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Removal of glycosaminoglycans present in tannery saline soak wastewater using integrated biological reactor and amylase immobilised reactor

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ABSTRACT

The present investigation offers the scope for the elimination of glycosaminoglycans (GAGs), the amino sugars, present in tannery saline wastewater (also known as soak liquor) generated during the soaking of raw materials for the removal of common salt. The extracted salt tolerant amylase from *Providencia Rettgerri sp.*, had very high amylase activity, 1320 U/ml. The modified NPAC(m-NPAC) was effectively produced by covalent incorporation method using ethylenediamine/glutaraldehyde for the immobilisation of amylase. The salt tolerant amylase immobilised nanoporous activated carbon (Am-NPAC) was used for the elimination of GAGs from soak liquor having chlorides of 4–5% (w/v). Various factors such as time, 120 min; pH, 6; temperature, 30°C; amylase concentration, 250 Units/g of m-NPAC and salinity, 3–5% of the medium that favour the maximum immobilisation of amylase onto m-NPAC were optimized. The instrumental techniques such as FT-IR spectroscopy, TGA-DSC, SEM and XRD analyses have confirmed the immobilisation of amylase. The optimum conditions such as time, 1 h; pH, 6; and temperature, 40°C for the elimination of GAGs from soak liquor were investigated. The amylase immobilised nanoporous carbon matrix (Am-NPAC) was very effective for the cleavage of GAGs into glucuronic acid and glucosamine bio-molecules. The removal of GAGs was confirmed through FT-IR, UV-visible and fluorescence spectroscopies.

Keywords: Amylase; GAGs; Glycosaminoglycans; Immobilisation; Mucopolysaccharides; Nanoporous activated carbon; Saline wastewater; Tannery soak wastewater

1. Introduction

Leather industrial sector plays a crucial function inside the financial system of a country and provides employment to the weaker section of the public since time immemorial. The tanning of skins/hides intends to transform them into solid and imputrescible (resistant to microbial attack) leather and its products. About 300 kg of organic/inorganic compounds are used in leather processing to convert one ton of skins/hides into leather. Many sectional operations such as preservation, soaking, liming, unhairing, deliming, bating, pickling, depickling, tanning and post tanning operations are followed in leather processing [1,2]. The leather sector suffered a setback in the recent past due to environmental damage rendered to the soil, water and air. Consequently, a full-size strain from the pollutants manipulate bodies arises to modify and decrease the pollutant loads made from the leather industries [3–6]. The soaking process, the first step in leather manufacturing practice, comprises of soaking the skins/hides in water (about 12 L/kg of raw material) containing small quantities of wetting agent (0.4% w/w) for rehydration of collagen fibres and

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solubilization of low molecular weight skin proteins. It is also used to remove the salt adhered onto the raw material and also for the effective elimination of the residuals of blood, animal excreta, soil attached to the epidermal surface of raw materials. Soaking process is commonly carried out in a soaking pit or paddle drum with agitation. The soak wastewater also known as soak liquor is characterised predominantly by dissolved bioorganic compounds and inorganic compounds, high concentration of suspended solids and halophilic and halotolerant microorganisms [7–10]. The soak liquor contains bioorganic compounds (majorly proteins, lipids, and glycosaminoglycans) besides chlorides in the range 40000–80000 mg/L. Hence, the soak liquor discharged onto soil without appropriate treatment influences the water potability, aquatic life and plant life [11–16]. Hence, it has become mandatory to treat soak wastewater for the removal of organic matter and salt from it. It is known that wastewater with more than 1% salt cause plasmolysis of bacterial cell wall and reduced the extra cellular/intracellular enzyme activity and consequently the efficiency of effluent treatment plants is retarded [17,18]. Hence, the strategies followed in conventional biological wastewater treatment plant have become inappropriate for the treatment of salt laden wastewater discharged from industries [19,20]. Moreover, the operational cost of thermo chemical processes and advance oxidation processes (AOP's) are increased by many folds due to the presence of high total dissolved solids. Hence, the current method followed is to evaporate the saline wastewater in solar evaporation pans and separate the evaporated residue due to lack of effective treatment systems.

The residue scraped out from the solar evaporation pans doesn't meet the characteristics for the reuse option in leather industry or any other industry and thus they are heaped up in leather industry. The accumulated salt residue leaches into groundwater resources during runoff season causing groundwater pollution. Treatment of soak liquor in modified biological reactor to remove the suspended solids and followed by fragmentation of bio-organic compounds by halo tolerant enzyme immobilised carbon matrix. This may be regarded as the novelty of the present investigation. The salt resistant/tolerant enzymes were extracted from the halophilic bacteria with specific substrates and were used to prepare enzyme immobilised modified nanoporous carbon matrix. The presence of amino sugars and lipids in soak wastewater have a significant effect on the solubility of bio-organics in water, causes turbidity to soak wastewater. Moreover, it may be the reason for the partial removal of pollution parameters from soak wastewater. Thus, it become essential to eliminatem ucopolysaccharides for the increase in biodegradability index salt laden wastewater for its effective treatment [21–28]. The presence of unbranched oligomers or polymers linked together by glyosidic linkages are identified as glycosaminoglycans. Mucopolysaccharides are associated with lipids, peptides, or proteins and their degradation becomes complicated owing to the presence of high TDS in the effluent. The current investigation focuses on the degradation of glycosaminoglycans into glucosamine and glucuronic acid by amylase (extracted from salt tolerant bacteria, *Providencia Rettgerri sp.*) immobilised nanoporous carbon matrix (Am-NPAC) for the effective treatment of soak wastewater.

2. Materials and methods

2.1. Materials

The chemicals such as soluble starch, DNS (dinitro salicylic acid), sodium hydroxide, sodium potassium tartarate, phenol, acetylacetone, ethanol, sodium carbonate were purchased from Hi-media (India). The chemicals used for the modification of nanoporous activated carbon surface consisting of EDTA and glutaraldehyde were obtained from Merck, India. The salt tolerant amylase was extracted from *Providencia Rettgerri sp.* It was screened from the soil collected from tannery cluster area, Chennai which was acclimatized with tannery soak wastewater.

2.2. Isolation of halophilic organism and the amylase production

The halophilic bacteria isolated from the tannery soak wastewater acclimatized soil was grown in the minimal media containing animal fleshing (1% w/v) and trace elements solution (1% w/v). The 16SrDNA analysis confirmed that the organism was *Providencia Rettgerri sp.* as shown by phylogenetic tree. The ANFL was dispersed in water byusing Ultra sonicator at 23 KHz. The optimization studies were carried out with minimal media of volume 50 ml and culture broth, 2 ml at different incubation time (12–72 h), pH (3–10), temperature (20–60°C), ANFL (0.5–5% w/v) and the salinity (0.5–5%) for the production of amylase [29–31]. Biomass in the reaction mixture was separated using refrigerated centrifuge followed by acetone precipitation. The DNS method with slight modifications was used for the determination of amylase activity of the collected enzyme.

2.3. Modified nanoporous activated carbon (m-NPAC), preparation and surface functionalization

Pre-carbonization and subsequent chemical activation were followed for the preparation nanoporous activated carbon from the raw material, rice husk as detailed by our earlier research work [32–35]. The functionalization of NPAC was performed with the addition of ethylenediamine to produce amine-modified matrix. The amine-modified NPAC was condensed with glutaraldehyde (named as m-NPAC) to immobilise saline tolerant amylase. The m-NPAC was dried at 103°C for 4 h to facilitate the condensation reaction. The immobilisation of saline tolerant amylase was facilitated through anchoring with the free aldehyde end groups in m-NPAC. The amylase immobilised NPAC is named as Am-NPAC. The process parameters such as time, concentration of amylase, mass of m-NPAC, bulk solution pH, salinity and temperature were optimized for the maximum immobilisation of salt tolerant amylase and the results are presented in supplementary materials 1.2.

2.4. Collection and degradation of tannery soak wastewater

The hypersaline soak wastewater used in this study was collected from the commercial tannery cluster in Chennai, Tamilnadu. The tannery soak wastewater was screened to remove the floating solids such as animal hair and skin trimmings and it was allowed to settle for approximately

two hours to remove coarse solids. The settled soak liquor was passed through the Sequential oxic-anaerobic bioreactor (SOAR) for the removal of total suspended solids (TSS) and for the partial hydrolysis of GAGs in soak liquor. SOAR with the dimensions 25 × 15 × 15 cm was made-up using polyacrylic sheet. It consisted of three compartments, including anaerobic and oxic zones in each compartment with total reactor volume of 3.6 L (compartment I, 1.2 L; compartment II, 1.2 L and compartment III, 1.2 L). The compartments were filled with corrugated high density cylindrical shaped commercial grade polyethylene plastic media with specifications: diameter, 21 mm and height, 25 mm. The plastic media supported the growth of biofilm on its active surface area and facilitated the removal of suspended solids and partial mineralisation of dissolved organic compounds present in soak liquor. The anaerobic zones in each compartment of the reactor were packed with plastic media and maintained the anaerobic conditions as mentioned in our previous work [36,37]. The samples were collected from the outlets provided in each compartment at oxic and anaerobic zones for characterization. The provision for maintaining oxic and anaerobic conditions supported the growth of microorganisms onto the plastic media and also for the maximum removal of organic compounds in soak liquor.

The hydrolysis of GAGs content of soak wastewater was completed by the application of Am-NPAC under batch mode, and the parameters such as time, pH, and temperature were optimised. The SOAR treated soak wastewater (20 mL) was incubated with Am-NPAC, 0.2 g for different time periods varied from 0 min to 180 min in shaking incubator at 80 rpm and at ambient temperature. The impact of pH (3–10) on degradation of GAG's was determined at the fixed incubation time (1 h). The pH of the solution was adjusted by using 0.01 N HCl/ 0.01 N NaOH to the required set conditions. The effect of temperature on degradation of GAGs was determined by maintaining the temperature from 20°C to 60°C in a constant temperature shaking incubator. The degradation of GAGs in pre-treated soak liquor was determined under continuous mode. A glass column reactor of diameter, 3 cm and height 23 cm was considered for the hydrolysis of GAGs in soak liquor under continuous mode.

2.5. Analytical techniques

The parameters such as COD, BOD_5, TS, TDS and TSS were determined in accordance with the procedures summarized in standard methods for analysis of water and wastewater, APHA, 2012 [38]. The GAGs were determined by taking the sample of volume 0.5 ml to which 0.5 ml of aqueous phenol, 0.5% and sulphuric acid, 2.5 ml were added and incubated for 20 min. The absorbance was measured at λ_{490} nm [39]. The glucosamine content was determined as follows. The aliquot of sample containing glucosamine of 10–50 µg was transferred to capped tubes containing 10 ml of freshly prepared Acetyl acetone, 2% (v/v) in 0.5 mM sodium carbonate and placed in boiling water bath for 15 min. After cooling in tap water, 5 ml of ethanol, (95% v/v) and Ehrlich's reagent, 1 ml were added and mixed very well. The pink colour was changed into purple colour at the end of the reaction and the absorbance was measured at λ_{585} nm. The glucuronic acid was determined by mixing 1 ml of the sample containing 5–20 µg of glucuronic acid with sulphuric acid reagent, 5 ml (0.025 M sodium tetra borate in sulphuric acid medium) in glass capped tube at ice cold condition under slow shaking in the beginning and followed by vigorous shaking. Then the tubes were placed in boiling water bath for 10 min and cooled to room temperature. It was followed by the addition of carbazole reagent, 0.2 ml (0.125% carbazole in absolute alcohol) and mixed thoroughly. It was heated in a boiling water bath for 10 min and cooled. Then the absorbance was measured at λ_{530} nm.

2.6. Instrumental analysis

The FT-IR analysis was carried out for the confirmation of functional groups present in m-NPAC and Am-NPAC and it was also used to determine the extent of degradation of GAGs content of soak wastewater (Perkin Elmer infrared spectrophotometer, USA). The pore size distribution, pore volume and surface area of NPAC were evaluated using automatic adsorption instrument (Quanta chrome Corp. Nova-1000, USA). The elemental composition of NPAC sample was analysed by CHNS analyser (CHNS1108, model Carlo–Erbaanalyser, Germany). The crystalline nature of m-NPAC and Am-NPAC was determined by X-ray diffraction (XRD) analysis (Rich Siefert 3000 Diffractometer) using Cu-Kα1 radiation (λ = 0.1541 nm). The surface morphology of support matrix before and after the immobilisation of enzyme was evaluated using SEM analysis (JEOL JM 5600 electron microscope at 20 kV, Japan). TGA from 30 to 800°C (TGA Q50) and DSC analysis from 30°C to 300°C (V20.6 Build 31, USA) were carried out under nitrogen atmosphere with a temperature gradient of 10°C min^{-1}. The degradation of GAGs content was confirmed by UV-visible absorption spectroscopy (CARY 5E UV–VIS-NIR Spectrophotometer, USA). The fluorescence spectrophotometric study was carried out for soak wastewater before and after treatment with Am-NPAC in UV-Visible range (Cary Eclipse, USA).

3. Results and discussion

3.1. GAGs contents of tannery soak wastewater

Mucopolysaccharides are the bioorganic compounds widely present in the salt laden tannery wastewater (soak wastewater) owing to leaching from the raw materials (animal skins/hides) used in manufacturing of leather. Mucopolysaccharides or glycosaminoglycans are the long unbranched polysaccharides, they have repetitive units of glucosamine and glucuronic acid in the chain as shown in Fig. 1. The presence of small amount of fat (about 6–7%)in the raw material affects the leaching of MP's and protein from the inter fibrillar spaces into soak wastewater. Glycosaminoglycans have high degree of heterogeneity with respect to molecular mass, disaccharide construction, and sulfation. The complete elimination of organic components of soak wastewater will be a challenging issue if the degradation/fragmentation of MP's were not carried out. Hence, the biodegradability of soak wastewater can be increased through the degradation/fragmentation of macro molecules into smaller units so that treatment of soak wastewater with high efficiency can be achieved. In this study, the

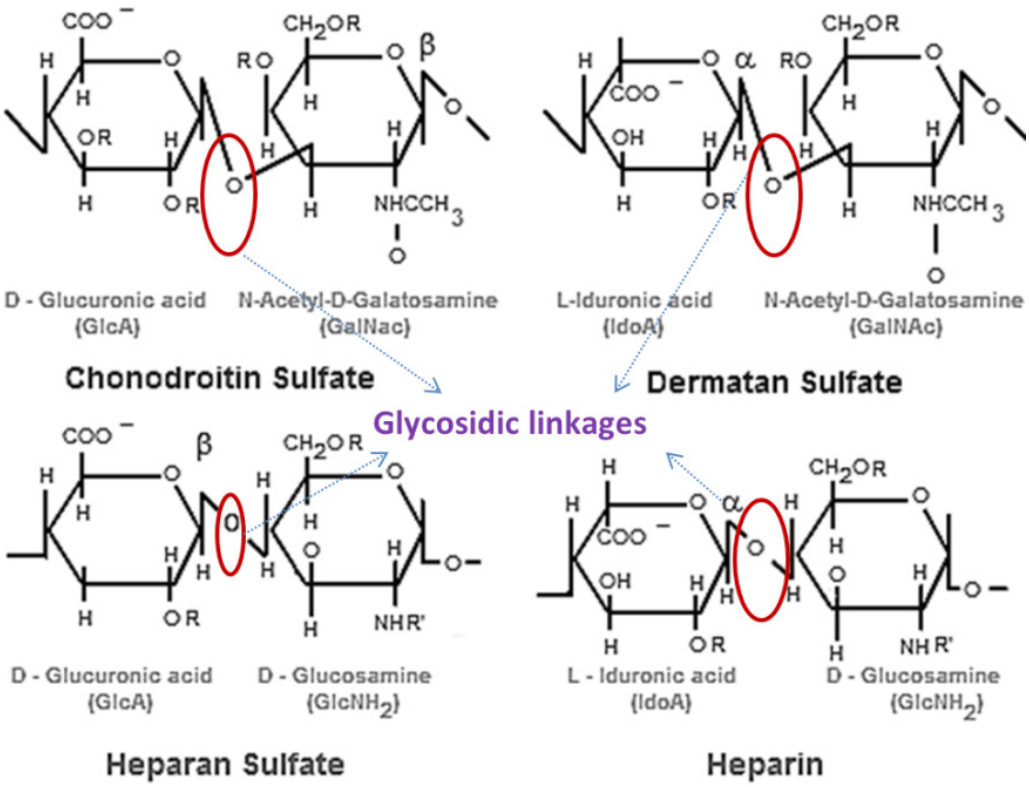

Fig. 1. Types of mucopolysaccharides/glycosaminoglycans present in skin/hide matrix.

validation was done on the formation of glucuronic acid and glucosamine by the degradation of MP's of soak wastewater by using Am-NPAC.

3.2. Production of salt tolerant amylase using Providencia Rettgerri sp.

The halophilic bacteria were isolated from the tannery soil acclimatized with soak wastewater and identified as *Providencia Rettgerri sp.* by the application of 16SrDNA sequencing analysis as presented in Fig. 2. The halophilic bacteria, *Providencia Rettgerri sp.* produced the saline tolerant amylase with the support of animal fleshing (2% w/v) as the substrate in minimal media along with the addition of growth factors in the medium. The most suitable growth conditions were maintained for the production of amylase, and the conditions are mentioned in Table 1 [40,41]. The amylase was precipitated by acetone precipitation technique from the reaction media and followed by dialysis to remove the alkali and alkaline earth ions and stored in the refrigerated condition at 4°C after the centrifugation at 20000 rpm for 20 min. The activity of purified amylase was observed to be 1320 U/ml and was used in further studies.

3.3. Optimization studies for immobilisation of amylase onto m-NPAC

The characteristics of nanoporous activated carbon are presented in the supplementary materials 1.1. and the significance of the parameters were discussed in our earlier studies [32–35]. The effect of time for the immobilisation of amylase was determined by validating the minimum amylase concentration obtained in the medium after the reaction between saline tolerant amylase and m-NPAC. The immobilization capacity of m-NPAC was calculated using the Eq. (S1) and (S2). The contact time required for the maximum immobilisation of amylase onto m-NPAC was found to be 120 min as shown in Fig. S1a. The pH optimization study concluded that the addition of acid or base for the adjustment of pH of the reaction medium impaired the immobilisation capacity of the matrix and thus there was no need for the adjustment of pH for the degradation kinetics as shown in Fig. S1b. The effect of temperature on immobilisation of amylase onto m-NPAC was determined by conducting the experiments from 20°C to 30°C with an incremental increase of 2°C. It was observed that the maximum immobilisation of amylase onto m-NPAC was obtained at 28°C and it remained the same at 30°C (approximately 90% of the initial concentration of amylase) as shown in Fig. S1c. Hence, the optimum temperature was selected as 28°C for the maximum immobilisation of amylase for the elimination of GAGs present in tannery soak wastewater. The immobilisation capacity of the applied matrix, m-NPAC was evaluated for the maximum immobilization of amylase enzyme. Hence, the effect of mass of m-NPAC and the effect of amylase concentration for maximum immobilisation were studied and shown in Fig. S1d and Fig. S1f. The results obtained from the experiments concluded that the immobilisation attained the maximum at 250 amylase units/g of m-NPAC. It was observed that a linear relationship was established between the mass of m-NPAC and the activity of amylase

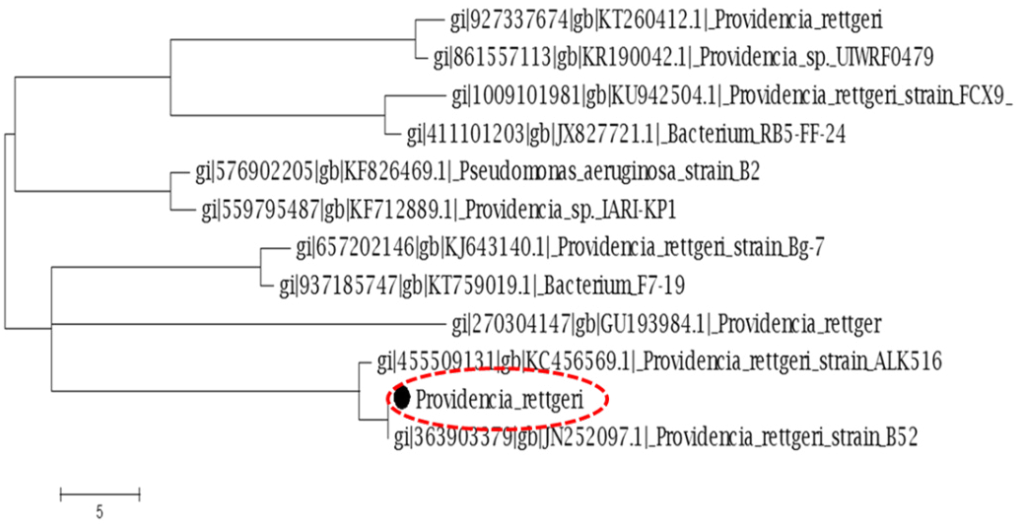

Fig. 2. Phylogenetic tree for the identification of halophilic organism *Providencia Rettgerri sp.* isolated from contaminated tannery soil for the production of saline tolerant amylase for the degradation of MP's.

Table 1
Optimization studies for the production of saline tolerant amylase from *Providencia Rettgerri sp*

S. No.	Factors	Optimum conditions
1.	Time (h)	60
2.	pH	7
3.	Temperature (°C)	40
4.	Salinity (%)	4
5.	Substrate concentration (%)	2

concentration. Hence, this study may be considered as an important tool for designing the Am-NPAC packed reactor for the removal of GAGs from soak liquor. The effect of salinity of the soak wastewater on denaturation of immobilised amylase was determined by varying the salinity of soak liquor. It was observed that the increase in the salinity of the soak wastewater ensured no disruption on the immobilised amylase in Am-NPAC during the process of eliminating GAGs in soak wastewater discharged from leather industry as shown in Fig. S1e.

3.4. Instrumental analysis

3.4.1. TGA analysis

TGA of m-NPAC and Am-NPAC are shown in Figs. 3a, b. Fig. 3a shows that there was a steady weight loss occurred in the temperature range from room temperature to 800°C. The weight loss at 150.20°C and 153.8°C are due to the movement of water molecules and hydrated organic molecules held on m-NPAC and Am-NPAC. The weight lossess at 432.24°C and 446.1°C are due to the liberation of ammoniacal nitrogen based organic compounds held on m-NPAC and Am-NPAC [34]. The movement of amine compounds from m-NPAC and Am-NPAC may be originated from ethylene diamine and glutaraldehyde which were added during the functionalization of NPAC and thereof for the immobilisation of amylaseto produce Am-NPAC. The final residue of m-NPAC and Am-NPACwere 72.02% and 63.08% respectively at the temperature of 800°C. The difference in weight loss was observed between m-NPAC and Am-NPAC, it might be owing to the elimination of volatilic components in the form of amine-based compounds present in the immobilised amylase. The activation energy of m-NPAC (for binding of EDA and Glutaraldeyde condensed layer) and Am-NPAC (for binding of enzyme layer onto m-NPAC) was calculated. The activation energy was maximum (1.3576 kJ/mol) for Am-NPAC than m-NPAC (0.8760 kJ/mol) which may be attributed to the presence of additional inter molecular bonds in Am-NPAC [40].

3.4.2. Differential scanning colorimetry analysis (DSC analysis)

The thermograms of differential scanning colorimetry form-NPAC and Am-NPAC were recorded from room temperature to 300°C as (Figs. 3c, d). Fig. 3c shows the presence of an endothermic peak in the thermogram at 114.25°C and 195.73°C respectively, may be atrributed to the energy absorption for the removal of water of crystallization and the removal of nitrogen based compounds. The expulsion of nitrogen based compounds may be from the bound layer of ethylene diamine and glutaraldehyde which were added during functionalization of NPAC. The energy of absorption at 114.25°C and 195.73°C was in the range of –3.27 mW and –4.34 mW for m-NPAC. The DSC thermogram of Am-NPAC shows the energy absorption of –9.12 mW at 114.44°C and –4.47 mW at 202.58°C which was comparatively more than the energy of absorption bym-NPAC [41]. The increase in energy of absorption confirms the firm bonding nature of amylase onto m-NPAC through primary bonding. Thus, the firm bonding of amylase onto m-NPAC established a localisation of amylase without dis-

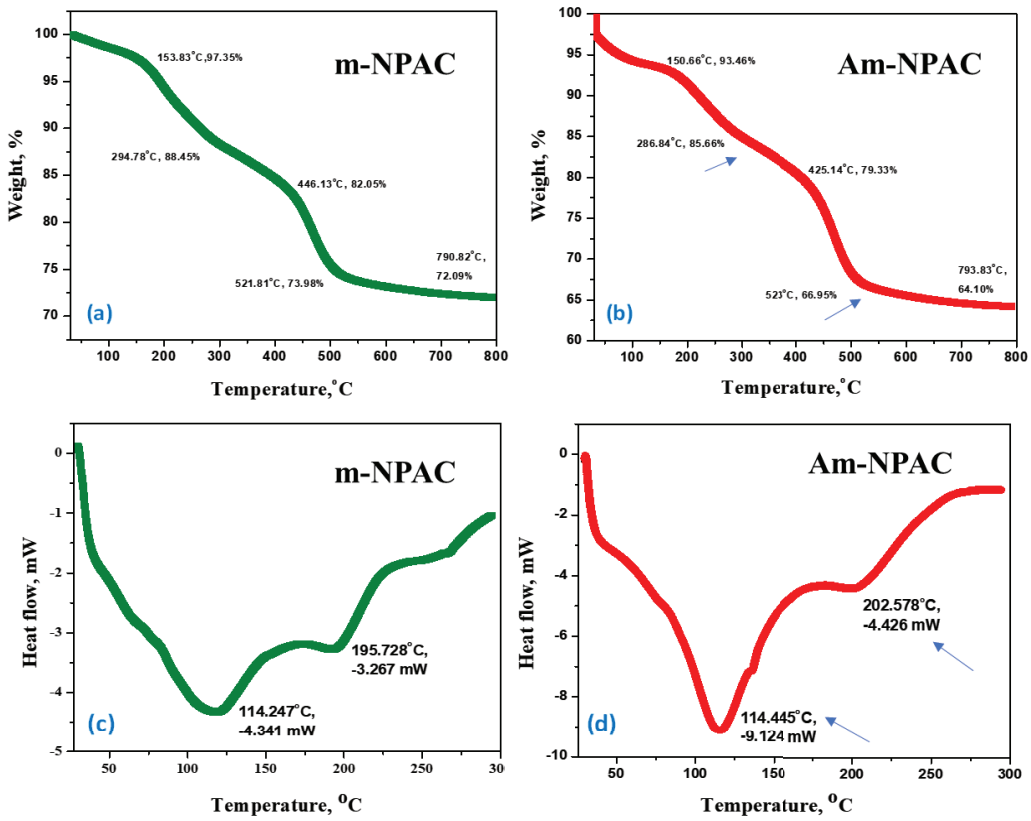

Fig. 3. (a, b) TGA and (c, d) DSC spectra of m-NPAC and Am-NPAC.

lodging from m-NPAC during the hydrolysis of GAGs in soak liquor.

3.4.3.SEM and XRD analyses

The surface morphology of m-NPAC and Am-NPAC was scanned to confirm the immobilisation of amylase onto m-NPAC. Figs.4a-bshow that the amylase was immobilised inside the pores of m-NPAC. The aldehyde group in the modified carbon matrix (m-NPAC) may support the binding of the saline tolerant amylase to locate at the outer pore surface area of m-NPAC through primary interactions [42,43]. The assembling/clustering of amylase molecules as observed in SEM images support the proposition of strong interaction requirement for the firm holding of amylase onto m-NPAC without dislodging into the reaction medium. The peaks spotted in the XRD spectrum of m-NPAC at 2θ values of 21° and 42° represent the amorphous nature and the formation of small noise due to the aldehyde functionalization onto the surface. The XRD spectra of Am-NPAC shown in Fig. 4c, 4d indicate the additional crystalline peak values at 2θ values of 26.5° and 39.8°, may be attributed to the presence of amylase onto m-NPAC [44].

3.4.4. FT-IR spectroscopic studies

FT-IR spectra (Fig. 4e,4f) of m-NPAC and Am-NPAC were recorded at the wavenumber ranges from 4000 cm^{-1} to 400 cm^{-1} which were used to confirm the presence of functional groups and types of bonding present in the materials. The presence of doublet peaks at 3200 cm^{-1}–3500 cm^{-1} correspond to the existence of asymmetrical and symmetrical N-H stretching vibrations of primary or secondary amine groups associated with m-NPAC. The absorption peaks at 2900 cm^{-1}–2800 cm^{-1} designate the presence of C-H stretching of alkyl chain present in both m-NPAC and Am-NPAC. The peaks present at the region of 1650 cm^{-1}–1700 cm^{-1} corresponds to –C=O stretching vibrations of amide group exist in m-NPAC and Am-NPAC. The peaks at 1254 cm^{-1} and 1046 cm^{-1} can be attributed to the presence of asymmetric and symmetric C-O-C stretch of the $–OCH_3$ groups. The peaks at 1601 cm^{-1} and 1501 cm^{-1} indicated the presence of C=C ring stretch and the peak at 699 cm^{-1} represents the out of plane C=C bending vibrations of aromatic groups present in the samples. The increase in the intensity of peaks at 3200–3500 cm^{-1} and 1400–1500 cm^{-1} regions confirm the immobilisation of amylase. The C-N stretching and a broad out of plane bending vibrations was observed at 1432 cm^{-1} and 700 cm^{-1}–600 cm^{-1} confirmed the covalent interactions between amylase and m-NPAC.

3.5. Adsorption isotherm models for the immobilisation of amylase on m-NPAC

The Langmuir, Freundlich, and Dubinin–Radushkevitch adsorption isotherm models were developed based on the postulation of mono/multi-layer interactions for the immobilisation between the surface of m-NPAC and the

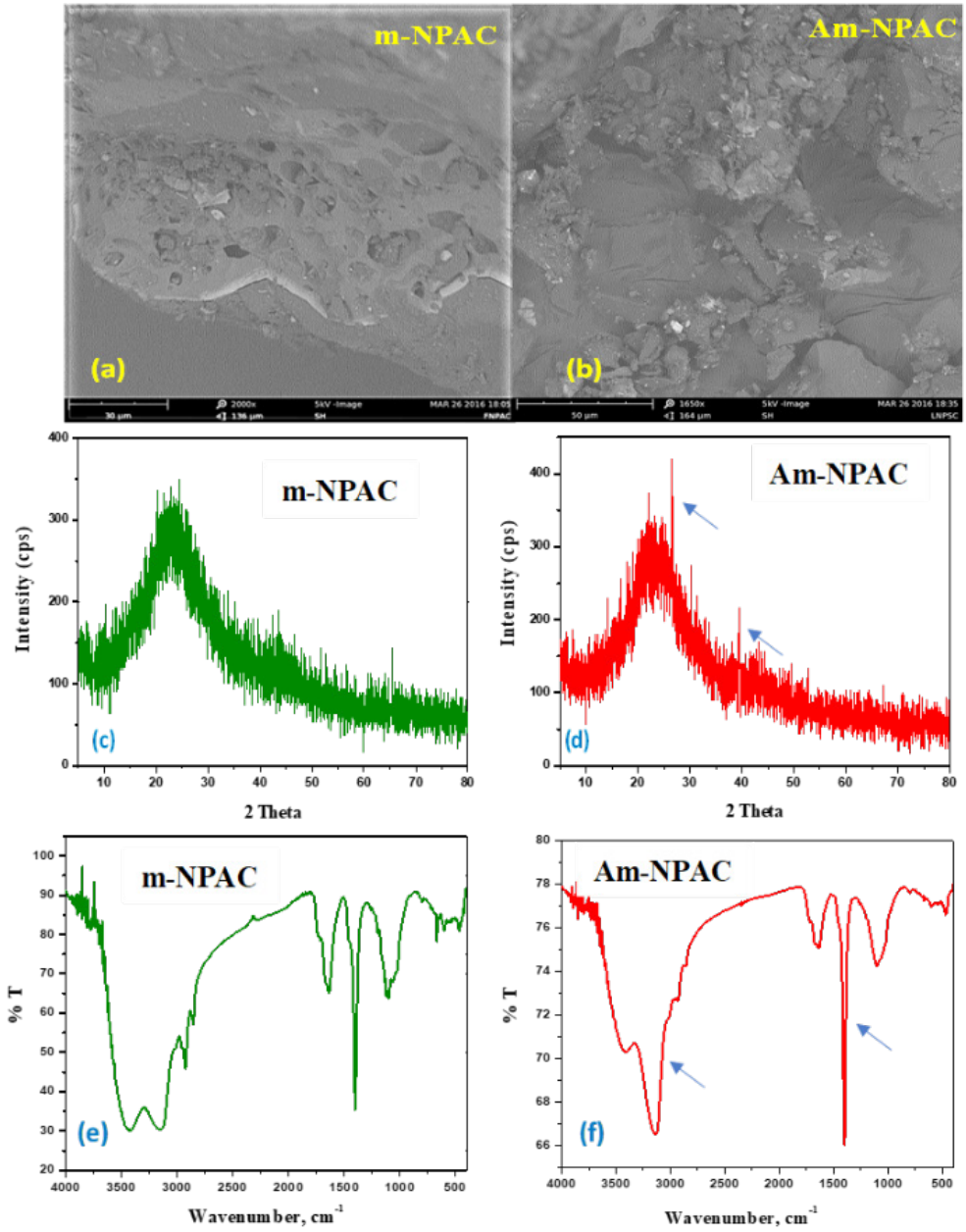

Fig. 4. (a, b) SEM, (c, d) images, XRD spectra and (e, f) FT-IR spectra of (a) m-NPAC and (b) Am-NPAC.

amylase with uniform energies of adsorption [35,45]. The following equation defined the linear expression of Langmuir adsorption isotherm model of immobilisation,

$$\frac{c_e}{q_e} = \frac{1}{k_L} + \frac{a_L}{k_L} c_e \tag{1}$$

where q_e (U/g) and C_e (U/L) are the amount of amylase adsorbed per unit weight of m-NPAC and equilibrium liquid phase concentration of amylase (U/L) respectively. k_L (L/g) and a_L (L/U) are the Langmuir equilibrium constants. The Freundlich adsorption isotherm (multi layer model) was applied to the adsorption onto heterogeneous surfaces and is expressed by Eq. (2)

$$\log\left(q_e\right) = \log K_F + \frac{1}{n}\log c_e \tag{2}$$

where K_F is the Freundlich constant and n is the Freundlich exponent. The Dubinin–Radushkevitch isotherm model which is expressed based on the Polanyi theory and is expressed in linear form by Eq. (3)

$$\ln q_e = \ln q_D - 2B_D RT \ln\left(1 + \frac{1}{c_e}\right) \tag{3}$$

The R^2 values for adsorption isotherms were found to be 0.9652, 0.9813 and 0.9375 with respective isotherm models as shown in Figs. 5a–c. Among them, the Freundlich iso-

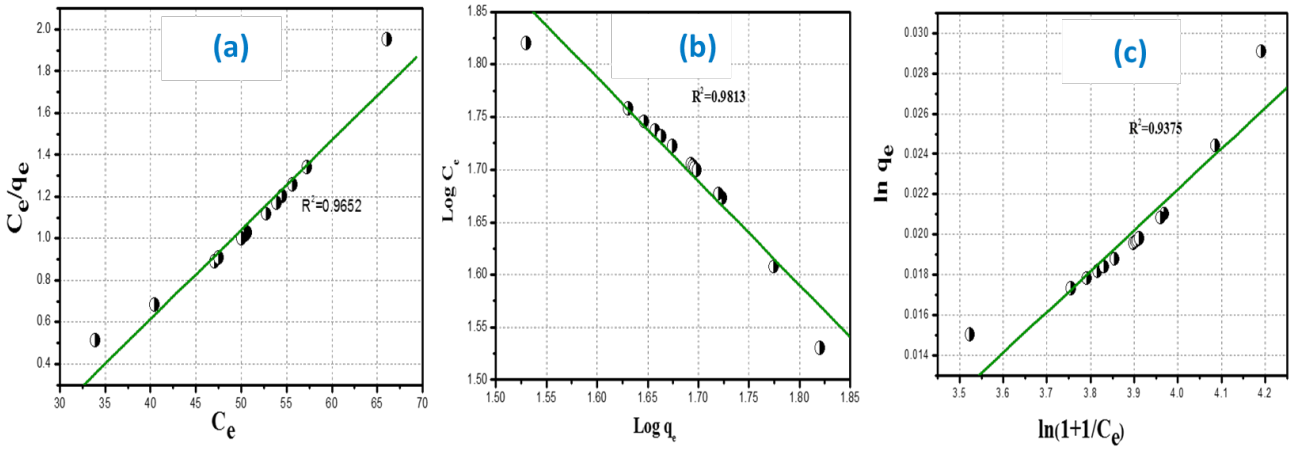

Fig. 5. (a) Langmuir (b) Freundlich and (c) Dubnin-Radushkevitch adsorption isotherm models for the immobilisation of saline tolerant amylase onto the surface of m-NPAC.

therm model obeyed best for the experimental data based on regression coefficient (0.9852). It indicates that there was the formation of multilayer physical interactions between m-NPAC and the saline tolerant amylase during immobilisation. Specifically, the Van der Waals forces play a major role in adsorption between amylase and m-NPAC.

3.6. Removal of GAGs from salt laden tannery wastewater

The concentration of organic constituents (proteins, lipids, and glycosaminoglycans) and total dissolved solids content of tannery soak wastewater are presented in Table 2. The high salt concentration of soak liquor inhibited the microbiological degradation of organic pollutants in it and, thus, conventional biological treatment of wastewater was very difficult. The total suspended solids (TSS) of tannery wastewater was removed initially by using sequential oxic-anaerobic bioreactor (SOAR) to facilitate the removal of organic constituents in soak wastewater by Am-NPAC. The SOAR was inoculated with mixed consortia of salt tolerant microorganisms and the filamentous bacteria for the removal of TSS. The hydraulic retention time (HRT) was optimized and it was fixed as 12 h. SOAR removed COD, 35.64%; BOD, 22.3%; total suspended solids, 77.5% besides the removal proteins, lipids and glycosaminoglycans by few odd percentages at the high saline environment. The structural deformation and cleavage of proteins, lipids, and glycosaminoglycans were evidenced through the formation of metabolites in SOAR. It was confirmed through the estimation of biological oxygen demand$_5$ (BOD_5) in raw soak wastewater and in the treated wastewater samples. The increase in BOD_5:COD from 0.328 to 0.394 is attributed to the enhanced removal of GAGs [36,37]. The SOAR treated soak wastewater was selected for the degradation studies under batch and continuous mode operations. The optimization studies were carried out with batch mode by taking the reaction mixture (SOAR treated tannery TDS wastewater and Am-NPAC) at the contact time from 10 min to 3 h. The aliquot of samples were analysed for GAGs, glucosamine and glucuronic acid. The glycosaminoglycans

Table 2
Characteristics of soak liquor before and treatment with Am-NPAC

Parameters	Soak liquor	After SOAR process	Am-NPAC reactor outlet
pH	7.42	7.15	6.82
Chemical Oxygen Demand (COD)	6340	4080	3340
Biochemical Oxygen Demand $(BOD)_5$	2080	1610	1480
BOD_5: COD	0.328	0.394	0.443
Total Dissolved Solids (TDS)	44350	43940	43120
Total Suspended Solids (TSS)	1420	320	240
Total Solids (TS)	45770	44260	43360
Protein	408	247	238
Lipid	147	104	98.5
GAGs	305	245.2	6.2
Amino acid	4.0	18.4	21.4
Fatty acid	10.4	21.8	23.2
Glycerol	0.29	6.6	8.5
Glucosamine	12.7	7.05	165.4
Glucuronic acid	0.2	8.4	38.9

All the parameters except pH, BOD_5: COD are expressed in mg/L.

of tannery wastewater (245.2 mg/L) was removed by 90% during the treatment with Am-NPAC in 1 h. The formation of glucosamine, 165.4 mg/L and glucuronic acid, 38.9 mg/L were recorded. The removal of GAGs was not at the noticeable level on increasing the reaction time for more than 1 has shown in Figs. 6a, b. So, the optimal hydraulic retention time (HRT) for the GAGs removal was considered as 1 h. The effect of pH on the reaction was determined by subjecting the reaction mixture at different pH conditions, from pH 3 to pH 10 at optimised time, 1 h. The aliquot of sam-

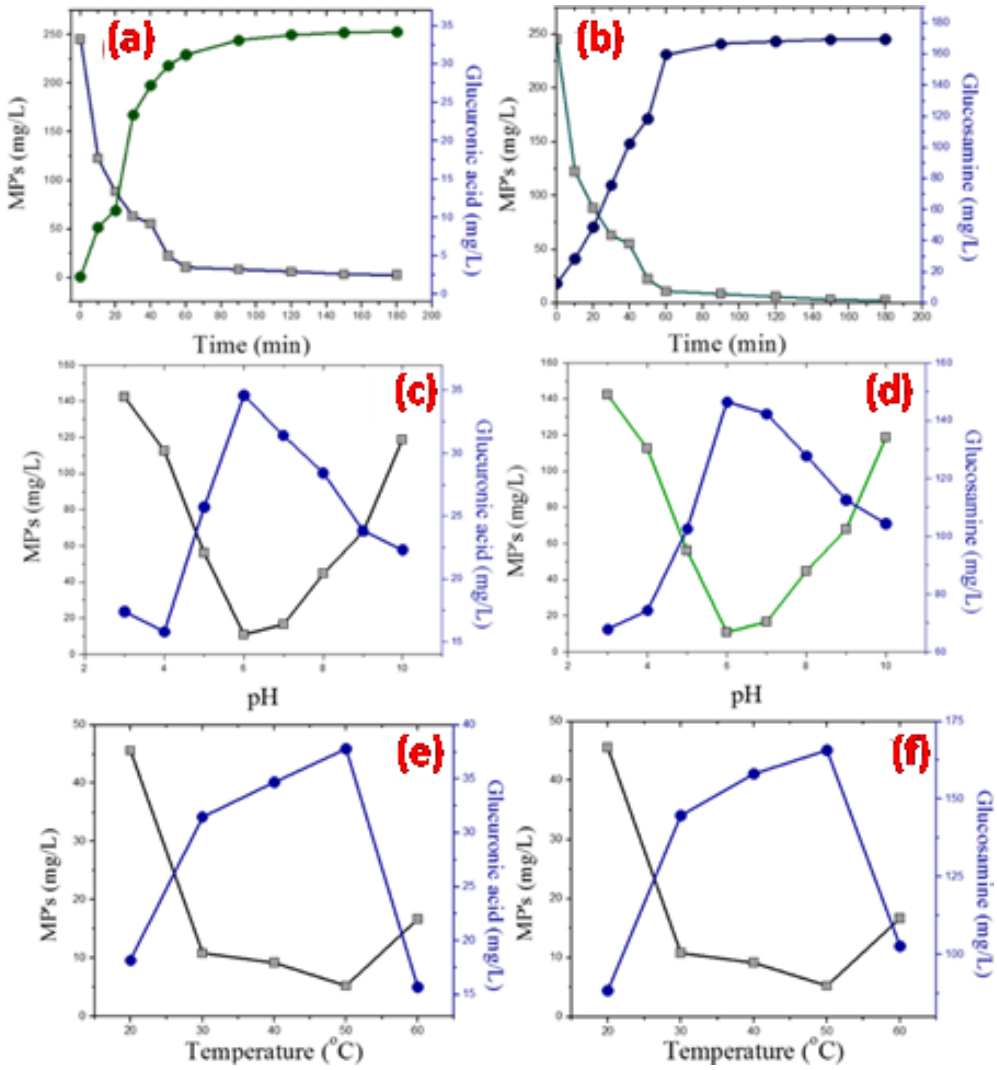

Fig. 6. Optimization of (a, b) time (c, d) pH and (e, f) temperature for the degradation of mucopolysaccharides content of soak liquor concerning the formation of (a) glucuronic acid and (b) glucosamine.

ples were collected and analysed for GAGs, glucosamine and glucuronic acid. The removal of glycosaminoglycans was very minimum (less than 40%) at the lower acidic pH conditions (pH 3 and pH 4). It was found that the hydrolysis of GAGs was increased from pH 5 and the removal was 90% at pH 6 and pH 7 as shown in Figs. 6c, d. The catalytic efficiency of Am-NPAC was decreased at high alkaline conditions (pH 9 and pH 10). The effect of temperature on the hydrolysis of GAGs by Am-NPAC was evaluated by carrying out the reaction at the temperature ranges from 20°C to 60°C at optimal conditions (reaction time, 1 h and pH 7). The removal of glycosaminoglycans was increased from 30°C and the removal was 99.2% at 50°C as shown in Figs. 6e, f. Several researchers reported that the effect of high temperature on the cleavage of (-C-O-C) bonding present in glycosaminoglycans. This may favour the fragmentation of GAGs into glucosamine and glucuronic acid even at high temperature conditions.

Hence, the application of Am-NPAC for the removal of GAGs was active up to 50°Cand sustained at alkaline conditions. At optimized conditions, the Am-NPAC packed bed reactor was performed under continuous mode for the degradation as mentioned in Fig. 7. The COD was reduced from 4080 mg/L to 3340 mg/L and the total suspended solids was removed from 320 mg/L to 240 mg/L. The increase in BOD_5:COD value up to 0.443 may favour the subsequent biological treatment methods. The Am-NPAC packed bed column reactor was operated under continuous mode at the optimized conditions. Hence, the present study concluded that the removal of GAGs from high TDS tannery wastewater was feasible by Am-NPAC. The elimination of GAGs in soak liquor could reduce the viscosity of the medium which amplified the accessibility of organic components to the biocatalyst and consequently the biodegradability of tannery soak wastewater was enhanced. The fragmentation of GAGs content would support the degradation of metabolites in soak liquor by halophilic bacteria in biological treatment [46]. Hence, the present study concludes the complete destruction of fragmented metabolites into safe end-products (CO_2, NH_3, and SO_4) by using halophilic bacteria after

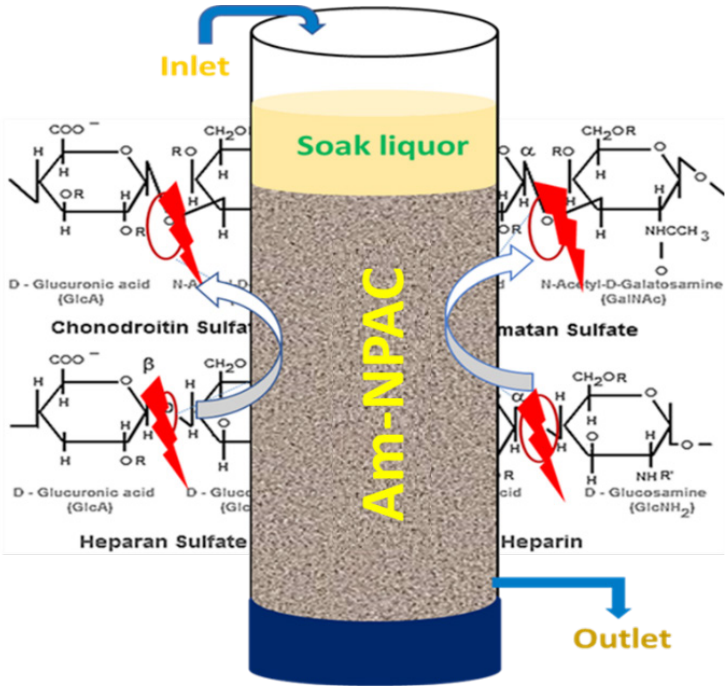

Fig. 7. Schematic representation of the degradation of mucopolysaccharides of soak liquor using Am-NPAC packed bed reactor.

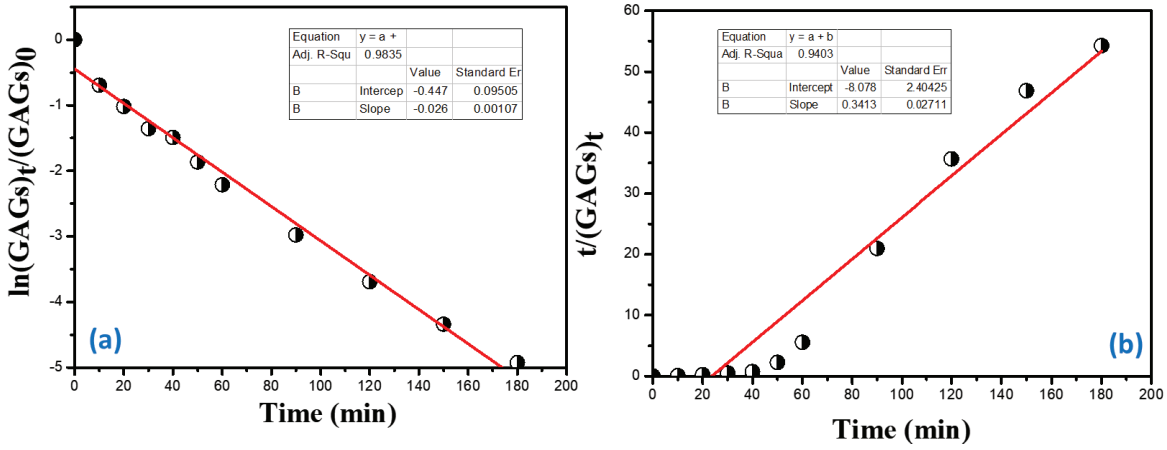

Fig. 8. (a) *Pseudo* first order kinetics and (b) *Pseudo* second order kinetics for the degradation of mucopolysaccharides content of soak liquor.

the removal of suspended solids by SOAR and enzymatic hydrolysis in Am-NPAC packed reactor.

3.6.1. Kinetics for the elimination of GAGs content of tannery soak wastewater

The kinetics for the elimination of GAGs content of soak wastewater using Am-NPAC was evaluated by applying *pseudo* first order and *pseudo* second order kinetic models as presented in Eqs. (4) and (5)

$$ln(GAGs)_t / (GAGs) = -k_1 t \quad (4)$$

$$1/(GAGs)_t = 1/(GAGs)_0 + k_2 t \quad (5)$$

where $(GAGs)_0$ is the initial concentration of GAGs content of soak wastewater (mg/L), $(GAGs)_t$ is GAGs content (mg/L) at time t, k_1 and k_2 are the *pseudo* first order and *pseudo* second order rate constants. The kinetic study for the elimination process was carried out and the results are shown in Figs. 8a, b. The results demonstrate that the GAGs elimination of soak wastewater by Am-NPAC followed the *pseudo* first order rate kinetic model with R^2 value of 0.9835 with rate constant (k_1), 1.78×10^{-4} min^{-1}. This confirms that the rate of the elimination of GAGs majorly depends upon its concentration in soak wastewater.

3.6.2. Instrumental evidence for the degradation of mucopolysaccharides

The degradation/fragmentation of GAGs content of soak wastewater was evaluated using UV-visible, fluorescence and FT-IR spectroscopic studies (Figs. 9a, b). UV spectrum shows the peaks at λ_{280}nm and at λ_{220}nm, may be correlated to the presence of MP's component of soak wastewater. The intensity of the respective peaks was decreased and also the peaks were shifted to red region after the treatment in Am-NPAC packed column at different time intervals from 10 min to 180 min. The fluorescence spectrum of initial soak wastewater shows the high intensity peak at λ_{622} nm and it was decreased gradually with increase in reaction time for the Am-NPAC treated samples as shown in Fig. 9b [41]. This confirmed the hydrolysis of GAGs contents of soak wastewater.

The peaks in the region of 3400 cm^{-1}–3200 cm^{-1} are attributed to N-H stretching vibrations, may be related to nitrogen related components in soak wastewater as shown in Figs. 9c, d. The symmetrical C-H stretching of methyl groups are correlated with the peaks recorded at 2500 cm^{-1} [47]. The presence of peaks in the region 1600–1650 cm^{-1} specifies the occurrence of amide bond (-C (=O)-NH), related to the peptide content of soak wastewater. The peak recorded at 1200 cm^{-1} may be attributed to the carbonyl group stretching in –COOH functional group of glycosaminoglycans. The presence of peaks in the region of 700–600 cm^{-1} relates to -N-H bending vibrations of the nitrogen components present in tannery soak wastewater. FT-IR spectrum of Am–NPAC treated soak wastewater shows the peak at 1401 cm^{-1}, may signify –C=O stretching of the glucuronic acid resulting from the cleavage of bonds in MP's present in soak wastewater. The peaks in the region of 1250–1150 cm^{-1} corresponds to -C-C (=O)-O bond vibrations of the glucuronic acid. Hence, FT-IR spectrum confirmed the elimination of MP contents present in the soak wastewater. The elimination of MP contents of soak wastewater and increase in the concentration of glucuronic acid and glucosamine units were observed in the saline medium, however, it lacks the dischargeable standards. The undegraded components of soak wastewater may cause the environmental issues. Hence, the present investigation was further extended to complete the mineralization of glucuronic acid and glucosamine units by the use of effective microorganisms inoculated aerobic fluidized bed reactor.

4. Conclusions

The present investigation concluded that the mucopolysaccharide contents in hypersaline soak wastewater were effectively eliminated by salt tolerant amylase immobilised nanoporous activated carbon (Am-NPAC). The amylase extracted from *Providencia Rettgerri sp.* shows high amylase activity, 1320 U/ml at saline medium. EDA/Glutaraldehyde

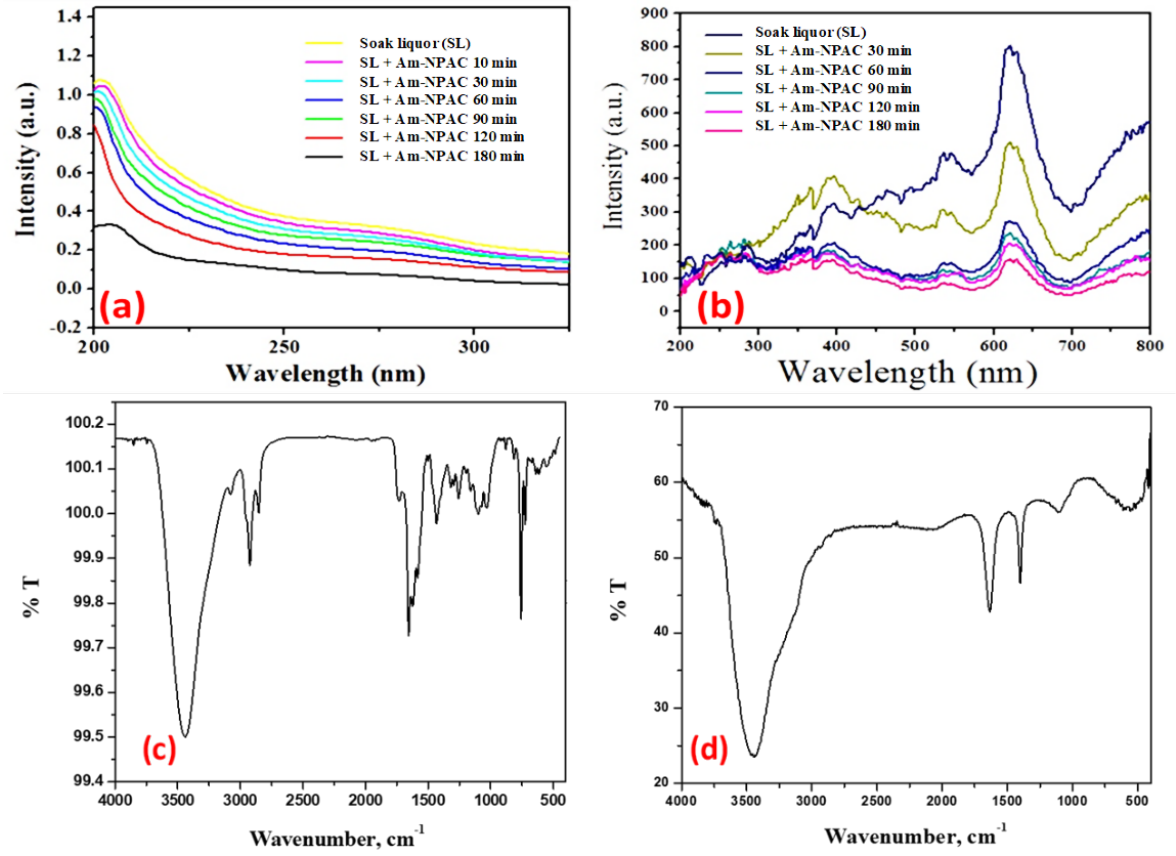

Fig. 9. (a) UV-visible and (b) UV-fluorescence spectra and (c) FT-IR spectra of degraded mucopolysaccharide contents of soak liquor using Am-NPAC.

modified nanoporous activated carbon was used for the immobilisation of saline tolerant amylase. The instrumental methods such as FT-IR, SEM, XRD and TGA-DSC confirmed the strong immobilisation of amylase in m-NPAC. The immobilisation of saline tolerant amylase onto m-NPAC followed Freundlich adsorption isotherm model and confirmed that the adsorption process was multilayer physical interactions. The elimination of mucopolysaccharide content of soak wastewater was achieved by using Am-NPAC at optimized conditions. The degradation of GAGs followed first order kinetics with rate constant 1.78×10^{-4} min^{-1}.

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Supplementary materials

1. Results and discussion

1.1. Characteristics of NPAC used for amylase immobilization

The attributes of NPAC used in this study for the immobilization of saline tolerant amylase because of its characteristics such as surface area, 379 m^2/g; pore volume, 0.188 cm^3/g; pore diameter, 39.36 Å; CHNS (41.5%, 2.85%, 0.7%, 0.612%) and free electron density, 1.60×10^{22} spins/g reported in our previous studies. The maximum reflectance measured at λ_{800}nm in UV-visible spectrophotometer correlates with the energy gap (Eg) of 1.55 eV for NPAC shows extrinsic semiconductor property.

1.2. Optimization studies for the saline tolerant amylase immobilised onto m-NPAC

The optimum time, pH, temperature, salinity, mass of m-NPAC and amylase concentration were studied and mentioned in Fig. S1a–Fig. S1f. The immobilization capacity of m-NPAC was calculated using the following Eq. (S1),

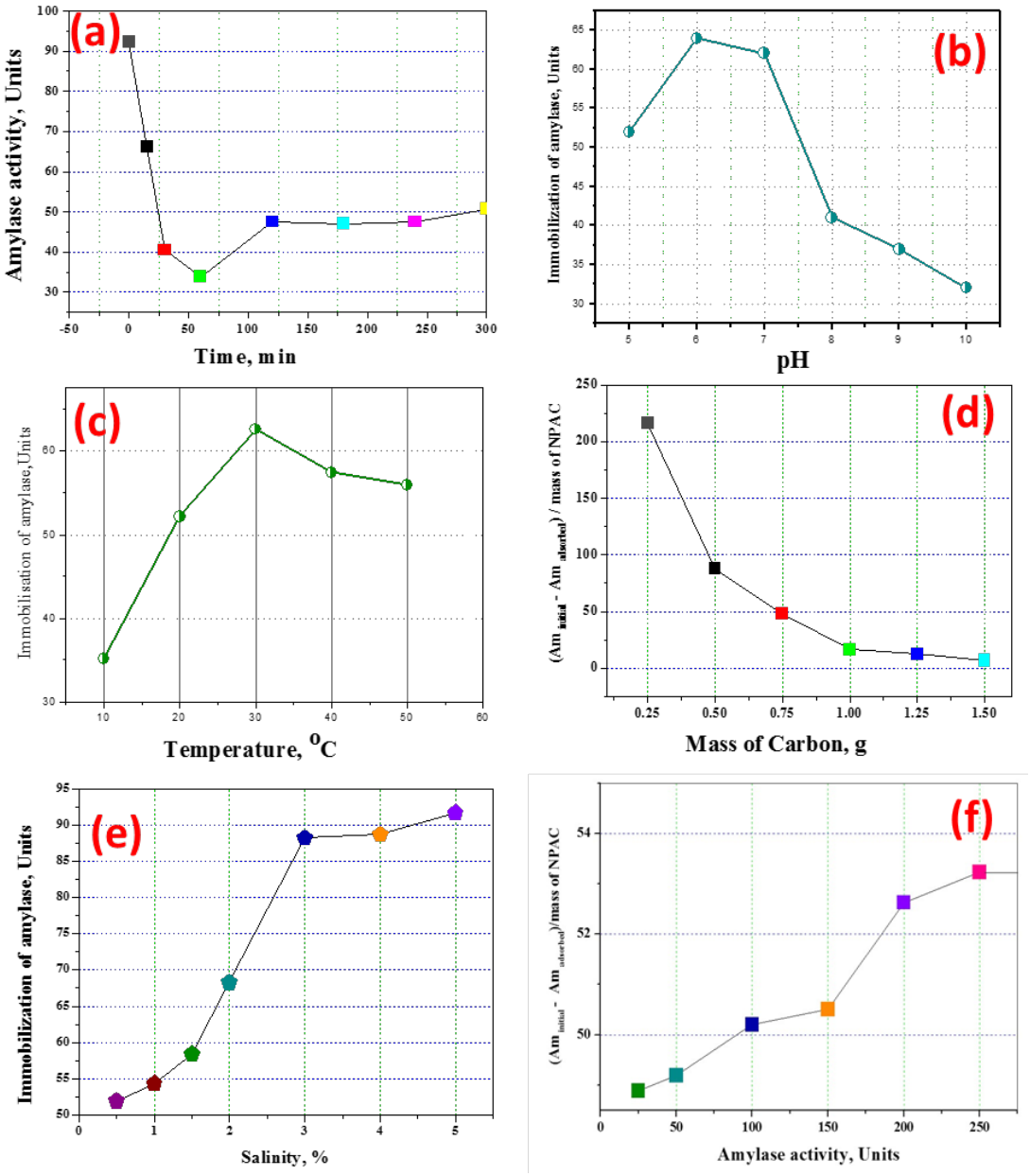

Fig. S1. Optimization studies on the effect of (a) time (b) pH (c) temperature (d) mass of carbon (e) salinity and (f) amylase concentration on the immobilization of amylase onto m-NPAC.

$$Immoblization\ capacity\ of\ m-NPAC, \left(\frac{U}{g}\right)=\left(\frac{A_0-A_t}{M}\right)$$

× Volume of the sample (mL) S1

where, A_0 is initial amylase concentration (U/ mL), A_t is immobilized amylase concentration (U/mL), and M is mass of m-NPAC. The percentage of immobilization of saline tolerant amylase onto m-NPAC was determined by the following Eq. (S2),

$$Amylase\ immobilization, \% = \left(\frac{A_0-A_t}{A_0}\right)\times 100 \quad (S2)$$

where, A_0 be the initial amylase concentration (U/mL), A_t be the immobilized amylase concentration in m-NPAC.