio (g)
1	Crude	2.73 ± 0.01^{a}
2	Purified	2.57 ± 0.02^{b}
3	Acid hydrolyzed	2.23 ± 0.05^{c}
4	Base hydrolyzed	2.49 ± 0.08^{d}
5	Enzymatic hydrolyzed	2.31 ± 0.04^{e}

The values are mean ± SD (n = 3)
Means with different letters differ significantly at ($P \leq 0.05$). Comparisons are made within the column for each guar fractions to evaluate the swelling ratio (LSD value =0.0228)

***Determination of sugar composition*:** HPLC analysis of the crude and acidic hydrolyzed guar gum confirmed that crude (1.25 & 0.22%) and acidic hydrolyzed (1.18 & 0.14%) samples of guar gum were composed of mannose and a very small amount of arabinose respectively (Fig. 2a-2b). Cunha *et* al. (2005) also detected comparatively greater concentration of mannose than arabinose in guar gum samples. Debon and Tester (2001) have also characterized guar gum and obtained similar monossacharide composition as detected in present research work. Similar findings were also reported by Chauna *et al.* (2009) and Jahanbin *et al.* (2012)

***Haemolytic activity*:** Haemolytic activity of crude and hydrolyzed guar gum is demonstrated in Table 2. Partially hydrolyzed guar gum has undergone extensive toxicity testing and found to be safe. Dietary levels up to 10% PHGG were tolerated without any signs of toxicity (Takahashi *et al.,* 1994). Similar findings were also reported by Salvin and Greenberg (2003). The hemolytic activity of the studied samples exhibited negligible toxicity against the human erythrocytes. Moreover, it was also verified from our results with no significant change in the haemolytic activity of native and hydrolyzed samples of guar gum. This PHGG has additional physiological benefits of fiber consumption.

Table 2. Hemolytic activity of gum samples against the human erythrocytes

Sr.	Guar gum sample	Haemolysis activity (%)
1	Crude GG	1.17 ± 0.09^{b}
2	Purified	1.09 ± 0.04^{b}
3	Acid hydrolyzed	1.31 ± 0.28^{b}
4	Base hydrolyzed	1.32 ± 0.24^{b}
5	Enzyme hydrolyzed	1.34 ± 0.26^{b}
6	PBS	0.00^{c}
7	Trixton-X-100	100^{a}

The values are mean ± SD (n = 3)
Means with different letters differ significantly at ($P \leq 0.05$). Comparisons are made within the column for each guar fractions to evaluate the hemolytic activity (LSD value =0.3342)

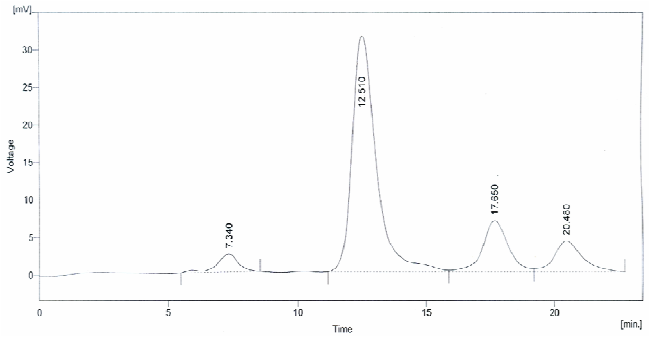

Figure 2a. Chromatogram of monosaccharide composition of crude guar gum

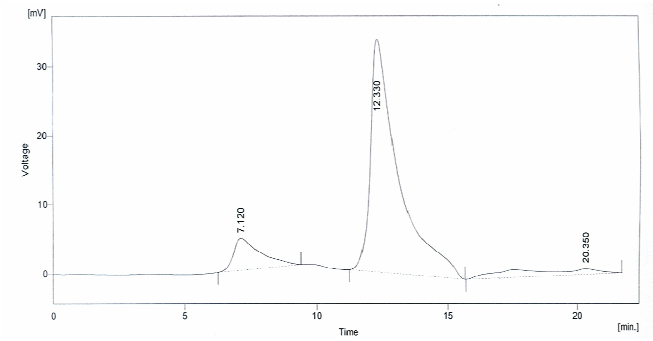

Figure 2b. Monosaccharide composition of acid hydrolyzed guar gum

***Fourier transforms infrared spectroscopy (FTIR)*:** Infra-Red Spectroscopy provides information regarding the evidences of the presence of different functional groups as well as molecular structure. FTIR spectra of crude, purified and hydrolyzed guar gum were recorded to compare the changes in their chemical structure and observed characteristic IR wave number. From the spectrum of crude guar gum (GG) (Fig. 3a) a broad peak at 3298 cm^{-1} due to O-H stretching vibration of polymer and water involved in hydrogen bonding while a peak at 2916 cm^{-1} might be due to C-H stretching modes of methylene group of sugar. In the same spectrum a peak observed at 1739 cm^{-1} was due to the presence of C=O stretching vibrations, associated water molecule resulted in the band at 1652 cm^{-1} in the spectra. The region around 1373 cm^{-1} due to CH_2 deformation was also observed, however the peaks observed between 800 and 1200 cm^{-1} represented the highly coupled aldehyde and ether stretching modes of polymer backbone. In addition to that, a peak observed at 1217 cm^{-1} which might be due to the presence of aliphatic amines. In the purified spectrum of guar gum (PGG) (Fig. 3b) O-H stretching vibrations were observed at 3257 cm^{-1} as well as C-H stretching vibrations of CH_2 at 2920 cm^{-1}. In purified gum, sharpening of absorption band around 2358 cm^{-1} showed its increased association with water molecule, which could be a justification of its improved solubility compared to native guar gum, while a peak at 1217 cm^{-1} as missing in purified sample. A slight modification can be observed in the well defined spectrum of purified guar gum. Similar behavior of purified and hydrolyzed guar gum samples were also reported previously (Huang *et al.,* 2007; Sharma and Lalita, 2011; Dodi *et al.,* 2011). In case of purified hydrolyzed guar gum (PHGG) using acid (Fig. 3c) C-H stretching vibration was observed at 2923 cm^{-1} and O-H stretching at 3290 cm^{-1}. But in case of basic hydrolysis (Fig. 3d) absorption bands of C-H and O-H stretching vibrations were not as sharp as in acidic hydrolysis. Except these two bands, all other bands are sharp in basic sample. It is considered that crystallinity of polymer is represented by the region between 700 and 500 cm^{-1}. Enzymatically hydrolyzed samples using cellulase (Fig. 3e) showed O-H and C-H stretching vibrations near 3300 cm^{-1} and 2900 $cm^{-}1$. C-H band was very sharp and clear in cellulase hydrolyzed sample comparatively. In cellulase hydrolyzed sample, sharpening of absorption bands indicate the association of gum with water molecules especially of C=O stretching vibrations observed at 1733 cm^{-1} but in case of cellulase very sharp and intense peak appeared. These results revealed that there are no major functional group transformations during enzymatic hydrolysis of guar gum but there is just increase in association of water molecules as reported by Sen *et al.* (2010).

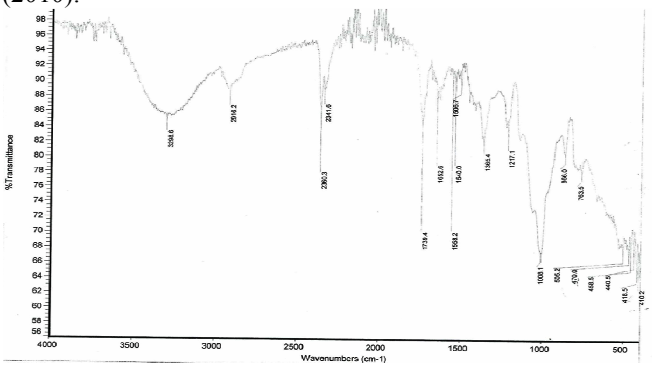

Figure 3a. FTIR spectrum of crude guar gum

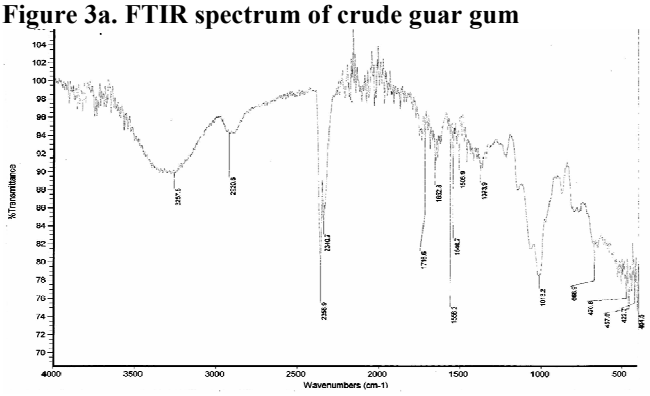

Figure 3b. FTIR Spectrum of purified guar gum

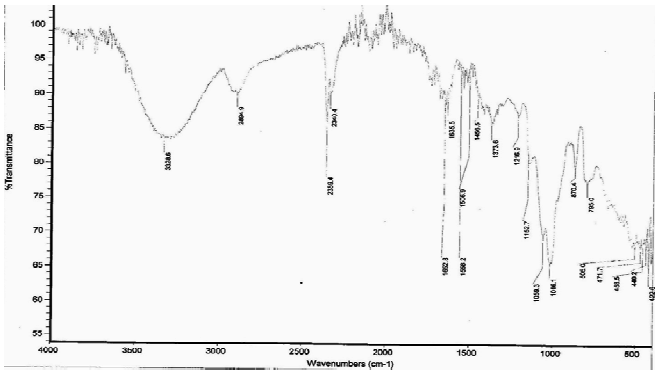

Figure 3c. FTIR spectrum of acid hydrolyzed guar gum

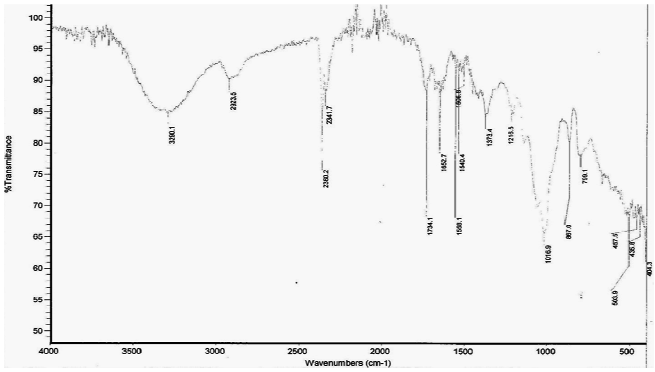

Figure 3d. FTIR spectrum of base hydrolyzed guar gum

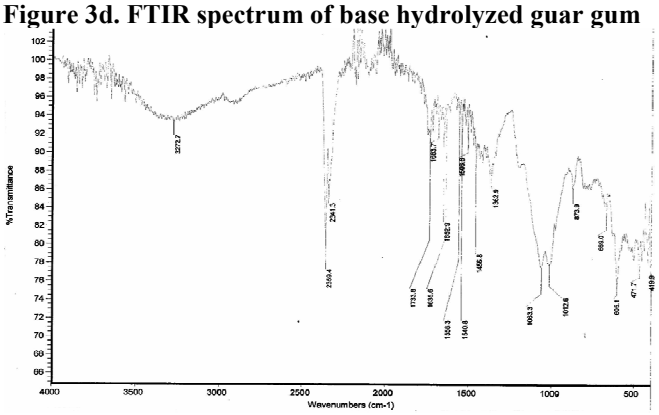

Figure 3e. FTIR spectrum of cellulase hydrolyzed guar gum

***X-ray diffraction analysis (XRD)*:** X-ray diffraction (XRD) is a powerful nondestructive technique for characterizing crystalline materials. Crude, purified and hydrolyzed guar gum showed different behavior in regular structure. Crude guar gum was largely amorphous and two peaks were observed at the scattering angle (2θ) at 17.5° and 20.4° (Fig. 4a). The biopolymer purified guar gum (PGG) was of low crystallinity, possibly due to intramolecular interactions and the 2θ observation was at 6.2° and 20.2° (Fig. 4b). The crystalline regions of hydrolyzed (Cellulase) guar gum were seen in the ranges of 20.7° to 43.1° (Fig. 4c) whereas the crystalline regions of hydrolyzed (base) guar gum were seen at 20.4° and 48.9° (Fig. 4d). This means that enzymatic hydrolysis of guar gum resulted in negligible change in XRD curve. However, it was observed that enzymatic hydrolysis slightly increased the crystallinity of partially hydrolyzed guar gum (PHGG). Acidic hydrolysed gum showed same patternt as basic hydrolyzed gum. Singh *et al.* (2009) have also reported the increase in crystalline character of guar gum. Present results are in accordance with the results reported by Dass *et al.* (2000), Cunha *et al.* (2005) and Mudgil *et al.* (2012) who reported the increased crystalline behavior of the guar gum upon partial hydrolysis.

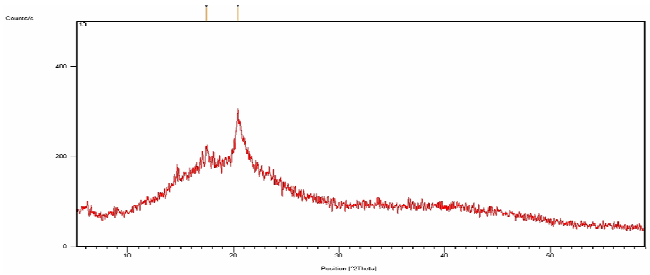

Figure 4a. X-ray diffraction spectrum of crude guar gum

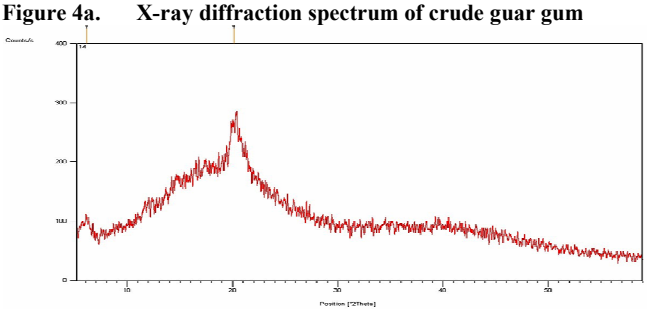

Figure 4b. X-ray diffraction spectrum of purified guar gum

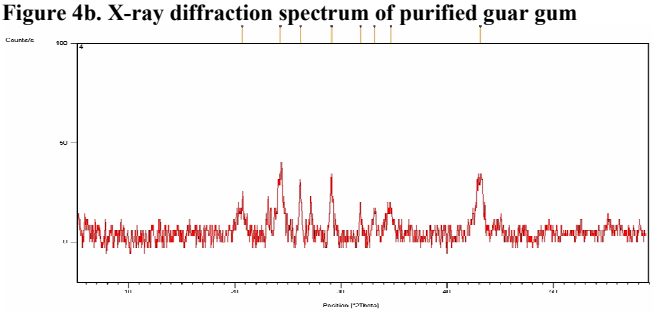

Figure 4c. X-ray diffraction spectrum of crude cellulase hydrolyze

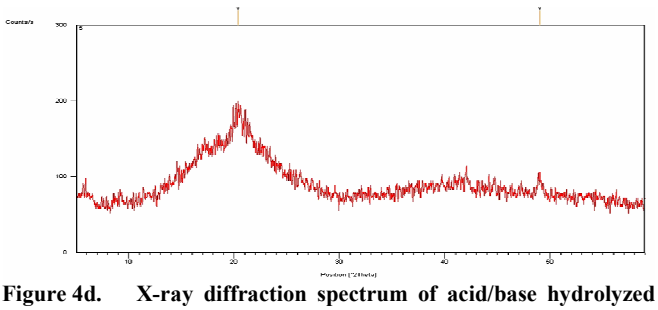

Figure 4d. X-ray diffraction spectrum of acid/base hydrolyzed guar gum

***Scanning electron microscopy (SEM)*:** Scanning Electron Microscopy was carried out to observe the surface morphology of crude, purified and hydrolyzed guar gum in order to verify visible morphological changes in the structure of the material and to verify an eventual rearrangement of the structure. Two facts are to be mentioned: first, normal guar gum particles were small and having the rough surface morphology, which would help in attaining highly viscous aqueous solution. Guar gum usually existed in a granular form and there was no cross linking between the granules. Scanning electron microscopy revealed the significant changes in the surface morphology of the guar gum after hydrolysis (Fig. 5a-e). It was observed that when water molecules were released during the lyophilization of guar gum solution, a soft structure was formed comparable to the results of Cunha *et al.* (2005). The morphological changes were brought about by hydrolysis as deposits of the hydrolyzed copolymers. TFA has strongly affected the morphology of hydrolyzed guar gum. In cellulase hydrolysis, a well defined porous structure was detected; the interconnection between pores could be assigned to the network formed by the reaction with cellulase enzyme. The average pore size, the pore size distribution, and the pore interconnections are considered as important factors of the hydrolyzed samples (Hoffman, 2002). The present finding are comparable to the findings reported previously (Sharma and Lalita, 2011; Kothiyal and Sharma, 2012).

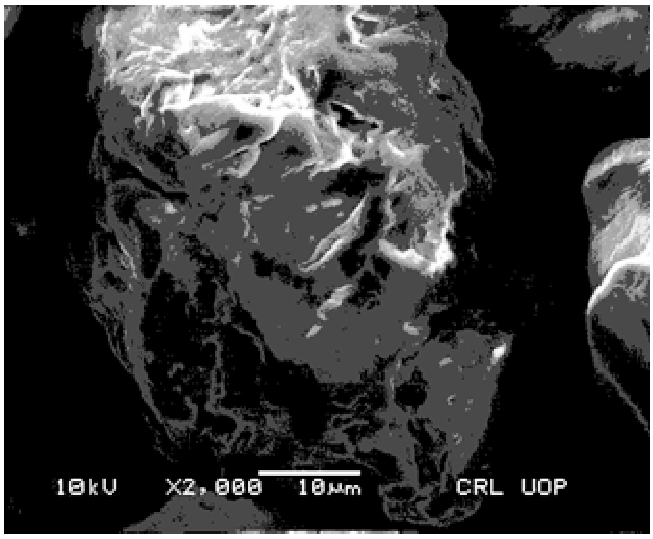

Figure 5a. **Scanning electron micrographs of crude guar gum**

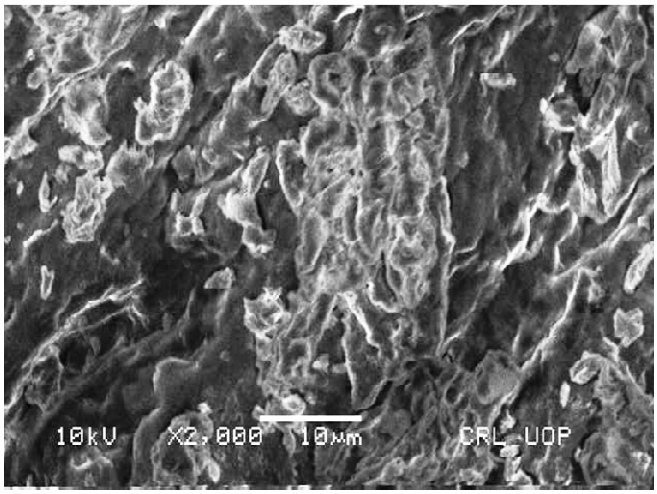

Figure 5b. **Scanning electron micrographs of purified guar gum**

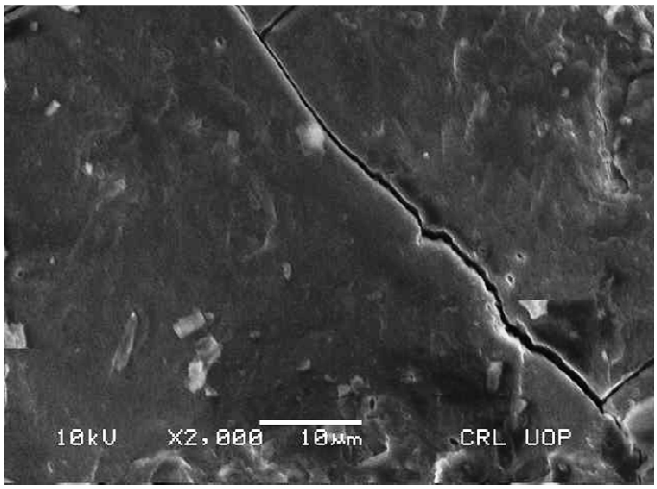

Figure 5c. **Scanning electron micrographs of acid hydrolyzed guar gum**

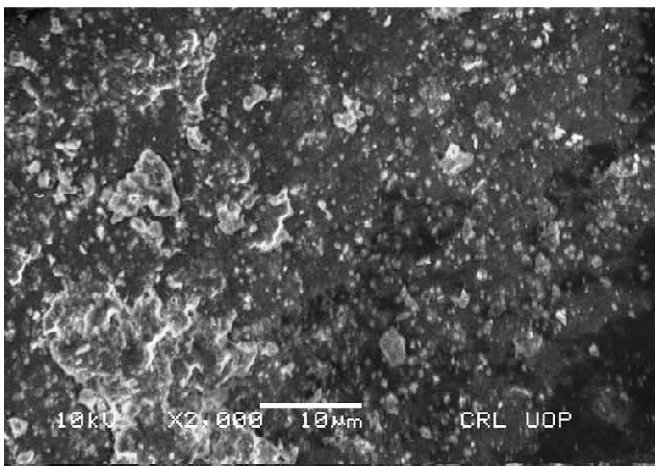

Figure 5d. **Scanning electron micrographs of cellulase hydrolyzed guar gum**

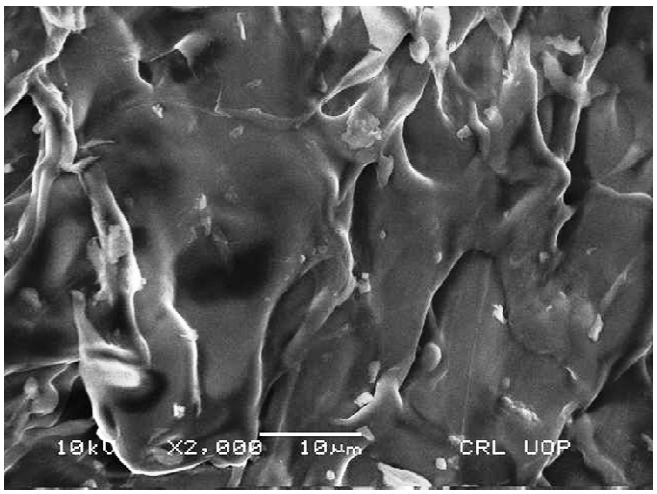

Figure 5e. Scanning electron micrographs of base hydrolyzed guar gum guar gum

***Elemental analysis*:** Electron dispersive spectroscopy (EDX) was carried out to detect the elements present at the surface of a particular area of guar gum. Galactomannan guar gum (crude) was analyzed to determine the percentage (w/w) composition of elements. From the spectrum (Fig. 6), it was clear that a higher percentage of carbon (C, 59.03%) and oxygen (O, 40.97%) was present in guar gum sample comparable to the findings of Pal (2008), Sharma and Lalita (2011) and Kothiyal and Sharma (2012).

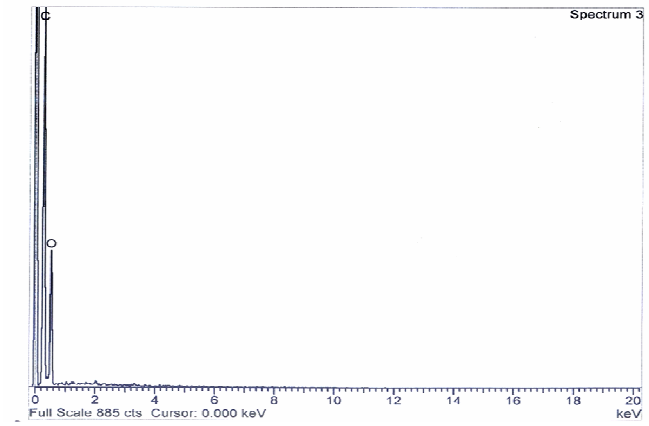

Figure 6. Electron dispersive spectrum (EDX) of guar gum

***Thermogravimetric analysis (TGA)*:** Thermal stability of the polymer is an important property that could make the material fit for food applications where material is thermally processed using unit operations such as sterilization and baking. Thermal stability analysis of polymer material is helpful in the selection of materials with the best properties for specific used. Thermal analysis of guar gum samples (crude, acidic, basic and enzymatically hydrolyzed) were carried out with thermal gravimetric analysis (TGA) technique. Guar gum degrades mainly by dehydration and depolymerization. TGA curve of GG showed that first decomposition begins at 100°C comparable to the results reported by Cunha *et al* (2005) and Singh *et al* (2009). Three dominant zones in TGA curve of GG which represent the loss in weight. The early weight loss was from 80-120°C, moisture might be a reason behind it (Fig. 7a-d). The second zone starts from 230-335°C which might be due to the degradation of polymeric backbone containing functional units of primary alcohol and the third zone was from 335-990°C represents breakdown of polymer backbone containing primary alcohol $–CH_2OH$. This is caused by sequence of processes in which galactose and mannose cleave from the guar gum backbone and then decomposition occurs (Sharma and Lalita, 2011). The change in heat flow of native and partially hydrolyzed guar gum was analyzed over a temperature range of 30°C to 1200°C at 10°C/min. TGA profile of residual mass demonstrated increased stability of partially hydrolyzed guar gum with no major change in chemical structure at higher temperature range than native guar gum. This statement was also accounted for the results of Sen *et al.* (2010).

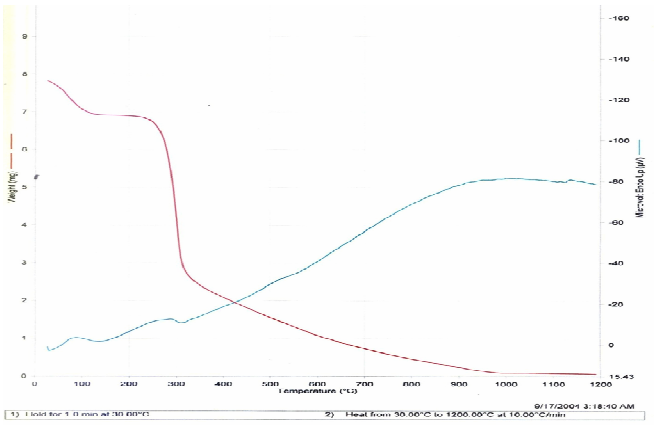

Figure 7a. Thermo gravimetric analysis of crude guar gum

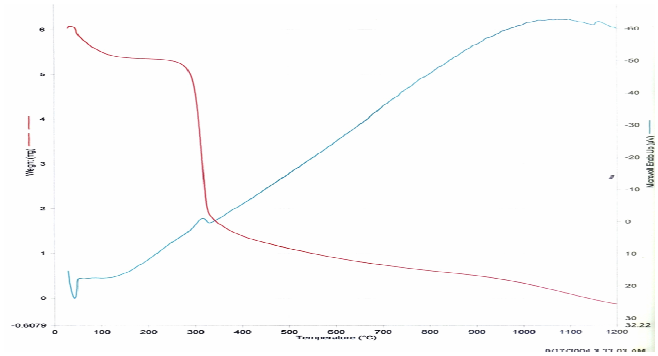

Figure 7b. Thermo gravimetric analysis of acid hydrolyzed guar gum

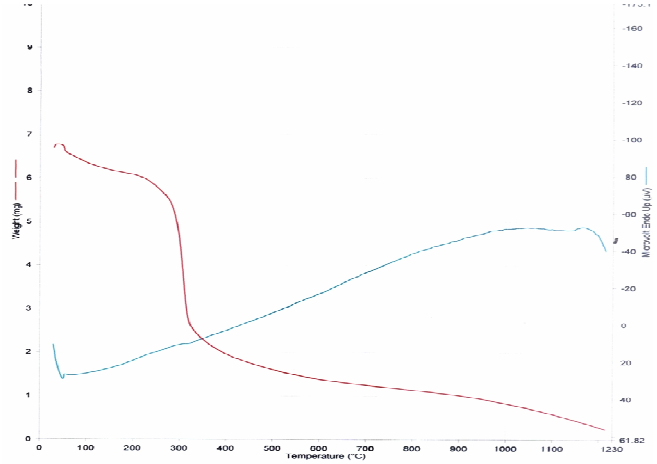

Figure 7c. Thermo gravimetric analysis of base hydrolyzed guar gum

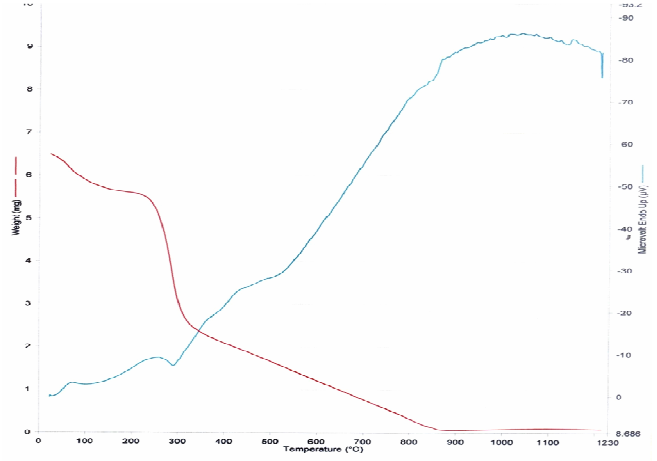

Figure 7d. Thermo gravimetric analysis of cellulase hydrolyzed guar gum

***Conclusions*:** To increase the commercial potential of guar gum, partially hydrolyzed guar gum obtained by acid, base and enzymatic hydrolysis showed a remarkable decrease in viscosity or swelling behaviour and molecular weight. HPLC analysis of hydrolyzed and unhydrolyzed samples showed the monosaccharide composition in guar gum indicating no change in functionality and structure but just association of water molecules. Characterization of crude, purified and hydrolyzed guar gum with FTIR, XRD, SEM/EDX and TGA demonstrated no major changes in structure of the molecule but increase in crystalinity thus increase in thermal stability of the guar gum.

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