Alinteri J. of Agr. Sci. (2021) 36(2): 156-165
e-ISSN: 2587-2249
info@alinteridergisi.com

http://dergipark.gov.tr/alinterizbd

http://www.alinteridergisi.com/
DOI:10.47059/alinteri/V36I2/AJAS21129

RESEARCH ARTICLE

Isolation of Alkalophilic Pectinolytic Bacteria and their Bio Retting Effect on Kenaf Fiber Compositions

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ARTICLE INFO

Article History:
Received: 08.06.2021
Accepted: 10.07.2021
Available Online: 26.07.2021

Keywords:
Bacterial Retting
Kenaf Bast Fiber
Non-cellulosic Gums
Pectinolytic Bacteria

ABSTRACT

Retting is the most limiting process of high-quality cellulosic kenaf bast fiber production which facilitating the separation of useable fiber from the plants' cell wall matrix. Existing traditional water retting approach confronts ineptitude and eutrophication related complications. Aiming to enhance the kenaf bio-retting process, sixty-seven alkalophilic bacterial colonies were isolated from paddy land soil sediments and kenaf retting water. These isolates were subsequently screened, of that two isolates were selected based on hyper qualitative and quantitative pectinolytic enzymatic measures. 16s rDNA gene sequence analysis revealed that both two strains were closely related to *Bacillus pumilus* species and designated as KRB56 and KRB22. These strains were applied in augmented non-sterile kenaf tank retting to investigate their kenaf retting efficiency and yielded fiber were analyzed for chemical compositions. Results revealed that, stains KRB56 and KRB22 significantly improve the retting process by degradation of 82.78% and 75.28% non-cellulosic gums, respectively comparing with uninoculated treatment niche (62.12%). These bacterial treated fiber samples showed thinner, smooth, and cleaner fibers surface morphology by SEM indicates sufficient non cellulosic gums (NCGs) removal comparing with URKF. Moreover, yielded fibers were examined for chemical composition, FTIR, XRD test. Results revealed that compare to un retted and un inoculated kenaf fiber, bacterial treated kenaf fiber increases cellulose portions, and their crystallinity index increases 35.50-41.30 % due to sufficient NCGs removal. This study's findings indicate that isolated alkalophilic bacterial strains KRB56 and KRB22 were effectively to be used as kenaf bio retting agents to produce quality kenaf fiber.

Please cite this paper as follows:

Hossain, M., Siddiquee, S. and Kumar, V. (2021). Isolation of Alkalophilic Pectinolytic Bacteria and their Bio Retting Effect on Kenaf Fiber Compositions. *Alinteri Journal of Agriculture Sciences, 36*(2): 156-165. doi: 10.47059/alinteri/V36I2/AJAS21129

Introduction

Demands of fast biodegradable commodities have increased many folds over the last decades due to injudicious utilization of slow degradable synthetics and their tremendous pollution hazards. In this regard, bast fiber has rapidly become a highly alternative source for supplying a continuous flow of biodegradable materials.

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In 2018 globally, more than eight million households are involved in producing six million mt of bast fibers (Opperskalski et al., 2019). Of that, kenaf (*Hibiscus cannabinus*) has great potential for industrial fibre and adapts in broad geographical areas. Genetically, kenaf and allied cellulosic bast fibers glued with other non-cellulosic components onto its plant's cell walls matrix. Extraction of

cellulose fibers from these plants' sources requires a post-harvest process called retting or degumming, facilitating the separation of useable fiber The majority of bast fiber crops provide a particular fiber bundle on their stems formed from a group of individual long fiber or crude fiber, possess an outstanding array of mechanical properties, which mostly inclined by growing conditions, retting, and other processing(Zimniewska et al., 2011). Selective biodegradation of non-cellulosic gummy substances (NCGs) is the central processing task of natural cellulosic fiber production from kenaf bast fiber plants(Narkpiban & Poonsawat, 2020). This obligatory post-harvest processing confers a wide range of advantages to its fibres. with desired quality. Among different retting approaches, biological retting of kenaf and allied bast fibers favours some advantageous features over chemical and others augmented process. Certain portions of these gummy substances are insoluble in water and require specific enzymatic steps to decompose these NCGs such as pectin, hemicellulose, and lignin. Microbial enzymes' combined actions, mainly bacterial extracellular enzymes and solvents-derived maceration are the critical biological retting components. Microbial community analysis revealed that the biological retting process was prevalent in the bacterial domain (Duan et al., 2020; Visi et al., 2013; Zhao et al., 2016).

The traditional water retting process has long been practiced and the only means of bast fiber in the many the hub of bast fiber producing South and Southeast Asian countries, which is feasible to handle a massive bast fiber volume with minimum input cost. It generally occurs in open field conditions by naturally grown mixed microbial populations confronts many issues related to efficiency and fiber quality. A proper level of retting within a minimum time is essential to achieve a high quality of fiber, whereas the traditional retting approach requires more extended retting periods, which eventually may decompose the basic cellulosic fibers. In mixed microbial endogenous populations, some microbes secrete specific enzymes, and the rest of them acts as non-producers, but all the microbes take nutrients from the sources for their metabolic activities. The non-enzyme producers are described as microbial cheaters and showed that higher costs of enzymes production favor cheaters and lower rate favored producers(Allison, 2005). The co-existence of cheaters and producers is highly organized but releases intermediate-level enzymes, which ultimately lingers the process and, consequently, over retting, under retting yields inconsistence low-quality fibers(Fernando et al., 2019). The selective microbial applications in the bio retting process arise because of these tumbling efficiencies; however, this process encompasses aquatic ecosystems pollution and in recent years faced severe water scarcity problems from open sources of water bodies(Majumdar et al., 2019; Zawani et al., 2015). Moreover, changing climatic conditions, socioeconomic trends, and inefficiency the whole bast industries become vulnerable for such retting difficulties. Therefore, alterations of retting approaches are now an agenda for enduring bast industries, and incorporation of bacterial agents could remediate these constraints in a biocompatible manner.

Biological retting of kenaf and allied fibers are the functions of enzymatic activities, whether direct microbial applications or their extracted enzymatic formulations. Even though multiple enzymes are required to degrade heterogeneous cell wall components, whereas pectinase is the main retting enzyme determining the retting magnitude and applications of high efficiency pectinolytic bacterial strains is advantageous in decreasing the retting period enhancing fiber quality(Datta et al., 2020; Hasan et al., 2020). In addition to pectic substances, pectinase enzyme activities are also associated with xylanolytic and cellulolytic activities(Brühlmann et al., 1994). Hence, bacterial strains prevalently possess pectinase potentials with hemicellulolytic (Xylan and mannan) activities favored to use as retting agents.

Among several methods of retting, conventional water retting (CWR) considered superior in terms of uniform fibre production and cost but has some limitations. CWR inaugurates as uncontrolled manners in open ditch by combine actions of naturally grown microbes and maceration. Hence, microbial water retting is of alkalophilic bacteriological interest(Kavuthodi & Sebastian, 2018) as those secrete alkali tolerant pectinases which prevent other microbial contamination and additionally permits adopting an open retting system(Zhang et al., 2008). Initial microbial loads in CWR are of an abundant microbial biological mixture that leads over retting or under retting of fibres, takes unlimited time and creates uncertainty to complete the process. Over retting and under retting are detrimental to fibre as under retting results low fibre yield and over retting leads to rot internal fibre, affect the crystalline form of cellulose and consequently gave low tensile strengths fibre. The quality of kenaf bast fibre to a large extent depends on the removal of these gummy pectineus substances by bacterial alkaline pectinase enzymatic activities.

Incorporation of bacterial strains in non-sterile retting process is limited for controlling contamination of endogenous strains, therefore alkalophilic enzymatic potentials are considered and thought to be suppressed the unwanted endogenous microbial populations. In application, alkaline pectate lyase is preferred for efficiently removing pectin, since pectin is more soluble in alkaline solution (Chiliveri & Linga, 2014; Khan et al., 2018; Zhang et al., 2013) and alkali tolerant enzymes generally produced by alkalophilic bacteria.

Augmentation of the retting process by bacterial strains gave substantial gum loss and influential fiber quality in many studies, but the success in open field conditions demands special requirements as there might have a chance to contaminate with endogenous microbial flora. The cementing pectic substances are generally are acidic, and in aqueous solution with lower pH (3.5) are more stable, whereas at higher pH the methoxyl, acetyl and neutral sugar groups tend

to be eliminated, and its polymer backbone is cleaved by dissociation of glycosidic linkages(Satapathy et al., 2020). This phenomenon is more prevalent in highly esterified kenaf bast tissue-associated pectins (Aritkhodzhaev et al., 1995), and more susceptible to alkaline catalyzed reactions or alkali tolerant enzymes. On the other hand, dissolution of pectin through enzymatic degradation is gaining importance as it happened in region-selective depolymerization in mild conditions and incase of retting the basic cellulosic structure remain intact, and consequently yield cellulose fiber with less deformities. The bacterial retting initiates the aerobic spore forming bacteria and after that anaerobic strains play vital role for conversion of non-cellulosic cell wall polysaccharides by secretes of enzymes(Lee et al., 2019). The chemical compositions of kenaf fiber consequently determine its others mechano-physical quality and end uses(Komuraiah et al., 2014). Therefore, hyper isolated pectinolytic alkalophilic bacterial strains with facultatively anaerobic growth characteristics can be superior as starter inoculum and could complete the retting process as demanded for quality fiber. In this study, alkali tolerant pectinolytic bacterial colonies were aimed to isolate and evaluated for kenaf retting process in target of greater cellulosic compositions quality kenaf bast fibers, with minimum NCGs.

Materials and Methods

A. Isolation, Screening, and Identification

To obtain alkaliphiles, samples were collected from two differential sources viz. wet paddy land soil sediments and kenaf retting effluents (B 6°.10 N 116.12° E) following standard procedures. After enrichment, 100 µL diluted suspension were spreads on basal alkalophilic Horikoshi-H1 nutrient media, prepared by maintain pH 9.5. and incubated at 34°C for 24 to 48 h the composition (g/L) were Glucose-10 g, Poly-peptone 5 g, Yeast Extract 5 g, KH_2Po4 1 g, $MgSO_4.7H_20$ 0.2 g, Na_2CO_3 10 g, and agar 20 g. Subsequently the grown isolates were grown on pectinolytic bacterial media (Nawawi et al., 2017): Peptone 5.0 g, Yeast Extract 5 g, $MgSO_4.7H_20$ 5 g, K_2HPO_4- 0.2 g, Agar 15 g, supplemented with 1% Pectin (sigma) and finally screened on sole carbon yeast extract pectin (YEP) media, yeast Extract 1%, Pectin 0.25%, and agar 20 g. Colonies were separated based on distinguished morphology and subsequently pure cultures were maintained in glycerol stocks to preserves at -80°C for further uses. Pure culture of bacterial strains was identified by 16S rDNA gene sequence analysis by extraction of total genomic DNA using conventional CTAB methods. The full-length gene were amplified by universal primers 27F (agagtttgatcmtg gctcagtac) and 1492R (tacggttaccttgttacgactt), amplicon was confirmed by gel electrophoreses and stain with ethidium bromide and purified PCR product sequenced by Sanger sequencing sanger methods in our lab. The nucleotide sequence results were subjected to search for similarity analysis in NCBI BLAST database and phylogenetic tree were constructed using MEGA X software with GenBank 16S data bases. Details of bacterial strains were presented in table1 and molecular evolutionary identifications were presented figure 1.

B. Determination of Enzymatic Potentials of the Isolates

The isolates' pure cultures were streaks on YEP agar plates and grown overnight at 34°C, adjusted pH 9.5 with 2m Na_2Co_3. For the screening of pectinase enzymes, an alternative set of sole carbon YEP media was prepared. From stock culture, a pinch of the bacterial colony was spotted on this screening media using cork borer and incubate at 32°C; overnight. The Petri plate was then supplemented with 2% cetrimide (Alkyltrimethylamonium bromide) solution and set at the same temperature for one hour to observe the colony's clear zone and measured. The ratio between the bacteria colony diameter (mm) and holo area diameter (bacterial colony + clear area) was expressed as an enzymatic index (EI). The EI of each strain against a white background (of the medium) indicates the ability of isolates to produce pectinase.

Polygalacturonase enzyme activity of the bacterial strains was estimated by using the modified method (Silva et al., 2005). In brief, 10 mL of overnight produced specific strains effluent was collected, followed by centrifugation (10000 g for 15 min at four (4) °C) and filtration with 0.22-micron filter. A 2 mL of filtrate was added to an equal volume of 0.5% apple pectin (Sigma) solution in a 0.1 M acetate buffer of pH 5. Then the mixture was incubated at 45° C for 30 minutes. About 1.5 mL of Dinitro salicylic (DNS) reagents was added and heated at 100°C for 15 minutes. Then the mixture was diluted to 25mL with ddH20. The mixture's absorbance at 530 nm was measured using a spectrophotometer to determine the amount of reducing sugars, D galacturonic acid as standard. For accuracy, OD of the samples maintained 0.1 to 0.9 with buffer solution, and ddH_20 were used as the reagent blank. Each enzyme activity unit was defined as the amount of enzyme that liberates one m mole of galacturonic acid per minute under assay conditions. tables.

C. Kenaf Plants, Solvents, and Retting Conditions

Kenaf (KE-3) plants were grown in field conditions at TFS, Biotechnology Research Institute, Universiti Malaysia Sabah, for 110 days. Harvested raw plant materials were retted in artificial covered plastic tanks in open field condition using ~50cm long, central parts of the whole kenaf plants, maintained 1:15 plants materials, fresh pond water ratio in ambient temperature. The isolated bacterial stains Bacillus sp. KRB56 and Bacillus sp. KRB22 has grown for 16 h in yeast extract pectin liquid media and eventually used as inoculum by maintaining 3.5×10^8 CFU/mL densities. Prior to applications both strains were evaluated invitro conditions for

optimal growth and enzymatic activities (Data not shown). Therefore, required inoculum of the total initial volume of the retting liquor were added as starter inoculum in to the 1 kg raw kenaf plants and retted for 144-hour retting periods. An uninoculated control tank simultaneous adapted and each treatment were replicated for three times. After retting, individual fibre and stick washed, dried, and stored for analysis. Retting liquor was collected aseptically after 24 hours to end at regular intervals and immediately kept at -20°C for further uses. After treatment, the fibre was separated, washed, dried, and stored for further analysis.

D. Determination of Extractable and Residual Gum Content

Before retting, the extractable gums content of kenaf estimated by taking 20 g of constant weighted raw dried whole plants parts of kenaf; after grinding, added five volumes of 96% ethanol, and boiled for 30 minutes. Then the slurry was filtered with suction glass, and the insoluble material washed with 70% ethanol; the remaining solid was then dried with acetone and left overnight at 40°C. Calculate the dried samples in contrast to the initial weighted and expressed them as a percentage. Residual gum loss from the samples were determined using the following equation number 1.

$$Rg = (Mo-M1) / M0 \times 100 \quad (1)$$

Where,

Rg = residual gum content as percentage

M0 = whole kenaf plants weight before retting

M1 = Fiber weight + stick weight after retting

E. Determination of Chemical Composition of Fibres

The α-cellulose, hemicellulose, and lignin (acid soluble + acid insoluble) content of retted and un retted fibers were measured according to(George et al., 2014; Guo et al., 2019; Punyamurthy et al., 2012). The pectin content of the fiber samples was determined by gravitational method as Ca- pectate using the following equation number 2.

$$\% \text{ Pectin (Ca-pectate)} = (\text{Weight of Ca-pectate (g)} \times 500) / (\text{Filtrate taken (mL)} \times \text{weight of sample for estimation (g)}) \times 100 \quad (2)$$

F. SEM Images of Kenaf Fibers

The longitudinal features and surface morphology of the fibers were investigated using Hitachi S3400-N scanning electronic microscopic technology with gold coating. Prior to investigation randomly selected fibres bundles combed and representatives were fixed overnight in 4% (v/v) glutaraldehyde prepared in 0.1M phosphate buffer (pH 7.2) at 4°C. Thereafter washed and dehydrated with ethanol for several times and dried specimen mounted on to a SEM. Then the samples were washed in the same buffer for three times of each 10 minutes followed by washing in distilled water for two times of 10 minutes. After that, the samples were sequentially dehydrated with 50% for 15 minutes, 75% for 15 minutes, two times in 95% for each 15 minutes, and three times in 100 % ethanol for each 20 minutes by ethanol. The dehydrated samples were immersed in 1 mL of HMDS (Hexamethyldisilane) for 10 minutes. Thereafter decanted in the desiccator and dried specimen mounted on to a SEM stub with gold coating to view SEM and captured images.

G. FTIR Spectra

The FTIR spectra of the bacterial treated and uninoculated raw kenaf fibers were investigated on a Cary 630 Spectrophotometer (Agilent Technologies) and subsequently compared with un retted raw kenaf bast fibers. Scanning of grounded fiber samples (<1mm) continued at a resolution of 4 cm-1 to characterize the fiber's constituents change upon retting treatments. Identifying different functional groups of the fiber constituting components to interpret the qualitative and quantitative aspects of the yielded kenaf fiber quality indicates its chemical structures with natural cellulosic fibers references.

H. X-Ray diffraction Analysis of Fibers

X-ray diffraction analysis X-ray diffraction (XRD) analysis was conducted to identify the proportion of crystalline and amorphous phases of the raw and macerating solvents treated kenaf fiber samples. Experiments were conducted using an X-ray diffractometer (Panalytical) scan ranges from of 10° to 40°. The relative amount of crystalline material (CrI), calculated according to the Segal empirical method as shown in Eq. 3(Segal et al., 1959).

$$CrI = (I002 - Iam)/I002 \times 100 \quad (3)$$

(1) In Eq. 3, CI is the crystallinity index, I_{002} is the maximum intensity of the crystalline fraction, and I_{am} is the lowest intensity peak of the amorphous region.

I. Statistical Analysis

One-way analysis of variance (ANOVA) for the fiber yields, chemical composition, and gums removal data of un retted and retted raw fibers was analyzed using the statistical software Sigma plot 14. Means were separated at 1% probability level based on the LSD test, and each treatment was replicated at least three times(Gomez & Gomez, 1984), and means with standard error were presented in parenthesis.

Results and Discussions

A. Isolation and Morpho-physiological Characteristics

The sixty-seven bacterial colonies which has grown on alkalophilic nutrient growth media (Horikoshi- H1), of

adjusted pH (9.5) considered as alkalophilic stains. The aliquots of soil suspension and retting water serially diluted at 10^{th} scale serial dilution, spread to find (30- 300) counting numbers of bacterial colonies. Among two sources, retting effluents comprise higher bacterial loads compare with wet paddy land soil sediments. Two enzymatically potentials bacterial strains KRB56 and KRB22 colony and microscopic cell view were shown in Fig. 1. Simultaneously, their morpho-physiological Characteristics are shown in table 1.

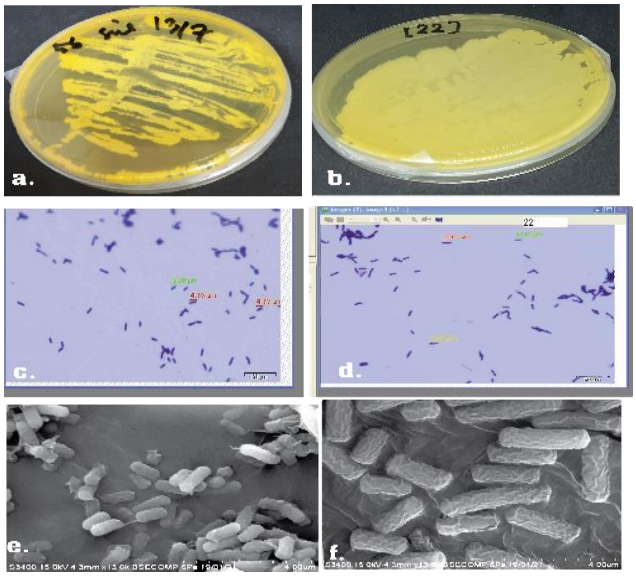

Fig. 1. Colony view of a. KRB56 b. KRB22, Microscopic view of c. KRB56 and d. KRB22 and SEM view of e. KRB56 f. KRB22

Most of the isolates were spore-former, whitish, or cream colors, dry to moist surfaces adhere to the nutrient media and sown differential growth patterns. In microbial retting bacterial as retting agents should have specific growth characteristics to survive in harsh environment conditions such as the aerobic strain were predominant in the earlier stages of retting if the O_2 since the and therefore anaerobic strains covers the niche. Water-based biological retting occurs in the harsh environmental conditions, where different acidic cell wall components disperse into the retting niche. Hence, synchronization of actual retting conditions, facultatively anaerobic alkaliphiles, are selected based on their hyper xylano-pectinolytic enzymatic activities. Endospore forming aerobic *Bacillus* species are highly efficient inoculant to unfavorable environmental conditions because of their higher survival rate ((Hu & Mahillon, 2011; Radhakrishnan et al., 2017), and facultatively anaerobic strains additionally helps to survive up to the end of the retting process. The finding of previous studies

Table 1. Colony, cell, and physiological characteristics of Bacillus sp. KRB56 and Bacillus sp. kRB22

Items	KRB56	KRB22
Cell	Spore forming rod, size (2-3) µm	Spore forming rod, size (4- 5 µm)
Colony character on nutrient agar	Medium Creamy white, mucoid	White, entire, Entire, irregular form
Gram staining	+ve	+ve
Aerobic + F. anaerobic growth	+	+
Temperature range (optimum)	20 - 45°C (34°.5C)	18 - 45°C (32°C)
pH range (optimum)	6.5 -11 (9.5)	6-10 (9.5)
Carbohydrate fermentation	+	+
Source of isolation	Wet paddy land soil sediments	Kenaf retting water

B. Screening for Retting Potentials Strains

Isolated alkaliphiles were qualitatively screened on sole carbon pectin agar plate's ability to produce zone of hydrolysis. The ratio for diameter of holozone and diameter of bacterial colonies termed as enzymatic index gave the in sight of their ability to enzymatically degrade pectin. In this case the pectinase positive colonies produced clear zones after supplemented with 2% cetrimide solution prior to incubation. The pectinase positive colonies EI ranges from 0.34 to 2.42. Based on the EI, hyper producers were further selected for polygalacturonase enzymatic assay determination. The clear zone formation of two isolated bacterial strains KRB22 and KRB56 were shown in figure 2 and their EI and polygalacturonase enzymes assay activities are shown in table 2.

Table 2. Enzymatic potentials

Isolates	Pectinase Enzymatic Index	Polygalacturonase enzymatic activity (µg/mL)	Polygalacturonase enzyme activity U/mL
KRB56	2.42±0.24	1520.33±6.352	7.17±0.14
KRB22	2.20±0.21	1388.67± 6.034	6.54±0.27

Enzymatic potentials of Several previous studies reported that the relationships between pectinase enzymes activity among bacterial strains and retting efficiency of bast and allied fibers are positively corelated (Cheng et al., 2018; Nath et al., 2017). The determination of pectinase activity differs in view of assay conditions and incubation of pectinolytic extracts with pectic substrates for a fixed time, followed by determination of the liberated reducing sugars. Though, different researchers use quite different conditions for the assay(Biz et al., 2014) hence, varied results were obtained.

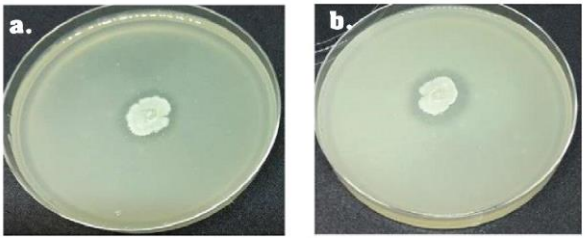

Fig. 2. Holozone formation of alkalophilic pectinolytic bacterial strain (a). KRB22 and (b). KRB56 on sole carbon pectin agar plates, supplemented with 2% cetrimide solution

for one hour incubation indicating the pectinolytic enzymatic potentials

These exo-polygalacturonases sequentially cleave pectin from the nonreducing ends, releasing mono or di-galacturonic acid residues. (Das et al., 2010), found the highest PG activity at alkaline pH (>8.5) in some isolated pectinolytic bacteria from jute retting water. The adaptive mechanism non-fermentative aerobic alkaliphiles in high external alkaline pH and describe it as the reversed gradient trans-membrane proton-motive force to boost ATP synthesis (Preiss et al., 2015). Although, multiple strategies are hypothesized to be involved in enabling alkaliphiles to avoid the challenge of a low bulk proton-motive force stimulating proton-coupled ATP synthesis at high pH. In bio retting process, PG is the main agent (Zhang et al., 2000). In this study two hyper pectinolytic alkaliphiles KRB56 and KRB22 further assessed for kenaf degumming process.

C. Identification and Phylogenetic Analysis

A molecular approach based on 16S rDNA gene sequence analysis was used to identify and distinguished closely related strains and the retrieved sequence were used to construct evolutionary relationships (Fig. 3) of the isolated KRB56 and KRB22 strains. The gene sequence of these isolates was subjected to validates for bioinformatic status to identify the nearest identical nucleotide sequence using NCBI-Blast analysis. A phylogeny was constructed following the neighborhood joining method with MEGA X software at 0.01 bootstrap value based on their nucleotide sequences. Results revealed that both isolates closely related to *Bacillus pumilus* species, whereas KRB56 formed a clade with S10 and KRB22 with MR52 with 98% sequence similarity. *Bacillus* species are widely utilized for retting purposes due to their extracellular enzymes among them the alkali tolerant species are considered more feasible to be used as non-sterile retting as they prevent to admixture unwanted endogenous colonies(Zhang et al., 2008). Previously no task was reported of alkalophilic *Bacillus pumilus* strain as kenaf bio retting process in ambient nonsterile conditions.

Some previous studies revealed that bacteria produce pectinase that withstands high pH and temperature(Hoondal et al., 2002) and pectinase from bacterial sources is feasible retting comparing with fungal sources as it is an extracellular product(Sohail & Latif, 2016).

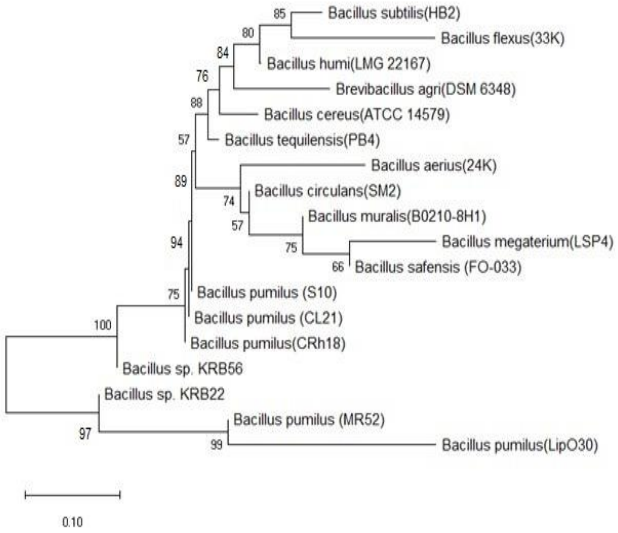

Fig. 3. Phylogenetic tree for identification of the isolates KRB56 and KRB22. All plotted sequences are representative of the genus Bacillus. The evolutionary tree was plotted using the 16S rRNA sequence retrieved from the NCBI GenBank following the neighbour-joining method. Bootstrap values based on 1000 replications are listed as percentages at the branching points; scale bar 0.1 is the number of nucleotide changes per sequence length.

D. Determination of Kenaf Bio Retting Efficiency Using KRB56 and KRB22 Strains

Before the commencement of retting treatment, the un retted raw fiber was subjected to chemical treatment to determine total extractable gums composition and it was found that maximum 29.45% of kenaf materials were losses, which presumably indicates that these NCGs gums' portions tends to be removed from the plant's portions through the any retting process. These indicators could help to interpret the degree of retting. The gum losses and fiber yield parameters were presented in table 3.

Table 3. Fibre and stick yield from 1000 gram of raw plants materials and gum loss treated by KRB56 and KRB22 bacterial strains for 144 hour retted fibre compared with uninoculated treatment

Treatments	Fiber weight (g)	Stem weight (g)	Fiber weight + stem weight (g)	Material Loss (%)	Gum loss %
KRB56	215.14±0.57	541.10±3.81	755.75±3.95	24.38±0.30	82.78
KRB22	224.45±1.63	554.84± 5.53	778.30± 3.97	22.17±0.41	75.28
Uninoculated	252.00±2.44	565.03±1.98	817.03±1.55	18.29±0.13	62.12

E. SEM Observation

Kenaf bast fibre surfaces observed through the SEM, for the gums adhere to the fiber. The utilization of potential bacterial strains in the kenaf bio retting process substantially

promotes gums loss without affecting basic fiber structure. Both bacterial strains treated fibre showed fewer gums on their surfaces than uninoculated and non-retted raw kenaf bast. Besides this, individual fibre diameter significantly reduces (Fig. 4) for the KRB56 treated fibre, attributing to higher gums removals (24.38%) from its surfaces; therefore, the average diameter had a lower measure compare with non-inoculated treatments.

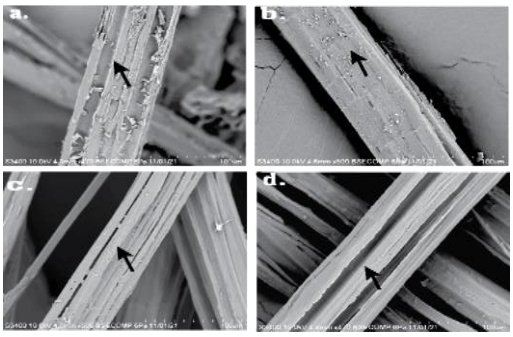

Fig. 4. SEM view of the kenaf fibre retted with selected bacterial strains compare with uninoculated and raw un retted kenaf fibres. The surfaces of the fibres at 100 µm magnification showing the presence and absence of gummy substances on the fibre's surfaces. The image a. showed un retted intact kenaf bast fibre with gums b. un inoculated kenaf retted fibre showed substantial gums presented on its surfaces after retting c. and d. showed smooth surfaces indicates the higher gums removal from the fibres retted with *Bacillus* sp. KRB56 and *Bacillus* sp. KRB22, respectively.

The individual fiber sizes of natural fiber were associated with the adjacent NCGs and inherent gums losses of natural cellulosic fibres though retting process is directly improving the individual fibre diameter fibre fineness and it is also reflective by reducing its fibres dimension(Beltran et al., 2002). Therefore, the fibres were also viewed by SEM at 100 mm magnification (Fig. 5) to observe that KRB56 bacterial treatments individual fiber sizes decreased following by KRB22 comparing with uninoculated treatments.

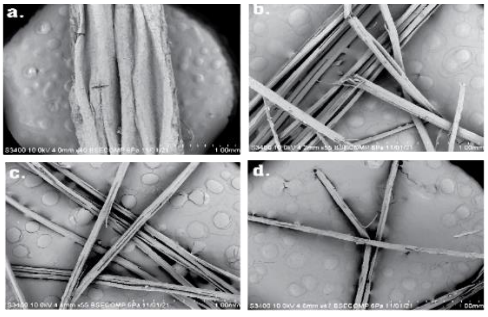

Fig. 5. SEM view at 100mm magnification showed the individual kenaf fibres sizes image a. showing higher width sizes of intact raw kenaf fibre before retting b. after retting by un inoculated bacterial strains possess comparably higher diameters indicates the presences of gums and c. retted with *Bacillus* sp. KRB22 and d. *Bacillus sp*. KRB56.

F. Chemical Composition Analysis

The selective chemical composition of un retted, un inoculated and bacterial treated kenaf bast fibres are shown in table 4. Results revealed that the KRB56 and KRB22 treatments losses sufficient NCGs comparing with un retted and un inoculated fibres samples. The KRB56 retted fiber gave highest 69.21% cellulose composition, followed by KRB22 67.42%, uninoculated 56.34% compare to un retted raw fiber (48.54%). Higher cellulosic composition of bast fiber positively increases its others quality parameters such as mechanical properties (Jankauskienė et al., 2015), while lower NCGs indicates successful retting completion. The basic cellulose of Kenaf is more resistant to hydrolysis than NCGs due to its crystalline composition, which retains its structure intact during the retting process; consequently, overall chemical compositions of kenaf fiber determine its others mechano-physical quality and end uses(Sisti et al., 2018).

Table 4. Chemical composition and gum removal rate of un retted, and KRB56 and KRB22 treated kenaf (KE-3) fibre compare with uninoculated and un retted raw kenaf

Fiber treatment	α-cellulose	Hemicellulose	Lignin	Pectin
KRB56	69.21±1.04	9.75±0.41[a]	8.24±0.22[a]	0.22±0.24
KRB22	67.42±1.07	10.73±0.63[a]	9.85±0.26[a]	0.27±0.31
Uninoculated	56.34±2.41	16.60±1.05[b]	13.35±0.28[b]	4.76±0.47
Un retted raw kenaf bast	48.54±2.18	18.12±1.66	19.09±0.5	8.28±0.31

G.FTIR Spectra Analysis

The FTIR spectra of un retted raw fibers, uninoculated, and bacterial treated fibers were determined to find the chemical compositional changes during the retting process. The peak at ~3500 decreased after retting, whereas uninoculated treatment also showed broad peaks compared with KRB56 and KRB22retted fibers; the same pattern was observed at peak 2915. Peaks at 1408 almost disappeared for bacterial treated fibers compared with non-retted kenaf bast, indicating the removal of aromatic compounds lignin and gummy substances pectin removals patterns. The hemicellulose and cellulose representing peaks at 1023 gradually decreased for un retted, uninoculated, and KRB22 and KBR56 treated fibers samples, whereas prominent cellulosic stretch at 650 indicates its higher proportional presence for bacterial treatments. Thus, the overall, FTIR spectra indicates that incorporation of both bacterial strains substantially reduces non cellulosic gummy components from kenaf fibers comparing with uninoculated and un retted fibers.

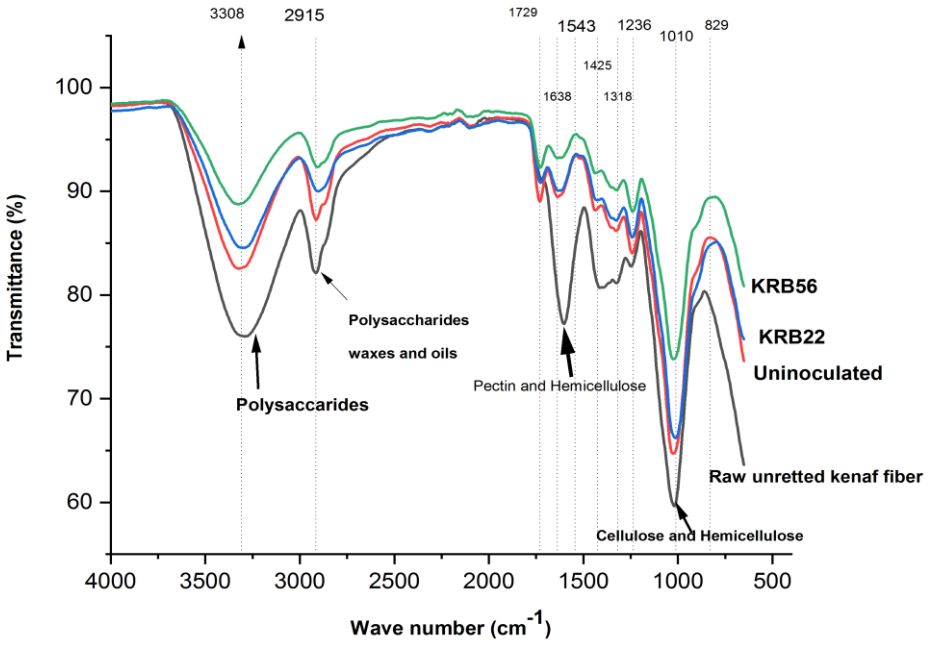

Fig. 6. FTIR spectra of un retted and uninoculated, KRB56 and KRB22 bacterial strains retted kenaf bast fiber

H.XRD Spectra Analysis

Xray diffraction spectra of the retted and un retted kenaf fiber samples are shown in figure 7 indicates the proportion of crystalline and amorphous materials exists in the fiber. The higher crystalline characters of kenaf fiber attribute to the cellulose of the fibers, in general, the natural cellulosic fibers higher crystallinity index considered the desired fiber quality, while retting improves the crystallinity for variable percentages, depending on how much NCGs have removed. The crystalline arrangements of kenaf fiber resulting by the intra and intermolecular arrangements though the hydroxyls (-OH) bonding and Van der walls interactions originating from the cellulose portions. In this case diffraction graph from 10° to 80°, at 2θ label showed major and minor peaks. The previous study of natural cellulosic kenaf fibers diffraction reflections determined for amorphous portions reflected in between 18° and 20°, 2θ label and crystalline parts in between 22° and 24°(Shi et al., 2011).

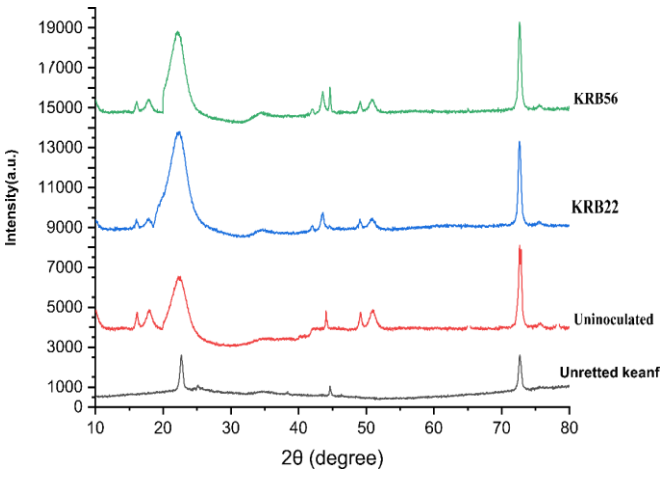

Fig. 7. XRD spectra of bacterial, uninoculated and un retted raw kenaf fiber

The major intensity peak at 2θ ranging of around 22° is quite like native cellulose I_{002} are the indicative distances between H bonding sheets while the lowest intensity around 18° (I_{am}) is the representing of amorphous lattice. KRB56, KRB22, un inoculated retting treatment kenaf fiber and un retted raw kenaf fibers crystallinity index determined by using the Segal equation and their relative crystallinity are shown in table 5.

Table 5. Crystallinity index of bacterial treated, un inoculated and un RETTED KENAF fiber

Kenaf fiber sample	Crystallinity index	Relative Crystallinity increases (%)
Un retted	49.20	-
KRB56	69.52	41.30
KRB22	66.67	35.50
Un inoculated	57.80	17.47

The determined crystallinity index illustrates the order of crystallite filling rather than the absolute state of the matter and used only for the comparison purpose (Beckermann & Pickering, 2008). The spectra as well as their Crl index implies that KRB56 and KRB22 retted fiber possess higher crystallinity index compare to un inoculated and raw kenaf fibers this is because of NCGs removal from the fibers.

Conclusion

Conventional water-based retting encompasses uncertainty and yield inconsistent fiber quality; therefore, the whole bast fiber industry faces multiple difficulties maintaining its productivity and promoting diversified

value-added bio composites preparations. These bacterial strains could benefit the bio-retting process by enhancing the bast industry's sustainability at the grower's level. Incorporation of *Bacillus sp*. KRB56 and KRB22 into the kenaf retting process resulted in enhanced gums degradation and fiber separation with quality fiber characteristics. Even though these strains gave superior retting efficiency, applications must follow optimized parameters to obtain the highest results. Furthermore, the yielded fiber was characterized by evaluating its chemical components, directly influencing the fiber's mechanical properties. Therefore, in this study, the isolated alkaliphilic pectinolytic bacterial strains are the potential bacterial agents used in the optimized kenaf bio retting process for getting desired quality kenaf bast fiber.

Acknowledgement

This research project was financed as part of foreign doctoral fellowship study No. NATP-2/PIU-BARC-44/ 2017/662(35) of the first author by the IFAD and World Bank-funded National Agricultural Technology project-2, (Project ID: P149553), of UMS research booth number of UMS L-24/2018.

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