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**INSIGHTS ON MICROBIAL AND BIOCHEMICAL  
ASPECTS OF RETTING FOR BAST FIBER PLANT  
PROCESSING IN A BIOREACTOR**

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## Abstract

Transition to a more sustainable bio-based economy, as a political consequence of the Kyoto protocol on global climate change (United Nations Framework Convention on Climate Change, 1997), includes a shift of feedstock for energy and chemical industries from petrochemical to renewable resources. The use of non-food crops as major source for renewable resources, however, requires careful consideration of the environmental impact. It is a good sign that also industries have by now recognised that the concept of "eco-efficiency" is an important way for businesses to contribute to sustainable development. As a major renewable resource lignocellulosic fibres derived from the structural plant tissues will play an important role in this transition. Fibre crops are -among the technical and non-food agricultural products- the commodities with the longest tradition. For example cellulosic fibres for textile and paper pulp production are still important commercial non-food commodities. The markets for fibre crops such as flax, hemp, jute and sisal have seen substantial erosion since the introduction of synthetic fibres after Second World War in textile industries. Actually, the ecological 'green' image of cellulosic fibres has been the driving argument for innovation and development of products in the past decade, such as fibre reinforced composites in automotive industries, building and construction materials, biodegradable geotextiles and horticultural products. But today the industrial demand of natural fiber didn't encountered yet an adequate offer able to soddisfying it. In fact, intensive cultivation of bast fiber crops as hemp, flax, kenaf and jute raised up while stalk processing for the extraction of bast fibers – the raw material for the industry - relyed on traditional method of retting.

The process of separation and extraction of fibers from non-fibrous tissues and woody part of the stem through separation, dissolution and decomposition of pectins, gums and other mucilaginous substances is called retting. The quality of the fiber is largely determined by the efficiency of the retting process. In retting, the most important aspect is that pectic materials are broken down and the fibers are liberated. Fiber quality is dependent on method of extraction applied in different natural conditions and duration of retting.

The major aim of this study, in collaboration with K.E.F.I. S.p.a., was to develop a ribbon retting process in bioreactor in order to improve the extraction of bast fiber from kenaf plant to industrial scale.

This study could be divided in three parts: the first part comprised the optimization of retting process in bioreactor by analyzing different kind raw materials (kenaf ribbons) and different variety of kenaf (Everglade and Tainung); in the second part the occurring bacterial microflora involved in plant maceration was deciphered and characterized; and in the third part a possible starter inoculum was identified in order to improve and standardize the retting process in bioreactor.

Ribbon retting is a particular method of retting based on a mechanical pretreatment of plant stalks that allowed reducing:

1. the requirement of water,
2. the length of retting time and
3. the level of environmental pollution to almost one-fourth

in comparison to other method that processed the whole plant.

In particular, in the first part of this study, the optimal tested conditions allowed assuring better quality kenaf fiber in terms of fiber strength, fineness, color, and overall absolutely barking free kenaf fiber.

The optimization of main parameters of maceration in bioreactor was achieved analyzing different loadings of kenaf ribbons and different varieties of kenaf.

The pilot plant was provided with air insufflation system and a retting liquor recycling apparatus which allowed processing perfectly kenaf ribbons in only 5-7 days. In particular, main parameters of bioreactor (pH, RedOx, oxygen content and temperature) were monitored and controlled during the process. The air insufflation system was very useful because controlled the excessive acidification of the liquor preventing over –retting risk with consequent damaging of cellulose (principal bast fibers component).

Microbiological analysis of retting liquor by enumerating on different culture media evidenced the development of pectinolytic bacteria versus heterotrophic bacterial populations occurring on plant stem and in water.

Moreover, the analysis of retting liquor evidenced the solubilization of phenolic compounds during maceration probably originating from lignin present in plant tissues. However, the phenolic content registered in retting liquor stabilized - during the process - to values influenced only by the loading of kenaf ribbons and kenaf varieties. The presence of phenolic compounds influenced negatively the development of bacterial populations by limiting the growth but also by inhibiting the enzymatic activities involved in the retting process.

In the second part of this work, the study of the bacterial populations responsible of the retting process was achieved in retting liquor by two distinct but complementary approaches: culture dependent and independent approaches.

During culture dependent approach and after screening of pectinolytic isolates through A.R.D.R.A. technique, strains belonging prevalently to genera *Bacillus* and *gamma-Proteobacteria* were identified. On the other side, culture independent approach such as D.G.G.E. analysis conducted on hypervariable V3 region of 16S rDNA of Eubacterial species confirmed the presence of those species.

Later on, five high pectinolytic bacteria were isolated among strains isolated from retting liquor: K2H1 *B. pumilus*, K2H2 *B. subtilis*, K2H3 *B. pumilus*, K2H7 *B. licheniformis* e K607 *Enterobacter* sp..

Most of high pectinolytic strains belonged to spore-formers bacteria which are well known in literature to produce pectinolytic esoenzymes.

On the other hand, RDA assay with tannic acid revealed that high pectinolytic isolates belonging to spore-formers bacteria were susceptible to phenolic compounds that inhibited their growth. Differently, K607 *Enterobacter* sp.'s growth was not affected by phenolic compounds. The presence of *Enterobacteriaceae* was normally encountered in wastewaters of pulp and paper industry suggesting that K607 *Enterobacter* sp. could be involved in the regulation of phenolic compounds content in retting liquor during the maceration.

In the third part of this study, the five high pectinolytic strains were tested as massive inoculum for trial of maceration in mesocosms in order to identify a possible starter strain able to improve retting process in bioreactor.

From the data obtained from trials of retting in mesocosms it was evident that K2H1 *B. pumilus* produced the best retted fibers in comparison to other strains and retted fibers from bioreactor. The conditions of retting applied in mesocosms could be considered as the worst retting conditions possible in bioreactor (no aeration, no recycle of retting liquor). For these reasons, it is possible to predict that K2H1 *B. pumilus* added as massive inoculum could improve the retting in bioreactor by reducing the time of retting without compromising the quality of retted fibers.

Later on, proteomic analyses of pectinolytic enzymes produced by K2H1 *B. pumilus* evidenced that this strain when induced by pectin produce a pectate lyase type C.

Pectate lyase acts preferentially on polygalacturonic acid that was the main component of pectin backbone, consequently pectin was disrupted in small soluble molecules.

Summarizing, in this study a pilot plant bioreactor was built up able to extract bast fibers of kenaf at industrial level both in term of quality and quantity. Furthermore, the environment of bioreactor was suitable to develop the retting process and to study and characterize the bacterial populations that allowed isolating a starter inoculum.

In the next future, the test of K2H1 *B. pumilus* as massive inoculum could improve the maceration by reducing time of retting from 5-7 days to 48-72 hours. Moreover, further studies on enzymatic activities of bacteria involved in retting of bast fiber crops could developed an enzymatic retting procedd that will be able to reduce time of retting from 48-72 hours to few hours.

## Sommario

La transizione ad un'economia bio-sostenibile ha previsto politicamente la coltivazione di biomassa per la produzione di energia e per quanto riguarda l'industria chimica l'impiego di materie prime rinnovabili in seguito al protocollo di Kyoto per far fronte ai cambiamenti climatici globali (Struttura della Convenzione sui Cambiamenti Climatici delle nazioni Unite, 1997). L'utilizzo delle piante coltivate per la produzione di materie prime rinnovabili e non per l'alimentazione umana ed animale richiede, però, una più attenta considerazione sulla base dell'impatto ecologico. Fortunatamente, anche a livello industriale è stato riconosciuta l'importanza del concetto di "eco-efficienza" indispensabile per uno sviluppo sostenibile. Le fibre naturali di origine vegetale rappresentano la maggiore fonte di materia prima rinnovabile e potrebbero giocare un ruolo importante in questa transizione. Le piante da fibra, tra le produzioni agricole tecniche e non destinate all'alimentazione, posseono una lunga tradizione nella produzione di beni di consumo. La valenza economica del mercato di piante da fibra come il lino, la canapa, la juta ed il sisal ha subito un calo dopo la Seconda Guerra Mondiale in seguito all'introduzione delle fibre sintetiche. Attualmente, la loro valenza ecologica ha implementato la ricerca e lo sviluppo

di compositi contenenti fibre rinforzate impiegate nell'industria dell'automobile, nella bio-edilizia e nella produzione di tessuti biodegradabili per la lavorazione della terra.

Tuttavia, l'aumento della domanda di fibre naturali non ha ancora trovato un'offerta adeguata in grado di soddisfarla. Infatti, nell'ultimo decennio si è registrato un aumento della coltivazione delle piante da fibra come canapa, lino, kenaf e lino non supportate da uno sviluppo di un processo di estrazione delle fibre che ad oggi si basa ancora su sistemi tradizionali di macerazione.

Il processo di separazione ed estrazione delle fibre dagli altri tessuti non fibrosi e dal midollo legnoso presenti nello stelo della pianta attraverso il distacco, la dissoluzione e la decomposizione delle pectine, gomme e le altre sostanze mucillaginose è chiamato *retting*.

L'obiettivo principale di questo studio, in collaborazione con K.E.F.I. S.p.a., è stato lo sviluppo del processo di estrazione delle fibre vegetali a livello industriale in bioreattore mediante *ribbon retting* del kenaf.

Il lavoro sperimentale può essere diviso in tre parti: la prima parte ha riguardato l'ottimizzazione del processo di *ribbon retting* nel bioreattore analizzando diverse tipologie di materie prime (i *ribbons* di kenaf) e differenti varietà di kenaf (Everglade e Tainung); la seconda parte si è occupata di decifrare e caratterizzare la microflora che naturalmente si è sviluppata ed è stata coinvolta nella macerazione delle piante; la terza parte è servita ad identificare un possibile inoculo starter capace di implementare e standardizzare il processo di *retting* in bioreattore.

Il processo di *ribbon retting* è un metodo particolare di macerazione basato sul pretrattamento meccanico degli steli che permette di ridurre:

- la quantità necessaria di acqua.
- il tempo di macerazione e
- l'inquinamento ambientale di circa un quarto

in confronto agli altri metodi di macerazione che trattano lo stelo intero.

In particolare, nella prima fase dello studio, le condizioni ottimali testate hanno permesso di garantire una miglior qualità di fibre di kenaf sia in termini di resistenza, finezza, colore e soprattutto hanno prodotto fibre libere da residui del midollo legnoso.

L'ottimizzazione dei parametri di macerazione in bioreattore è stata condotta analizzando diversi carichi del bioreattore e testando differenti varietà di kenaf.

La particolare impostazione del bioreattore, dotato di sistema di insufflazione di aria forzata ed di ricircolo del liquor di macerazione ha permesso di ottenere una macerazione spinta che ha prodotto in soli 5-7 giorni fibre di ottima qualità e perfettamente macerate. In particolare, la macerazione in bioreattore ha permesso di monitorare e controllare i principali parametri del processo (pH, RedOx, ossigeno disciolto e temperatura). Particolarmente utile si è dimostrata la regolazione dell'insufflazione dell'aria che ha permesso di mantenere il pH del liquor di macerazione a livelli vicini alla neutralità evitando l'eccessiva acidificazione ed il rischio di *over-retting* con conseguente danneggiamento delle fibre di cellulosa, principali costituenti delle fibre vegetali.

L'analisi microbiologica del liquor di macerazione mediante conta su diversi terreni di crescita ha evidenziato che durante il processo di macerazione popolazioni microbiche con attività pectinolitica si sono sviluppate sin dalle prime fasi del processo e hanno preso il

sopravvento sull'iniziale microflora eterotrofa derivante dalla contaminazione naturalmente presente sugli steli delle piante e nell'acqua impiegata per la macerazione. Inoltre, l'analisi del liquido di macerazione ha evidenziato il rilascio di sostanze fenoliche probabilmente originatesi dalla lignina presente nei tessuti vegetali. Tuttavia, il contenuto di composti fenolici si è stabilizzato nel corso della macerazione a valori che dipendevano dalla tipologia di kenaf e dal carico del bioreattore. La presenza di composti fenolici può influenzare negativamente lo sviluppo delle popolazioni batteriche inibendo la crescita ma anche limitando la loro attività enzimatica.

Nella seconda fase lo studio della microflora batterica coinvolta nel processo di macerazione è stato condotto sul liquor di macerazione attraverso due approcci differenti ma complementari: un approccio *culture dependent* ed un approccio *culture independent*.

Durante lo studio microbiologico del liquor di macerazione mediante l'approccio *culture dependent*, lo screening degli isolati pectinolitici mediante la tecnica A.R.D.R.A. ha evidenziato che la maggior parte degli isolati appartenga al genere *Bacillus* ed al gruppo dei *gamma-Proteobacteria*. L'approccio *culture dependent* mediante analisi D:G:G:E condotta sulla regione ipervariabile V3 del gene 16S rDNA degli Eubatteri ha permesso l'individuazione della quasi totalità delle specie batteriche presenti nel liquor di macerazione, ha confermato la presenza di tali specie.

In seguito, tra i batteri pectinolitici isolati dal liquor di macerazione sono stati individuati 5 ceppi con spiccata attività pectinolitica: K2H1 *B. pumilus*, K2H2 *B. subtilis*, K2H3 *B. pumilus*, K2H7 *B. licheniformis* e K607 *Enterobacter* sp..

La maggiorparte degli isolati con spiccata attività pectinolitica appartiene a batteri sporigeni conosciuti in letteratura per la produzione di eso enzimi pectinolitici.

D'altro canto, il saggio RDA con acido tannico ha dimostrato che gli isolate con spiccata attività pectinolitica sporigeni sono suscettibili alla presenza di sostanze fenoliche che influenzano negativamente la loro crescita a differenza dell'isolato K607 *Enterobacter* sp.. La presenza di *Enterobacteriaceae* è stata riscontrata anche nelle acque reflue delle industrie cartarie suggerendo che la presenza di questo ceppo possa essere legata alla regolazione del contenuto di sostanze fenoliche nel liquor di macerazione.

Nella terza fase dello studio, i ceppi con spiccata attività pectinolitica sono stati testati in macerazioni in mesocosmo come inoculi massivi per individuare un possibile inoculo starter in grado di implementare il processo di macerazione in bioreattore.

Dalle prove effettuate è emerso che il ceppo K2H1 *B. pumilus* ha ottenuto i migliori risultati paragonabili a quelli ottenuti dalla macerazione in bioreattore e, considerando le condizioni stringenti della macerazione in mesocosmo (senza aerazione e ricircolo in incubazione statica), è possibile prospettare che l'inoculo massivo di tale ceppo possa accelerare la macerazione in bioreattore riducendo il tempo di macerazione senza compromettere la qualità delle fibre.

Successivamente, dall'analisi proteomica degli esoenzimi pectinolitici prodotti e dall'attività enzimatica registrata durante la macerazione in mesocosmo dell'isolato K2H1 *B. pumilus* è emerso che l'attività pectinolitica principale è imputabile ad un esoenzima pectato liasi di tipo C che viene prodotto in elevate quantità dal batterio quando indotto dalla pectina.

La pectato liasi agisce sulla catena dell'acido poligalatturonico che è il principale costituente della pectina, distruggendone la complessità e solubilizzandola in molecole più piccole.

Riassumendo, in questo studio è stato approntato un bioreattore su scala pilota in grado di estrarre in termini di quantità e qualità fibre vegetali di kenaf su scala industriale. Inoltre, il processo in bioreattore si è dimostrato un ambiente ideale per la ricerca e lo sviluppo del processo di macerazione mediante la caratterizzazione della microflora macerante e l'individuazione di un inoculo starter.

In futuro, la sperimentazione dell'inoculo potrebbe ridurre i tempi di macerazione dai 5-7 giorni alle 48-72 ore. Inoltre, ulteriori studi delle proprietà enzimatiche dei batteri coinvolti nel processo di macerazione potrebbe permettere lo sviluppo di un processo di macerazione enzimatico riducendo ulteriormente la qauntità d'acqua necessaria al processo ed i tempi di estrazione delle fibre da giorni ad ore.



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# **1. Introduction**

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### 1.1. Bast fiber crops as a renewable resource

Transition to a more sustainable bio-based economy, as a political consequence of the Kyoto protocol on global climate change (United Nations Framework Convention on Climate Change, 1997), includes a shift of feedstock for energy and chemical industries from petrochemical to renewable resources (van Dam *et al.*, 2004). Renewable resources, which were important for the wealth of the people before the Industrial Revolution, are gaining more interest in our modern society due to their positive effects on agriculture, environment and economy. A significant advantage of renewable resources exists in their contribution to the conservation of finite fossil resources and their importance regarding the green-house effect (Kessler *et al.*, 1998).

The use of non-food crops as major source for renewable resources, however, requires careful consideration of the environmental impact. Data on emission reduction of greenhouse gasses are to be combined with the projections for the year 2050 on the demand for food, energy, and raw materials (van Dam *et al.*, 2004). For instance the impact of a growing world population and more distribution of resources have to be included. It is a good sign that also industries have by now recognized that the concept of "eco-efficiency" is an important way for businesses to contribute to sustainable development<sup>1</sup> (Ranalli *et al.*, 2004).

As a major renewable resource lignocellulosic fibers derived from the structural plant tissues will play an important role in this transition. Fiber crops are - among technical and non-food agricultural products - the commodities with the longest tradition (Small *et al.*, 2002). For example cellulosic fibers for textile and paper pulp production are still important commercial non-food commodities. The markets for fiber crops such as flax, hemp, jute and sisal have seen substantial loss since the introduction of synthetic fibers after Second World War in textile industries (FAO statistics). However, still a market niche has been maintained and numerous new markets are emerging for fiber crops. Especially, the ecological 'green' image of cellulosic fibers has been the driving argument for innovation and development of products in the past decade, such as fiber reinforced composites in automotive industries (Karus *et al.*, 2004), building and construction materials (Nykter, 2006), biodegradable geotextiles and horticultural products (van Dam *et al.*, 2004).

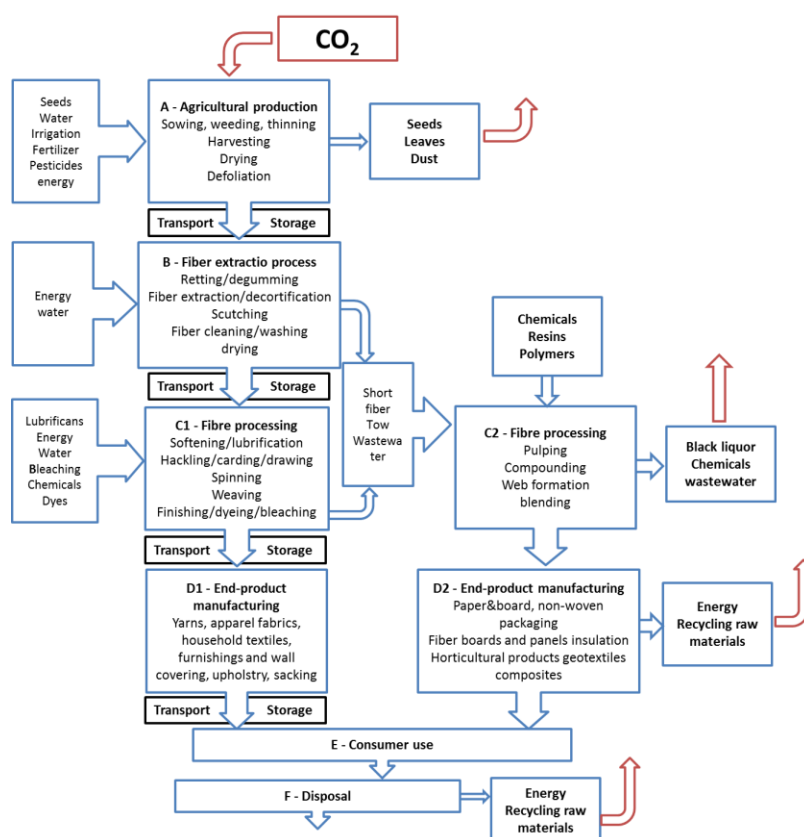
### 1.2. Life cycle assessment of fiber crops

Quantitative tools for comparing the environmental impact of processes and products are necessary as criteria for the selection of the most sustainable option. Since as early as the 1980's environmental affection due to products has been subject of systematic analysis. Methods such as life cycle assessment (LCA) of products were developed for comparing and classifying environmental effects (van Dam *et al.*, 2004). Standard LCA's, as defined

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<sup>1</sup> "Sustainable development meets the needs of the present without compromising the ability of future generations to meet their own needs." (United Nations. "Report of the World Commission on Environment and Development." General Assembly Resolution 42/187, 11 December 1987).

by ISO 14040-43, include the ecological implications of the whole life cycle of a product as well as its by-products. The positive or negative effects of by-product accumulation and use may have a strong influence on the total ecological impact of a product. The essence of LCA methodology is that it makes comparison possible between extremely diverse environmental effects. LCA focuses on the entire life cycle of a product from raw material acquisition to final product disposal, weighing environmental effects damaging ecosystems or human health. Most information on the environmental impact of agricultural production is available for energy crops and agro-residues used as fuel (Alexopoulou *et al.*, 2004). The cost of primary production and supply of biomass is considered with respect to energy consumption for cultivation, harvest and transport and is compared to the net energy yield. In the case of fiber crops this is more complicated because downstream processing, product performance and life time cycle need more detailed considerations (van Dam *et Bos*, 2004). In according with ISO 14040-14043 a general scheme of the production chain for fiber crops for textile production (Figure 1) was summarized by van Dam and Bos (2004), and on this flow chart was calculated the LCA of fiber crops for industrial applications.



The environmental impact is also influenced by the energy for operating agricultural machinery for sowing, and harvesting. Large differences between fiber crops can be observed, depending on the degree of mechanization in the crop production and local traditions. In developing countries manual labor to work the fields and harvest the crop is not uncommon (Webber III *et al.*, 2002a). Some fiber crops, and especially cotton, require substantial irrigation for obtaining good yields. In the post-harvest processing steps the fiber extraction process is consuming most (fossil) energy and water, yielding biomass waste and contaminated process water (Webber III *et al.*, 2002b). This forms a considerable risk of pollution of surface waters, when no measures are taken for waste water treatment (Mondal *et al.* Kaviraj, 2008). Utilization of residues and waste for generation of energy or other value added outlets, substantially enhances the overall ecological performance of a crop (Himmelsbach *et al.* Holser, 2008).

Comparison of the production phase of fiber crops with synthetic products or glass fibers (Table 1) is resulting in that the score of the fiber crops on CO<sub>2</sub> and greenhouse gas emission levels, consumption of fossil energy and resources is much better. For example, growing one ton of jute fibers require less than 10% of energy used for the production of propylene (FAO).

Materials/Process	Material	Eco-indicator [mPt/kg]
Fibers	Natural fibers	0.34
	Glass	2.31
Matrix materials	EP resin	10.2
	UP resin	9.45 <sup>1</sup>
	PP	2.99

<sup>1</sup>This value is for hand lay-up, for closed mould processing the eco-indicator would be 3.08 mPt/kg.

**Table 1.** Environmental impact of the various material (van Dam *et al.* Bos, 2004).

In fiber reinforced composites, blended textiles or non-wovens, the relative impact of synthetic resins and polymers on the LCA is large (Hepworth *et al.*, 2000). The weight reduction of natural fiber composites in automotive applications contributes more in terms of fuel savings than the impact of energy savings in the production phase of the panel (Karus *et al.* Vogt, 2004). When the same mechanical strength is desired the advantage of using lignocellulosic fibers over glass fibers in a composite may be lost because heavier constructive elements are required. However, when the same stiffness is required lignocellulosic fiber can give lighter constructive elements due to their higher specific stiffness (van Dam *et al.* Bos, 2004).

In the case of building materials the comparison of LCA needs to be made also with the costs of maintenance and replacement in relation to the performance of the material. The magnitude of the environmental advantage depends obviously on the kind of application.

In other words: the environmental gain is usually due to a secondary effect, such as weight saving, and is then not caused by the 'green' origin of the fiber (Nykter, 2006). It is therefore not possible to give a general rule of thumb of the advantages of the use of fiber crop products.

The economic advantage of fiber crops use in many applications is still small as long as costs of waste management are not included in the product costs. Feasibly, the increasing costs of petrochemical resources will enhance the competitiveness of fiber crops in the next future (van Dam et Bos, 2004).

### 1.3. Natural bast fiber plants and kenaf

Natural bast fiber plants can be divided geographically in two main groups: in the Western Countries most important fiber crops are cotton (*Gossypium hirsutum* L.), flax (*Linum usitatissimum* L.), hemp (*Cannabis sativa* L.) and recently kenaf (*Hibiscus cannabinus* L.), on the other side in Asiatic countries principal cultivations are jute (*Corchorus capsularis* L. and *Corchorus olitorius* L.), kenaf and roselle / mesta (*Hibiscus sabdariffa* L.) (Figure 2). The second ones constitute very important fibers and cash crops of the world in order of production (Dempsey, 1975).



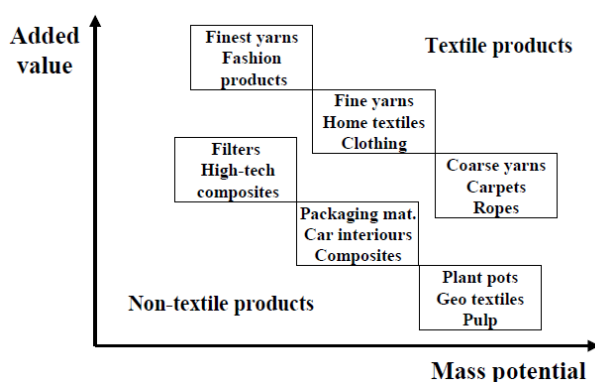
**Figure 2.** Representations of principal natural fiber crops: cotton (*Gossypium* spp.), flax (*Linum usitatissimum*), hemp (*Cannabis sativa* L.) and jute (*Corchorus* spp.).

In particular, the six producing member countries of the International Jute Organization (IJO), namely Bangladesh, China, India, Indonesia, Nepal and Thailand, account for about 95% of the total global production of about 3 million tons of jute and kenaf, harvested from about 2.5 million hectares. These countries except Indonesia are net exporters, accounting for about 98% of the world exports of raw fiber and finished products (Ahmed et Akhter, 2001).

Natural fibers from fiber crops, as a commodity, are facing competition on two fronts. They are facing stiff competition from synthetics at the consumer's side, while on the other, from more remunerative crops at the grower's side. In order to face the dual challenge the production of fiber crops have to adopted a strategy which consists of agricultural

research and development, industrial research and development and market promotion of both traditional and diversified fiber crops products (van Dam *et al.*, 2004).

As can be seen from the figure 3, textile products usually show higher mass potential and higher added value in comparison to technical applications (Kessler *et al.*, 1998). The demands in technical applications are much higher at lower costs. Man-made fibers (E-Glass, HS carbon, Kevlar™) can be designed specifically to the individual application and the quality can be guaranteed year by year (Fowler *et al.*, 2006). In addition, quality assessment is already standardized. Thus competition of bast fibers in this sector with man-made fiber will be tough on high level applications. At low level applications bast fibers compete normally with other renewable resources like wood, the cost benefit for the farmers is only profitable with high subsidies (Kessler *et al.*, 1998).



**Figure 3.** Added value versus mass potential of bast fiber products (Kessler *et al.*, 1998).

Moreover, International Year of Natural Fibers (2009) was promoted by FAO and UN with the aim to raise interest of natural fibers, because they are not only important for their environmental low impact, but also because they are of major economic importance to many developing countries. Among bast fiber plants, kenaf (*Hibiscus cannabinus*) is the best emerging and promising crop (Alexopoulou *et al.*, 2004; Danalatos *et al.*, 2004; H'ng *et al.*, 2009). In fact, kenaf cultivation is spread in all populated continents, characterized by fast growing, high biomass and bast fiber yields.

Kenaf is a multipurpose crop with various harvestable components: leaves and tender shoots are suitable for forage (Swingle *et al.*, 1978); the woody core has attributes for forest-product substitutes, absorbents, and structural materials (Fowler *et al.*, 2006; Nishimura *et al.*, 2002); and seeds have an oil and protein composition similar to cotton seed (Jones *et al.*, 1955; Mohamed *et al.*, 1995). The bast fibers, however, remain the primary economic incentive to grow kenaf (Ayre *et al.*, 2009).

The expansion of the commercial industry for kenaf will encompass an understanding of the diverse management systems including the production, harvesting, processing, and marketing kenaf as a fiber, feed, or seed crop, combined with directed research, focused development, and communication among diverse constituencies working closely for economic development (Fowler *et al.*, 2006). The initial step in managing this system is a

greater understanding of the variables within each segment of the kenaf industry (Alexopoulou *et al.*, 2004). The commercial success of kenaf has important potential economic and environmental benefits in the areas of soil remediation (Citterio *et al.*, 2003), toxic waste cleanup, removal of oil spills on water, reduced chemical and energy use for paper production (Ververis *et al.*, 2004), greater recycled paper quality (Ohtani *et al.*, 2001), reduced soil erosion due to wind and water (Webber III *et al.*, 2002b), replacement or reduced use of fiberglass in industrial products (van Dam *et al.*, 2004), and the increased use of recycled plastics (Fowler *et al.*, 2006). The activities of private industry augmented by public supported agricultural research continue to provide a diverse range of new kenaf products that suggests a bright future for the continued expansion of kenaf as a commercial crop (H'ng *et al.*, 2009). Beyond cordage, bast fibers obtained by kenaf are expanding into new markets of moldable, nonwoven fabrics, and reinforced composite materials in automotive, aerospace, packaging and other industrial applications (Karus *et al.*, 2004). This trend is in part due to the fiber's physical properties of light weight, competitive tensile strength and stiffness, and vibration damping properties, and also due to the fiber being a renewable and biodegradable resource (Ayre *et al.*, 2009).

### 1.4. Kenaf

#### Scientific classification

Kingdom:	<b><i>Plantae</i></b>
Division:	<b><i>Magnoliophyta</i></b>
Class:	<b><i>Magnoliopsida</i></b>
Order:	<b><i>Malvales</i></b>
Family:	<b><i>Malvaceae</i></b>
Genus:	<b><i>Hibiscus</i></b>
Species:	<b><i>H. cannabinus</i></b>
Binomial name:	<b><i>Hibiscus cannabinus</i> L.</b>



**Figure 4.** Representation of *Hibiscus cannabinus* L. with mature leaf, flowers and mature capsule of seed.

Kenaf [Etymology: Persian] (*Hibiscus cannabinus*) is a species of *Hibiscus*, probably native to Southern Asia, though its exact natural origin is unknown. The name also applies to the fiber obtained from this plant. Kenaf (*Hibiscus cannabinus* L., Malvaceae) is a warm season



annual fiber crop closely related to cotton (*Gossypium hirsutum* L., Malvaceae) and okra (*Abelmoschus esculentus* L., Malvaceae) and is one of the allied fibers of jute and shows similar characteristics (Figure 4).

### 1.4.1. Botany

#### 1.4.1.1. Plant Components

##### **Stalks.**

Kenaf is an annual or biennial herbaceous plant (rarely a short-lived perennial) growing to 1.5-3.8 m tall with a woody base. The stems are 1-2 cm diameter, often but not always branched (Figure 5). The length of the growing season, the average day and night temperatures, and adequate soil moisture are considered the key elements affecting kenaf yields (Webber III *et al.*, 2002a; Danalatos *et al.*, 2004). Stalk yields normally range from 11 to 18 tons (t)/ha, oven dry weight, depending on the previously listed production factors (Webber III *et al.*, 2002b). In addition to its low economic inputs and high stalk yields, suitable production areas for kenaf will depend greatly on the economics of the competing crops and the kenaf market. The kenaf cultivar 'Tainung #2' (photosensitive) has consistently surpassed other cultivars in yield evaluations (Webber III *et al.*, 2002a).



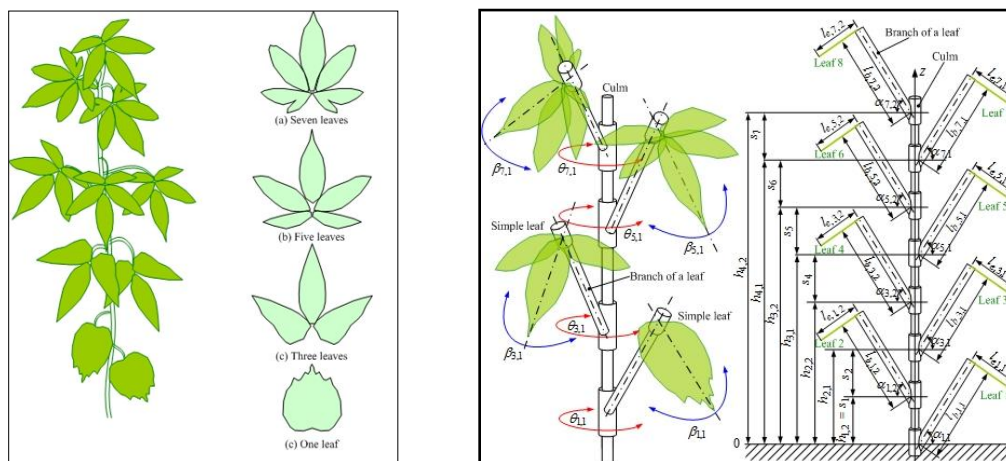
**Figure 5.** Example of performances of kenaf in stalks yields.

##### **Leaves.**

Kenaf plants produce simple 10-15 cm long leaves with serrated edges on the main stalk (stem) and along the branches. The position of these leaves alternate from side to side on the stalk and branches. Cultivar and plant age affect the leaf shape. Kenaf plants produce two general leaf types, divided and entire (Figure 6). The divided (split-leaf) cultivars have deeply lobed leaves with 3, 5, or 7 lobes per leaf. 'Everglades 71', 'Tainung

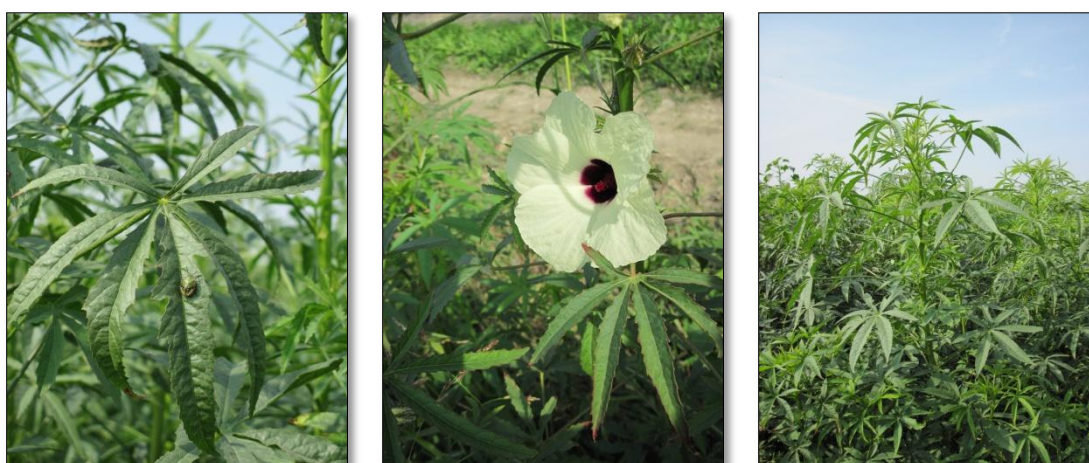


#1', 'Tainung #2', 'Guatemala 51', and 'SF-459' are examples of divided leaf cultivars. The entire leaf cultivars ('Everglades 41', 'Guatemala 4', 'Guatemala 45', 'Guatemala 48', 'Cubano', 'Cuba 108', 'Cuba 2032', and 'N7') produce leaves that are shallowly lobed, that are basically cordate (heart-shaped). The divided leaf characteristic was dominant and the entire leaf shape was recessive (Webber III *et al.*, 2002a).



**Figure 6.** Kenaf with distribution of different leaves. (a)(b) divided leaves and (c) simple leaf form of a kenaf. On the right, a models of leaves disposition and orientation in kenaf plant (Obara, 2009).

The juvenile or young leaves on all kenaf seedlings are simple, entire, and cordate. As the kenaf plant matures and additional leaves are produced, the newer leaves start to differentiate into the leaf shape characteristic of that particular cultivar. Divided leaf cultivars can produce 3 to 10 entire juvenile leaves prior to producing the first divided leaf (Webber III *et al.*, 2002a). Each leaf also contains a nectar gland on the mid-vein on the underside of the leaf (Dempsey, 1975).



**Figure 7.** Particular of mature kenaf leaves and their disposition along the stem in a real cultivation field.

The leaf and seed capsule nectar glands are visited in large numbers by wasps (*Campsomeris trifasciata*). Although these wasps may be present in large number during flowering, they seem to restrict their activity to the leaf and capsule nectar glands, rather than the flowers (Jones *et al.*, 1955).

Obara (2009) studied the conformation of leaves and their positions along the stem, because kenaf can be considered as a model of compact light reception system (Figure 6). Kenaf has simple leaves and divided leaves of two or more types in the same plant. The growth of a kenaf plant is faster than that of other plants, as an annual height of 1.5-3.5 m is reached. The growth rate of a plant is correlated with its high rate of photosynthesis (Archontoulis *et al.*, 2005). Kenaf shows assimilation rates in excess of 50-58 kg CO<sub>2</sub> ha<sup>-1</sup> h<sup>-1</sup> (32-37 µmol CO<sub>2</sub> m<sup>-2</sup> s<sup>-1</sup>) despite its belonging to C3 crops and uses CO<sub>2</sub>, solar radiation, water and nitrogen less efficiency than C4 crops (Cosentino *et al.*, 2004).

Since a plant's biomass is produced by photosynthesis, changes in the amount of the light received by a plant greatly influence plant's survival. Therefore, various mechanisms have evolved to increase the amount of the light received and to increase the rate of photosynthesis.

In other words, plants have evolved light reception systems through various methods throughout their evolutionary history. To reduce shading of photosynthetic surfaces, plants evolved phototropism and the ability to modify shoot configurations (see Figure 7): the shape and area of the leaf, leaf orientation, the lengths of branches and leaves, distribution of leaves, etc. Kenaf plants are the most perfect example of the adaptation of light reception system to growth and development of plant in order to maximize biomass production (26).

### **Flowers and Pollination.**

Kenaf plants produce large showy, light yellow, creamy colored flowers that are bell-shaped and widely open (Figure 8). The flowers of many cultivars have a deep red or maroon colored center. The flowers are 8 to 13 cm in diameter with 5 petals and are borne singly in the leaf axis along the stalk and branches.

The complete flowers are indeterminate; therefore the plant continues to produce additional flowers. Although the plants are highly self-fertile, generally considered self-pollinated, the plants can be cross-pollinated. The flowers open and close in a single day and are either cross-pollinated, primarily by domesticated honey bees (*Apis mellifera* L.), or self-pollinated by the twisting closing movement of the petals (Webber III *et al.*, 2002a).

### **Seed and Seed Capsules.**

Following pollination, a pointed, ovoid, seed capsule is formed that is about 1.9 to 2.5 cm long and 1.3 to 1.9 cm in diameter. The seed capsules are covered with many small, fine, loosely held, hairy structures that are very irritating when in contact with human skin. Each capsule contains 5 segments with a total of 20 to 26 seeds/ capsule (Dempsey, 1975). The slate-black, wedge-shaped kenaf seeds are approximately 6 mm long and 4 mm wide,

with 35,000 to 40,000 seeds/kg (Jones *et al.*, 1955). Once pollinated, the seeds require 4 to 5 weeks to maturation (Webber III *et al.*, 2002a).



**Figure 8.** Examples of white flowers of *Hibiscus cannabinus* L. with deep purple core.

### 1.4.1.2. Photosensitivity

Kenaf cultivars differ in their sensitivity and response to day-length, although it is actually the length of darkness that is the critical element that triggers the response. And it is the latitude north and south of the equator that determines the day-length for any particular time of the year. Understanding the influence of day length (latitude) is fundamental in selecting the optimum cultivar for the production location and the intended use of the crop. Dempsey (1975) divides kenaf cultivars into three maturity categories: ultra-early, early to medium (photosensitive), and late-maturing (photoinsensitive).

The ultra-early maturing cultivars were developed for use at latitudes greater than 37° north (Dempsey, 1975). These include the Russia and Korea cultivars that mature in 70–100 days. Even though these cultivars have high seed yields, the compressed growing season produces shorter plants with lower yields of fiber. These cultivars are not grown at lower latitudes (e.g. United States) because they will flower even earlier, and therefore produce even shorter and lower yielding plants (Webber III *et al.*, 2002b). The cultivars in the remaining two maturity categories, early to medium and late maturing, are normally simply referred to as photosensitive and photoinsensitive respectively (Dempsey, 1975).

Photosensitive (early and medium maturing) cultivars, classified as short-day plants, are typically preferred for fiber production. Two of these, 'Everglades 41' and 'Everglades 71', were developed by USDA researchers for the US to increase disease resistance and extend the vegetative growing period before the plants initiate flowering (Webber III *et al.*, 2002b). These photosensitive cultivars initiate flowering when the day length decreases to approximately 12.5 hours. It is advantageous to delay flowering in these cultivars, because the initiation of flowering causes a reduction in vegetative growth rate. As a result of late floral initiation and inability to produce mature seed prior to a killing frost, seed production for these cultivars is limited to certain geographical areas. If these cultivars, ideally suited for production between latitudes of 10° to 27° N or S, are grown in equatorial latitudes (0°

to 10° N or S) they will flower very early (60 to 80 days) and produce much lower yields than at the more northern latitudes (Webber III *et al.*, 2002a).

Photoinensitive, late maturing, (also referred to as day-neutral) cultivars are ideally suited for the latitudes surrounding the equator, 0° to 10° N or S. Although usually referred to as photoinensitive, these cultivars may still be responsive (semi sensitive) to the day length for flowering initiation (Dempsey, 1975). These cultivars (e.g. 'Guatemala 4', 'Guatemala 45', and 'Cuba 2032') because earlier floral initiation and the resulting seed production decreases the rate of vegetative growth resulting in lower stalk and fiber yields compared to the photosensitive cultivars (e.g. 'Everglades 41' or 'Everglades 71') (Webber III *et al.*, 2002a).

Photoinensitive kenaf cultivars may be ideally suited for use as forage or livestock feed crops (Dempsey, 1975). As a livestock feed, kenaf is usually harvested at an earlier growth stage than as a fiber crop; 60 to 90 DAP (days after planting) compared with 120 to 150 DAP. During a shorter growing season, photoinensitive cultivars (e.g. 'Guatemala 4', 'Guatemala 45') can produce dry matter yields equivalent to or greater than photosensitive cultivars, while using seed that can be produced in a larger geographic area (Webber III *et al.*, 2002a).

### 1.4.2. Diffusion and utilizations

Kenaf has a long history of cultivation for its fiber in India, Bangladesh, Thailand, parts of Africa, and to a small extent in southeast Europe. The stems produce two types of fiber, a coarser fiber in the outer layer (bast), and a finer fiber in the core. It matures in 100 to 1000 days (Webber III *et al.*, 2002b). About 9000 cultivars are produced (H'ng *et al.*, 2009). Grown for over 4000 years in Africa where its leaves are consumed in human and animal diets, the bast fiber is used for cordage, and the woody core of the stalks burned for fuel.

Today, principal farming areas are throughout China, India, and in many other countries including the following: Australia, USA, Mexico, and Senegal (H'ng *et al.*, 2009). This crop was not introduced into Southern Europe until the early 1900s. In particular, a network - called BIOKENAF - for implementation and development of this cultivation was established between Portugal, Italy and Greece in 1994, supported by UE incentives (Alexopoulou *et al.*, 2004). As result, in Europe, experimental cultivations of kenaf began to appear since 1995 (Mambelli *et Grandi*, 1995). Recently, many processing factories of natural fibers established and bore these cultivations by supporting breeders (Di Virgilio *et al.*, 2004). In the specific, in Italy, kenaf cultivations and processing factories are localized principally in the North, especially in the Po valley (Amaducci *et al.*, 2000).

The main uses of kenaf fiber have been rope, twine, coarse cloth (similar to that made from jute), and paper. Emerging uses of kenaf fiber include engineered wood, insulation, and clothing-grade cloth (Fowler *et al.*, 2006). Panasonic has set up a plant in Malaysia to manufacture kenaf fiber boards and export them to Japan, oil absorbent (based on patent issued to H. and C. Willett), soil-less potting mixes, animal bedding, packing material, organic filler for blending with plastics for injection molding (using the technology developed and patented by Fiber Packaging International, Inc.), as an additive for drilling muds, and various types of mats, such as, seeded grass mats for instant lawns to moldable

mats for manufactured parts and containers (Webber III *et al.*, 2002b). As the commercial use of kenaf continues to diversify from its historical role as a cordage crop (rope, twine, and sackcloth) to its various new applications including paper products, building materials, absorbents, and livestock feed, choices within the decision matrix will continue to increase and involve issues ranging from basic agricultural production methods to marketing of kenaf products (Alexopoulou *et al.*, 2004). However, the market of kenaf products relies principally on seeds for oil, fiber for paper and non-woven production and feedstock for animal breeding.

### 1.4.2.1. Seed production

Seed production strategies are affected by the cultivar, location-especially latitude, and cultural practices. The first issue to address is the cultivar photosensitivity, whether the cultivar is an ultra-early, an early to medium, or a late maturing cultivar (Webber III *et al.*, 2002a). The advantage of harvesting maximum seed yields from tall kenaf plants must be balanced with the disadvantage of decreased mechanical harvesting efficiency resulting from running large volumes of very tough, rope-like plant material through a combine (Dempsey, 1975).

Rather than producing the highest seed yields from plants that might be 6 m tall and/or greatly branched, it is more desirable to harvest single stalk plants of limited height, 1.8 to 2.4 m tall, and harvest less seed. This trend will likely continue until new and more efficient harvesting machinery is developed. Hand harvesting the seed is not a very realistic alternative, not only would hand harvesting be very labor intensive, but it would be very disagreeable work because of the presence of the many, small, loosely held, very irritating spines on the seed capsules (Webber III *et al.*, 2002a).

Researchers and seed producers have reported that the strategy for maximizing harvestable kenaf seed is very different than the production approach in maximizing kenaf fiber yields (Mullens, 1998). Cultural methods, such as planting date, fertilization, and plant populations, are altered to maximize seed yields while limiting the vegetative growth of the plant, especially plant height and excess branching. Mullens (1998) stresses the importance of having shorter plants, therefore reducing the volume of dry matter passing through the combine. Mullens (1998) states this is a more important consideration than the possible seed loss due to a later planting date. Soil fertility is also an important aspect of kenaf seed production. Contrary to what might be expected, seed production on less fertile soils or the use of less fertilizer is preferred for seed production (Mullens, 1998). Also, Mullens (1998) suggests only moderate fertilizer applications on marginal soils. It is very important to properly clean and condition the seed after harvest to maintain high seed viability (Dempsey, 1975). This is especially important because the seed normally contains at least 20% oil (Mohamed *et al.*, 1995). It may be necessary to immediately clean the seed to remove any green plant material to prevent the seed from heating up. This is particularly true if the seed crop was harvested prior to a killing frost that normally helps desiccate the plants. Although the maximum seed moisture content for bagging seed going into controlled temperature and humidity storage is 16.5%, it's recommended that the seed moisture content be 14% or less (Mullens, 1998).



Independently of the kenaf cultivar selected, seed storage prior to planting is an important consideration. Kenaf seed has high oil content, 21.4% to 26.4%, averaging 23.7% (Mohamed *et al.*, 1995), and therefore precautions should be taken to preserve the seed viability, especially when contemplating long-term seed storage. As with other crop seeds containing high oil percentages, seed viability decreases over time when stored at higher relative humidity (RH) and higher temperatures. Research on kenaf seed storage indicated that seed stored at 8% RH remained fully viable for 5.5 years when stored at either  $-10^{\circ}$ ,  $0^{\circ}$ , or  $10^{\circ}\text{C}$ , and fully viable for 5.5 years when stored at  $-10^{\circ}$  or  $0^{\circ}\text{C}$  at 12% RH (Webber III *et al.*, 2002a).

Kenaf seeds yield a vegetable oil that is edible and high in omega antioxidants. The kenaf oil is also used for cosmetics, industrial lubricants and as bio-fuel. Recently, kenaf alternative properties were outlined by Vicidomini (2007) that analyzed the presence and importance of interesting compounds (see 6.1. Appendix of principal phytoextract obtained by kenaf (*Hibiscus cannabinus*) page 109) existing in bark, leaves and especially in seeds of this plant. Main activity of most important kenaf phytoextracts were as follows:

1. Cyto-toxic effects versus *Colletotrichum, acutatum*, *C. fragariae*, *C. gloeosporioides* (Ascomycota: Phyllachorales), *Fusarium oxysporum vasinfectum* (Ascomycota: Hypocreales), *Oscillatoria perornata* (Cyanobacteria: Oscillatoriales), *Verticillium dahliae* (Ascomycota: Hypocreales); main cyto-toxic molecules identified were 5-CH<sub>3</sub>-furfural, benzen-acetaldehyde, n-nonanal, hibiscanal, O-hibiscanone, and defending peptide.
2. Anti-tumoral properties versus HeLa, Hep-2, A549; main carcino-cyto-toxic molecules were boehmenan-H, grosamine-K, pinoresinol, *threo*-carolignan-K.
3. Allelopathic, versus *Agrostis stolonifera* (Poaceae), *Amaranthus retroflexus* (Amaranthaceae), *Cucumis sativus* (Cucurbitaceae), *Lactuca sativa* (Asteraceae), *Lolium multiflorum* (Poaceae), *Lycopersicon esculentum* (Solanaceae), *Phaseolus vulgaris* (Papilionaceae)
4. Zoo-toxic, with the only bioassay dealing pinoresinol versus Hemipteran *Oncopeltus* (Lygaeidae) and *Rhodnius* (Reduviidae).
5. *Hibiscus cannabinus* have important anti-anemic properties, hepato-protective effects on liver and immunomodulatory effect on macrophage functions (pro-inflammatory suppression). Also anti-*Dracunculus* properties was reported (Nematoda). Kenaf has used in ethnobotany and folk-medicine in Gambia, Zaire and Camerun (Vicidomini, 2007).

### 1.4.2.2. Production for livestock feed

Although kenaf is usually considered a fiber crop, the entire kenaf plant, stalk (core and bark), and leaves, can be used as a livestock feed (Webber III *et al.*, 2002a). Crude protein in kenaf leaves ranged from 14% to 34%, stalk crude protein ranged from 2% to 12%, and whole-plant crude protein ranged from 6% to 23% (Webber III *et al.*, 2002a). Kenaf can be ensilaged effectively, and it has satisfactory digestibility with a high percentage of digestible protein. Digestibility of dry matter and crude proteins in kenaf feeds ranged from 53% to 58% and 59% to 71%, respectively (Swingle *et al.*, 1978). Kenaf meal, used as a

supplement in a rice ration for sheep, compared favorably with a ration containing alfalfa meal. It has also been determined that chopped kenaf (29% dry matter, 15.5% crude protein, and 25% acid detergent fiber) is a suitable feed source for Spanish (meat type) goats (Swingle *et al.*, 1978). The majority of the breeding programs have developed cultivars that are more suitable for fiber production (stalk yield, self-defoliating, greater stalk percentages, reduced branching) than for forage production (Webber III *et al.*, 2002a). The leaf yields and leaf biomass percentages are important considerations in selecting cultivars to be used for kenaf forage production, because the leaves are the primary source of protein (Webber III *et al.*, 2002b). The leaf biomass percentage and percent crude protein decreased as the kenaf plant increased in height and maturity. This composition and quality change occurred because the lower leaves senesce, often producing plants without leaves on the lower one-half to three-quarters of the plant stalk (Swingle *et al.*, 1978).

### 1.4.2.3. Fiber production

#### **Planting**

Kenaf can be planted in the spring once the soil has warmed to 13°C and the threat of frost is past. In most areas, kenaf can be planted as early as April or May. Planting can be accomplished by using standard planting equipment in a wide range of row spacings, and can be planted on raised beds or on flat ground. The slate-black, wedge-shaped kenaf seeds are approximately 6 mm long and 4 mm wide, with 35,000 to 40,000 seeds/kg. Kenaf and grain sorghum (*Sorghum bicolor* L.) seed are similar in size, and therefore kenaf has often been planted using grain sorghum planting plates in commercial planters. Kenaf seed is planted 1.25 to 2.5 cm deep, and normally emerges within two to four days after planting (Webber III *et al.*, 2002b).

#### **Plant Populations**

Final plant populations of 185,000 to 370,000 plants/ha (75,000 to 150,000 plants/acre) are desirable for maximum yields and the production of single stalk plants with very little or no branching. To achieve the middle range of plant populations will require about 8 kg/ha of seed (corrected to 100% germination). Research has shown that when plant populations drop below the 185,000 plants/ha the stalk yields usually also decrease (Webber III *et al.*, 2002b). At low plant populations the crop produces plants with multiple branches, rather than the more desirable single stalk plants that are easier to mechanically harvest. If kenaf is planted at upper plant populations of 370,000 plants/ha, the crop compensates through competition to available environmental resources (light, soil moisture, and nutrients) by reducing the total number of plants. Although basal stalk diameters may vary greatly within a given kenaf field, at satisfactory populations the average basal stalk diameters will be in the range of 1.5 to 3.8 cm. Plants along the field's outward border are usually larger and branched in the direction away from the kenaf field (Webber III *et al.*, 2002a).

### **Fertility**

One of kenaf's advantages as a crop, is it can be successfully grown in a wide range of soil types, from high organic peat soils to sandy desert soils (Dempsey, 1975). Although kenaf grows better on well-drained, fertile soils with a neutral pH, the crop can withstand late season flooding, low soil fertility, and a wide range of soil pH values (Dempsey, 1975). Kenaf also has shown excellent tolerance to drought conditions. Proper fertility maintenance, especially supplemental nitrogen application, is needed to optimize kenaf yields, and minimize production costs. As the result of the inherent differences between soil types with respect to soil fertility, soil texture, organic matter, and pH, there is a wide range of reported responses to fertilizer applications on kenaf crop production (Webber III *et al.*, 2002a). Previous studies reported inconsistent differences relative to the effects of N on kenaf stalk yields, because plants responded in different ways on the same soil in different year. Excess of N is clearly detrimental for stalk yield and fiber quality (Webber III *et al.*, 2002b).

### **Weed Control**

Though kenaf grows quickly and competes well with weeds, initial weed control is often required. Researchers have reported that kenaf is a good competitor with weeds once the plants are of sufficient size to shade the ground, yet weeds can significantly reduce kenaf yields. Weed control, therefore, becomes an important consideration in obtaining optimum kenaf yields (White *et al.*, 1970).

### **Crop Rotation**

As kenaf production continues to increase, it is essential to integrate this alternative fiber and feed crop into existing cropping systems. Including kenaf in a crop rotation with a legume crop is an excellent management strategy that has the potential to provide numerous crop production advantages, including reduced pest problems and increased soil fertility. Soybean (*Glycine max* L.) is a legume crop that is also grown throughout the same production areas where kenaf can be successfully produced. Research with kenaf-soybean crop rotations indicated that the crops were indeed compatible (Webber III *et al.*, 2002a). The research determined that stunt (*Tylenchorhynchus* spp.) nematode populations decreased as a result of kenaf production. The significant reduction in stunt nematodes benefited the next year's soybean crop (Vicidomini, 2007).

### **Kenaf paper**

The use of Kenaf in paper production offers various environmental advantages over producing paper from trees. In 1960, the USDA surveyed more than 500 plants and selected kenaf as the most promising source of "tree-free" newsprint. In 1970, kenaf newsprint produced in International Paper Company's mill in PineBluff, Arkansas, was successfully used by six U.S. newspapers. Printing and writing paper made from the fibrous



kenaf plant has been offered in the United States since 1992. Again in 1987, a Canadian mill produced 13 rolls of kenaf newsprint which were used by four U.S. newspapers to print experimental issues. They found that kenaf newsprint made for stronger, brighter and cleaner pages than standard pine paper with less detriment to the environment (Suihko *et al.*, 2009). Due partly to kenaf fibers being naturally whiter than tree pulp, less bleaching is required to create a brighter sheet of paper. Hydrogen peroxide, an environmentally-safe bleaching agent that does not create dioxin, has been used with much success in the bleaching of kenaf (Ohtani *et al.*, 2001). Various reports suggest that the energy requirements for producing pulp from kenaf are about 20 percent less than those for wood pulp, mostly due to the lower lignin content of kenaf (Yang *et al.*, 2001).

USDA kenaf expert Daniel Kugler predicts that kenaf will be widely used to make paper, and that it represents a promising cash crop for American farmers. One acre of kenaf produces 7 to 11 tons of usable fiber in a single growing season. In contrast, an acre of forest (in the USA) produces approximately 1.5 to 3.5 tons of usable fiber per year. It is estimated that growing kenaf on 5,000 acres (20 km<sup>2</sup>) can produce enough pulp to supply a paper plant having a capacity of 200 tons per day. Over 20 years, one acre of farmland can produce 10 to 20 times the amount of fiber that one acre of Southern pine (Webber III *et al.*, 2002b).

### 1.5. The natural fibers extraction

The process of separation and extraction of fibers from non-fibrous tissues and woody part of the stem through separation, dissolution and decomposition of pectins, gums and other mucilaginous substances is called retting. The quality of the fiber is largely determined by the efficiency of the retting process (Pallesen, 1996). In retting, the most important aspect is that pectic materials are broken down and the fibers are liberated. Fiber quality is dependent on method of extraction applied in different natural conditions and duration of retting (Ali *et al.*, 1976). Generally, the bottom portion of the plant is thick and hard which takes a longer time for retting than the upper portion (Ahmed *et al.*, 2001). Owing to over maturity, variety and improper retting, the bottom portions of these unretted bark materials are cut down in the mills and are known as cuttings. In addition to these, at the end of the process there are some fibers which are naturally spiky, course, harsh and less pliable (Ali *et al.*, 1976). These fibers are known as low-grade fibers and have less utility, which amount 30-40% of the total fibers, and are not suitable for spinning (Ahmed *et al.*, 2001).

Extraction of the fiber involves separation of the cementing material by mechanical method or its dissolution and decomposition by physical-chemical and microbiological method by which the fiber bundles are loosened from the adhering tissues and are removed by washing (Ali, 1958). The quality of the fiber produced and the simplicity with which it is spun into yarns fibers depends to the proper control of the different process (Pallesen, 1996).

### 1.5.1. Mechanical retting

Private industries have developed an assortment of mechanical harvesters and post-harvest equipment to separate the bark from the core material, and the bast fibers from the core fibers (Webber III *et al.*, 2002b). In the past, USDA's initial interest in kenaf as an alternative cordage source during World War II, a tractor-drawn harvester-ribboner was developed. This machine harvested green plants, removed the leafy, low fiber top portion of the plant, ribboned the bark, bundled the ribbons, and tied the ribbon bundles (Dempsey, 1975). Ribboning is the process of removing the bark from the core material and the portions of bark obtained is called ribbons. The same process is also referred to as decortication, the removal of the core from the bark. The original objective of the ribboners/decorticators was to harvest the bark for its valuable bast fiber and discard the unwanted core material. The main difference between ribboners and decorticators is that in ribboners the core material was crashed and crumbled by grooved rollers, instead the decorticators peel off the bark maintaining the core intact.

Newer ribboners/decorticators have been developed specifically for the kenaf industry (Chen *et al.*, 1995) or adapted from other fiber industries (hemp and jute). Unlike the older equipment, the newer ribboner/decorticator was built specifically for kenaf and actually intended to be an in-field harvest-separator. The objective is no longer to harvest only bark ribbons, but to separate and harvest the core material for other uses (Chen *et al.*, 1995). As with the earlier ribboners/decorticators, the newer equipment must also achieve a number of outcomes to produce positive economic advantages (Webber III *et al.*, 2002b). The advantages of these newer ribboner/decorticator harvesters over other types of kenaf harvesters, such as sugarcane-type or forage-type harvesters, include the ability to produce a cleaner separation between the bark and core components, quicker drying of the separated components, and greater flexibility in determining the cutting length of the fiber strands (Chen *et al.*, 1995).

Nevertheless, the mechanical methods always perform a separation of single tissues and a partial cleaning of the bark, without liberation of bast fibers (Ouajai *et Shanks*, 2005). Generally, the mechanical methods are applied to obtain raw material for low price kenaf fibers applications. But mechanical methods are very important as a pre-treatment before chemical or biological extraction of bast fibers in order to obtain raw material of high quality.

### 1.5.2. Physical-chemical retting

In chemical retting, the cementing material can be removed by dissolution with certain chemicals, usually strong alkali solution, at high temperature and pressure.

The wet oxidation process combines alkali solution with high temperature (till 320°C) and air insufflation (0,5-20 MPa) in controlled environment. In this process lignin and hemicellulose are removed, partially hydrolyzed and mineralized to CO<sub>2</sub> and H<sub>2</sub>O, gaining a solid fraction particularly rich in cellulose. The oxygen introduced in the controlled environment contributes to bleaching and sparkling of processed fibers (Bjerre *et Schmidt*, 1997).

The steam explosion process (STEX) relies on a sudden changes of pressure applied to the entire stem of plants. A pretreatment of stalks with chemical compounds precedes the incubation at high temperature with overpressure of vapor at 230 °C, then, after 30 minutes, the pressure is dropped down suddenly. This sudden change of pressure with the help of chemical compounds makes explode the plant stem dividing in its single components. Obviously, the explosion nature of the extraction process altered the fiber integrity and quality (Vignon *et al.*, 1995).

The fiber obtained by physical-chemical method of retting seems to be a little coarser, rough in the feel and stiff. The gravimetric fineness values do not differ very much from that of retted fiber by bacteria. The fiber strands after drying needs to be softened by rubbing with hand to open up the fiber and to remove the stiffness of the strand. A cationic softener may be used to the extent of 0.2% on the weight of the fiber. Ammonium oxalate and sodium sulphate was found to be suitable, as in chemical retting the fibers are extracted under controlled condition and the fiber properties are not affected by the treatment, the procedure may be adopted as a standard method of fiber extraction (Hepworth *et al.*, 2000).

However, these processes are considered expensive, due to the high consumption of chemicals and energy, and environmental unfriendly, furthermore, it produces low-quality fibers (Tamburini *et al.*, 2004).

### 1.5.3. Microbiological retting

Traditionally, two different types of microbiological retting were mainly adopted; those are dew and water retting (Tamburini *et al.*, 2004). Our knowledge about microbiological retting mainly derives from studies on flax (Donaghy *et al.*, 1990), hemp (Tamburini *et al.*, 2003; Di Candilo *et al.*, 2000), and jute (Ahmed *et al.*, 2001; Ali, 1958), while kenaf was less investigated.

In dew retting, the harvested plants are left in the fields and retting is carried out by filamentous fungi present both in soil and on plants. The process is speeded up by changes in environmental conditions: high humidity and low temperatures during the night and higher temperatures and drier conditions during the day. Several fungal species colonising the plant during flax dew retting have been isolated: *Aspergillus niger* (Ahmed *et al.*, 2001), *terricola* (Yadav *et al.*, 2009) and *sojae* (Henriksson *et al.*, 1997), *Macrophomina phaseolina* (Ahmed *et al.*, 2001), *Rhizopus oryzae* (Xiao *et al.*, 2008), *Mucor* sp. (Henriksson *et al.*, 1997), *Chaetomium* sp., *Phoma* sp. *Fusarium* sp. and several *Penicillium* sp. have been found to be good retting agents (Molina *et al.*, 2001; Booth *et al.*, 2004). Important properties of colonizing fungi are the high level of pectinase activity, ability to attack noncellulosic cell types without attacking cellulosic fibers, capacity to penetrate the cuticular surface of the stem, and efficient fiber release from the core (Henriksson *et al.*, 1997).

In water retting, bast fiber crops' straw is soaked in large tanks filled with water. In this process, plant absorbs water when steeped in it and swell, ultimately bursting occurs at several places and the soluble constituents which include sugars, glucosides and nitrogenous compounds are liberated creating the surrounding environment a good start

medium for the growth of micro-organisms present in water as well as in the plants (Ahmed *et al.*, 2001). These organisms gradually develop and multiply by utilizing free sugars, pectins, hemicelluloses, proteins etc. of the plants as nutrients. Specific enzymes secreted by the organisms cause degradation of the complex organic materials to simpler compounds which are then metabolized for their life processes (Munshii *et al.*, 2008). A series of biochemical reactions go on as a result of which the chemical composition and pH of the water continuously change during the retting process (Tamburini *et al.*, 2004). Decomposition of the free sugars takes place at early stages of the process, followed by the breakdown of pectins and decomposable hemicelluloses and nitrogenous compounds (chiefly proteins) are degraded at the later stage (Ahmed *et al.*, 2001). Analysis of retting water revealed the presence of decomposed products such as organic acids (acetic, lactic, butyric  $\alpha$ -ketoglutaric), acetone, ethyl alcohol, butyl alcohol and various gases (Ahmed *et al.*, 2001). If this process is continued beyond the optimum period, microorganisms begin to degrade the fiber cellulose. Such a condition is known as "over retting" (Pallesen, 1996).

Microbial retting is essentially a biochemical process where fiber bundles are steeped in water and subjected to controlled decomposition of biopolymers such as pectins, celluloses, and hemicelluloses that hold the bast cells to the rest of the stem (Munshii *et al.*, 2008). Attempts have been taken to isolate and identify the complex microbial community, which include hydrolytic, cellulolytic, fermenting, homoacetogenic, syntrophic, and acetate-utilizing microorganisms, necessary for dissolution and decomposition of these biopolymers (Ahmed *et al.*, 2001; Tamburini *et al.*, 2003). As retting is one of the most important factors responsible for determining the quality and yields of fiber, other than type of bast fiber plant, age of plant, temperature and pH of retting water, type and depth of water and activators, controlling the quality of water along with improving microorganisms used in the process are the keys to improved fiber quality (Munshii *et al.*, 2008).

Several aerobic bacteria of the genus *Bacillus*, viz., *B. subtilis*, *B. polymyxa*, *B. mesentericus*, *B. macerans* (Munshii *et al.*, 2008; Ali, 1958; Kapoor *et al.*, 2001; Tamburini *et al.*, 2003) and anaerobic bacteria of the genus *Clostridium*, viz., *C. tertium*, *C. aurantibutyricum*, *C. felsineum* etc... have been isolated from retting water (Munshii *et al.*, 2008; Tamburini *et al.*, 2003; Zheng *et al.*, 2001; Di Candilo *et al.*, 2000; di Candillo *et al.*, 2010; Donaghy *et al.*, 1990). The aerobic organisms grow first and consume most of the dissolved oxygen, ultimately creating an environment favorable for the growth of anaerobes. It has been stated that the greater part of decomposition is carried out by anaerobic species (Munshii *et al.*, 2008). In microbial retting, pectin and hemicellulose are decomposed into water soluble compounds by specific enzymes secreted by the microorganisms present in water and in plant. Microbiological retting conditions are difficult to maintain in actual retting operation (Di Candilo *et al.*, 2000). There is no test method to determine the end of retting period to extract the fiber. Biological retting is the cheapest and still universally practiced method for the commercial extraction of natural fibers from bast fiber plants (Ali *et al.*, 1976).

Different microorganisms have been identified as main retting agent of different fiber crops. Nevertheless, all these diverse microorganisms showed a common feature: a high

pectinolytic activity as described for dew retting process. Since fungi and bacteria are the main retting agents, their properties affect the course of the process and the quality of the product (Tamburini *et al.*, 2004).

The retting process is the major limitation to an efficient and high quality fiber production, thus being the key feature in any future expansion of these industrial crops (Pallesen, 1996). The industrial retting process needs to be enhanced by speeding up and controlling the process to improve fiber quality and reduce production costs.

A modification of water retting is enzymatic treatment, called also bioscouring, where degrading enzymes are directly added to tank water or in a bioreactor (Ouajai *et Shanks*, 2005). Despite the complexity of plant stem, the retting process was successfully carried out using purified enzymes.

Akin and colleagues developed an enzyme-retting process tested in small pilot plant for the treatment of flax (Akin *et al.*, 2000). Structural and chemical characteristics determine the effectiveness of retting of stems (Akin *et al.*, 2000). The main enzymatic activity necessary to free the fibers from other tissues are pectinases, and the addition of chelators with pectinase-rich enzyme mixtures to improve retting (Akin *et al.*, 2001). Same good results were obtained also with other fiber crops, as hemp (Ouajai *et Shanks*, 2005). Such an approach is thought to be particularly effective in degrading Ca<sup>2+</sup>-stabilized pectins primarily located in the epidermal regions (Akin *et al.*, 2001). Optimization of the retting method should be continued and further knowledge of the structure and chemistry of the bast tissues will reduce the cost of enzyme formulations and improve specific properties of the fibers (Foulk *et al.*, 2008; Foulk *et al.*, 2008).

However, at the present time, enzymatic retting is not yet feasible, due to the high cost of the process, the scarce availability of enzymatic mixture specific for retting (Tamburini *et al.*, 2004).

### 1.5.3.1. Factors affecting in the retting

The efficiency of retting depends on a number of factors (Ahmed *et Akhter*, 2001). Some of these factors are within the easy control of the cultivators, whereas other are dependent on nature. The factors are as follows:

- a) **Plant age:** As the kenaf plant grows older, the tissues become more and more matured; the structure of the decomposable matters like pectins and hemicelluloses may be modifies to more resistant forms and their quantity may increase. As a result micro-organisms take longer time to ret a plant. Usually, fiber loss is 17.3% if 75 days old plant is ribboned and 9.5% if plants of 120 days is ribboned (Webber III *et al.*, 2002b).
- b) **Fertilization of crop:** Higher dose of nitrogenous fertilizer applied to the crop has been found to reduce the retting period. The reverse happens when phosphatic fertilizers are used (Ahmed *et Akhter*, 2001).
- c) **Retting water:** Retting is a biochemical process in which various decomposition is carried out in stagnant water, accumulation of these products causes hindrance to the growth and activity of the causative micro-organisms. Vary fast moving water, removes these toxic substances quickly, but it carries away the microbial

population along with it resulting in uniform retting. Retting is best carried out in slow moving clear water (canal, river, etc.) with low content of materials as salts, iron and calcium content is preferable for good retting. It is desirable to change water to keep the pH about 7 and 35°C temperature. When retting water is soft, the quality of fiber is better than when hard water is used. The presence of iron, particularly ferrous iron, is not desirable as it imparts a dark color to the fiber. Good results are obtained when nearly 15 cm water is left over the top layer of the charge of plant bundles, bundles of stems to be retted are approximately 20 cm in diameter and plant water ratio is nearly 1:20. In laboratory trials, partial aeration may accelerate the retting indirectly by oxidizing the decomposition products formed.

- d) **pH and temperature:** Laboratory experiments have shown that two pH values are optimum for retting, one being in the acidic region (around pH 5.0) and the other in the neutral or slightly alkaline region and that the retting period is relatively shorter in acidic pH. Natural water from different sources have pH between 6.0 and 8.0 and are used for retting purposes (Ali *et al.*, 1976). The optimum temperature for retting was 34°C. Retting takes a longer time when the temperature deviates from 34°C.
- e) **Activators:** Retting is accelerated in the presence of several activators. Natural activators like dhaincha (*Sesbania aculeata*) and sunnhemp (*Crotalaria juncea*) plants are generally introduced into the fiber crops' stem bundles put for retting. These leguminous plants being rich in nitrogen content help the growth and activity of the retting microbes by supplying additional nutrients to them. Indian Central Jute Committee used tribasic calcium phosphate dibasic potassium phosphate, ammonium sulphate, ammonium oxalate, dibasic ammonium phosphate, calcium carbonate, monobasic potassium phosphate, sodium oxalate, calcium nitrate, potassium nitrate, bone dust and gelatin with retting water as activators of kenaf retting. Only bone dust and ammonium sulphate when added singly or in combination reduced the retting period considerably. Stems of sunnhemp or dhaincha when put in the bundles of retting kenaf shortened the retting period (Ahmed *et al.*, 2001).
- f) **Neutralization of retting liquor:** The retting speed can be accelerated and quality of fiber can be improved significantly if the retting liquors are neutralized by chemicals like  $\text{NaHCO}_3$ . During the process of flax retting, butyric, lactic and acetic acids were produced in the retting liquor. In *C. capsularis*, although the addition of sodium carbonate and sodium bicarbonate turned the retting liquor dark at the end, this color did not at all have any effect on the color of the fibers. This eliminates the usually common contention that whenever the retting liquor is dark the fibers are likely to be dark (Ahmed *et al.*, 2001). It might be suspected that the tannin-iron-reaction may be responsible for the dark color and of possibly operative only at a particular hydrogen-ion concentration of the retting liquor having different chemical properties. These properties do not exclude the possibility of the influence of phenolic compounds of kenaf plants. In *C. olitorius*, only the phenolic compound is responsible for the dark color. *C. capsularis* is far darker than that of *C. olitorius*.



- g) **Urea:** Retting water incorporated with urea ahead higher count of bacteria (both aerobic and anaerobic) maintained higher pH favorable to the bacterial growth and provide increase supply of total nitrogen which increase retting (Ahmed et Akhter, 2001).
- h) **Acidity and total nitrogen:** The retting water become acidic which impaired the proper multiplication and activity of retting bacteria of the retting water, since it is known that the optimum growth of most bacteria is towards the neutrality. The growth of retting bacteria is not favorable, will affect the quality of the fibers, neutralization of the acidity of retting liquor could be useful approach for conducting retting in more favorable conditions (Ali et al., 1976).
- i) **Harvesting time:** Considering the quality of the fiber it seems advantageous to harvest kenaf about 25 days earlier than the usual recommended stage of average flowering. The slightly lower yield obtained because of the early harvest is likely to be compensated by the improved quality of the fiber (Webber III et al., 2002b).
- j) **Fibrous matter:** Speed of retting depends on the non-fibrous matters and soluble carbohydrates contents of the stem which provide food to the retting microbes and eventually get decomposed into organic acids. Kenaf plants contain much more non-fibrous matter that can ret effectively. Imperfect retting cause defects in fibers which cause processing difficulties for the industry. Some of these defects are carried even to the finished goods and devalue them to the point of even rejection. The more common defects are described below (H'ng et al., 2009).
  - *Rooty fiber, centre root, runner, hunka:* In all these defects, fiber is masked by barks. In rooty fiber, barks remain at the bottom, in centre root at the central region, in runners along the entire half of the bottom and in hunka all over the stem. These defects may be due to unusual developments in some portions of the plant or to improper retting.
  - *Creppy and gummy fiber:* Fibers with top ends rough and hard are called creppy, while fibers which are held together by non-dissolved and non-decomposed gummy substances, they are called gummy.
  - *Sticky fiber:* If not properly cleaned after extraction, fibers may contain adhering sticks; these may create trouble in the processing machinery.
  - *Shamla fiber:* Presence of excessive iron in retting water or use of weighting materials rich in tannin, such as, banana stems, freshly cut mango trees, etc. may impart dark color to the fiber. This dark colored fiber is known as shamla jute (Ahmed et Akhter, 2001).

### 1.6. Pectinolytic enzymes

In nature, microorganisms produce an array of enzymes, which have been exploited commercially over the years. Pectinases are of great significance with tremendous potential to offer to industry (Jayani et al., 2005). They are one of the upcoming enzymes of the commercial sector (Kashyap et al., 2001), especially the juice and food industry

(Hoondal *et al.*, 2002) and in the paper and pulp industry (Ohtani *et al.*, 2001; Beg *et al.*, 2001).

Pectinolytic enzymes or pectinases are a heterogeneous group of related enzymes that hydrolyze the pectic substances, present mostly in plants. Pectinolytic enzymes are widely distributed in higher plants and microorganisms (Mohnen, 2008). They are of prime importance for plants as they help in cell wall extension and softening of some plant tissues during maturation and storage (Ridley *et al.*, 2001). They also aid in maintaining ecological balance by causing decomposition and recycling of waste plant materials. Plant pathogenicity and spoilage of fruits and vegetables by rotting are some other major manifestations of pectinolytic enzymes (Obi, 1981; Obi *et al.*, 1981).

It has been reported that microbial pectinases account for 25% of the global food enzymes sales. Almost all the commercial preparations of pectinases are produced from fungal sources (Henriksson *et al.*, 1997). *Aspergillus niger* is the most commonly used fungal species for industrial production of pectinolytic enzymes (Jayani *et al.*, 2005).

### 1.6.1. Pectinases and their industrial applications

Pectinolytic enzymes are of significant importance in the current biotechnological era with their all-embracing applications in fruit juice extraction and its clarification, scouring of cotton, degumming of plant fibers, waste water treatment, vegetable oil extraction, tea and coffee fermentations, bleaching of paper, in poultry feed additives and in the alcoholic beverages and food industries (Jayani *et al.*, 2005).

The industrial applications of pectinases are wide and comprise all the sectors described above, but, from a commercial point of view, they can be roughly divided by their origin in acidic ones (produced by fungi) and alkaline ones (produced by bacteria) (Kashyap *et al.*, 2001).

- a) Acidic pectic enzymes used in the fruit juice industries and wine making often come from fungal sources, especially from *Aspergillus niger*. The juices produced by these industries commercially include: Sparkling clear fruit juices as apple, pear and grape juices (also wine),
- b) Juices with clouds as citrus juices, prune juices, tomato juice and nectars, and
- c) Unicellular products where the intent is to preserve the integrity of the plant cells by selectively hydrolyzing the polysaccharides of the middle lamella (maceration of plant tissue, liquefacing and saccharification of biomass, and isolation of protoplasts).

The objectives of adding enzymes differ in these three types of fruit and vegetable juices. Alkaline pectinases are mainly used in the degumming and retting of fiber crops and pretreatment of pectic wastewater from fruit juice industries. These enzymes come mostly from bacterial sources (Hoondal *et al.*, 2002). In the industrial sector, alkaline pectinases, mainly from *Bacillus* sp. are applied for the following purpose:

- a) Retting and degumming of fibers crops (jute, flax, ramie, hemp, kenaf and coir from coconuts husks (Kapoor *et al.*, 2001; Akin *et al.*, 2000; Bruhlmann *et al.*, 1994).
- b) Treatment of pectic wastewater like the wastewater from the citrus-processing industry.



- c) Production of Japanese paper a traditional paper obtained by retting of Mitsumata bast.
- d) Paper making because pulp and paper mills are beginning to use enzymes to solve problems in their manufacturing processes.
- e) Oil extraction from rape seed (*Canola*), coconut germ, sunflower seed, palm, kernel and olives. In fact, cell-wall-degrading enzymes, including pectinase, may be used to extract vegetable oil in an aqueous process by liquefying the structural cell wall components of the oil-containing crop, in alternative to commonly used organic solvents as hexane, which are potential carcinogen.
- f) Coffee and tea fermentation. Pectic enzymes are added in order to remove the pulpy layer of the coffee bean, mainly composed by pectin. While, in tea fermentation these enzymes, carefully dosed, accelerate the process without damaging the tea leaves.

Supplier	Location	Brand name
C.H. Boehringer Sohn	Ingelheim, Germany	Panzym
Ciba-Geigy, A.G.	Basel, Switzerland	Ultrazyme
Grinsteevaeket	Aarhus, Denmark	Pectolase
Kikkoman Shoyu, Co.	Tokyo, Japan	Sclase
Schweizerische Ferment, A.G.	Basel, Switzerland	Pectinex
Societe Rapidase, S.A.	Seclin, France	Rapidase, Clarizyme
Wallerstein, Co.	Des Plaines, USA	Klerzyme
Rohm, GmbH	Darmstadt, Germany	Pectinol, Rohament

**Table 2.** Principal commercial pectinases or enzyme mixtures containing pectinases (Kashyap *et al.*, 2001).

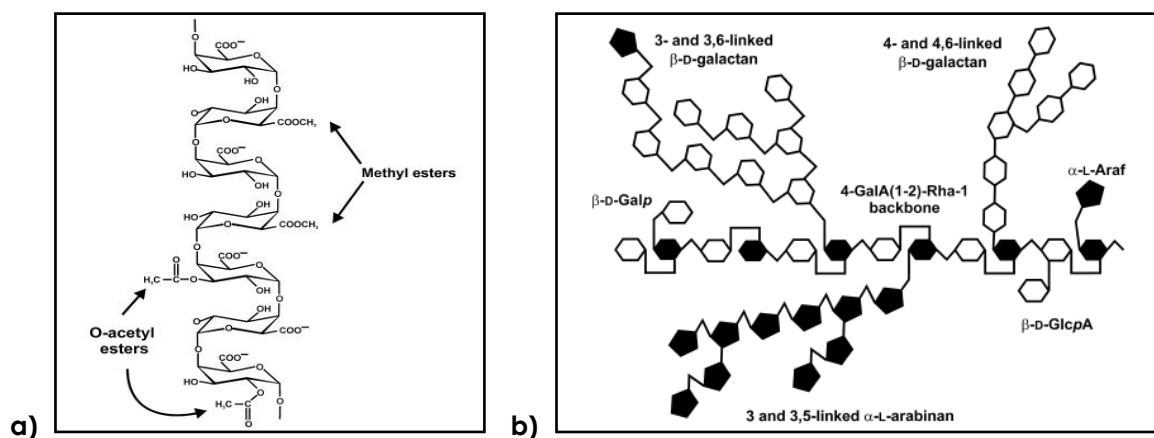
Pectinases or pectinolytic enzymes can be briefly described as those enzymes that hydrolyze pectic substances (Kashyap *et al.*, 2001). Listed in table 2, there are the most important commercial pectinases produced actually and commonly employed in processes cited before.

### 1.6.2. The substrate

Pectic substance is the generic name used for the compounds that are acted upon by the pectinolytic enzymes. They are high molecular weight, negatively charged, acidic, complex glycosidic macromolecules (polysaccharides) that are present in the plant kingdom. They are present as the major components of middle lamella between the cells in the form of calcium pectate and magnesium pectate (Ridley *et al.*, 2001). That the middle lamella is largely composed of pectic substances has been confirmed by the comparable uptake of ruthenium red by known pectic substances (Mohnen, 2008) and from the estimation of pectin by the use of alkaline hydroxylamine (Ridley *et al.*, 2001). Pectic substances account for 0.5–4.0% of the fresh weight of plant material (Jayani *et al.*, 2005).

Pectic substances mainly consist of galacturonans and rhamnogalacturonans in which the C-6 carbon of galactate is oxidized to a carboxyl group, the arabinans and the arabinogalactans.

These substances are a group of complex colloidal polymeric materials, composed largely of a backbone of anhydrogalacturonic acid units (Mohnen, 2008). The carboxyl groups of galacturonic acid are partially esterified by methyl groups and partially or completely neutralized by sodium, potassium or ammonium ions. Some of the hydroxyl groups on C2 and C3 may be acetylated. The primary chain consists of  $\alpha$ -D-galacturonate units linked  $\alpha$ -(1 $\rightarrow$ 4), with 2–4% of L-rhamnose units linked  $\beta$ -(1 $\rightarrow$ 2) and  $\beta$ -(1 $\rightarrow$ 4) to the galacturonate units (Figure 9). The rhamnogalacturonans are negatively charged at pH  $\geq$  5.



**Figure 9.** Principal structures in pectic substances: a) composition of backbone of pectin with sites of modification: methylesterification on C6, and acetylation of C2 and C3 carbon of primary chain; b) schematic raffiguration of possible side chain modifications present in pectic substances (Mohnen, 2008; Ridley *et al.*, 2001).

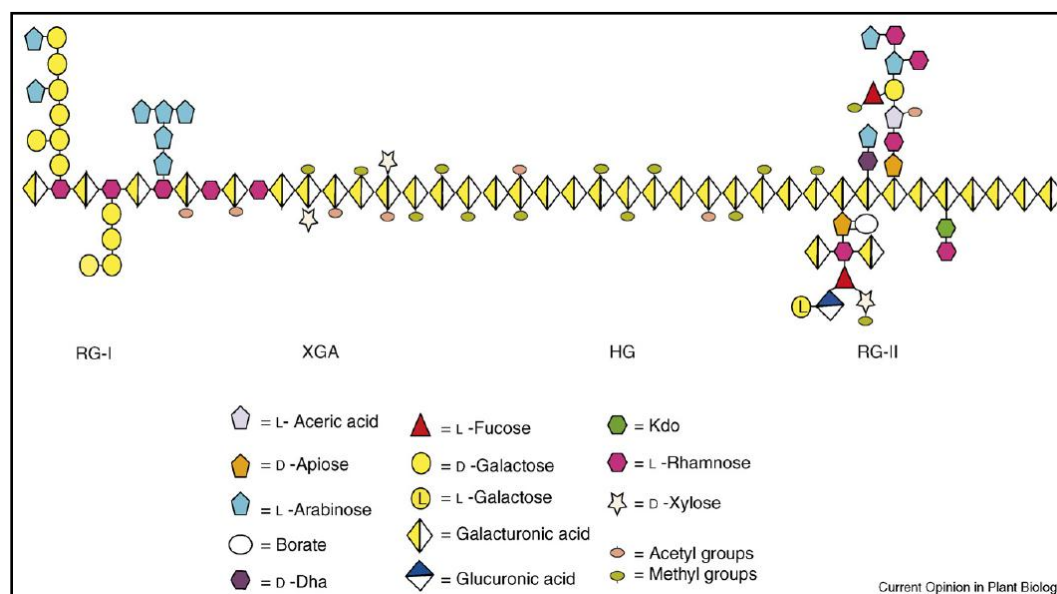
The side chains of arabinan, galactan, arabinogalactan, xylose or fucose are connected to the main chain through their C1 and C2 atoms (Ridley *et al.*, 2001). The above description indicates that the pectic substances are present in various forms in plant cells and this is the probable reason for the existence of various forms of pectinolytic enzymes (Mohnen, 2008).

The American Chemical Society classified pectic substances into four main types as follows (Jayani *et al.*, 2005):

- a) **Protopectin:** is the water insoluble pectic substance present in intact tissue. The features of the insolubility of pectin in protopectin are complicated, and include (i) bonding of pectin molecules to other cell wall constituents, such as hemicellulose or cellulose, (ii) binding of pectin molecules with polyvalent ions, such as Ca<sup>2+</sup>, Fe<sup>2+</sup>><sup>3+</sup>, and Mg<sup>2+</sup> leading to insolubility, (iii) secondary valence bonding between pectin molecules or with other cell wall constituents such as cellulose or hemicellulose, (iv) salt bridging between the carboxyl groups of pectin molecules and the basic groups of protein and, (v) mechanical entwining of the pectin molecules with each other and with other cell wall constituents (Sakai *et al.*, 1989). Protopectin on restricted hydrolysis yields pectin or pectic acids. A model for the chemical structure of protopectin has been proposed

(Yoshitake *et al.*, 1994) in which neutral sugar side chains are arranged in blocks (hairy regions) separated by unsubstituted regions containing almost exclusively galacturonic acid residues (smooth regions).

- b) **Pectic acid**: is the soluble polymer of galacturonans that contains negligible amount of methoxyl groups. Normal or acid salts of pectic acid are called pectates.
- c) **Pectinic acids**: is the polygalacturonan chain that contains >0 and <75% methylated galacturonate units. Normal or acid salts of pectinic acid are referred to as pectinates. Pectinic acid are the unique pectic substance able to form gel structure combined with sugars, acids and divalent ion (Hoondal *et al.*, 2002).
- d) **Pectin** (Polymethyl galacturonate): is the polymeric material in which, at least, 75% of the carboxyl groups of the galacturonate units are esterified with methanol. Pectin is the most structurally complex family of polysaccharides in nature (see Figure 10), making up 35% of primary walls in dicots and non-graminaceous monocots, 2–10% of grass and other commelinoid primary walls, and up to 5% of walls in woody tissue (Mohnen, 2008). Three pectic polysaccharides (homogalacturonan, rhamnogalacturonan-I, and substituted galacturonans) have been isolated from primary cell walls and structurally characterized (Ridley *et al.*, 2001).



**Figure 10.** Schematic structure of pectin showing the four pectic polysaccharides homogalacturonan (HG), xylogalacturonan (XGA), rhamnogalacturonan I (RG-I) and rhamnogalacturonan II (RG-II) linked to each other (Mohnen, 2008).

### 1.6.3. Classification of pectic enzymes

Pectinases are classified under three headings according to the following criteria: whether pectin, pectic acid or oligo-D-galacturonate is the preferred substrate, whether pectinases act by trans-elimination or hydrolysis and whether the cleavage is random (endo-,

liquefying of depolymerizing enzymes) or endwise (exo- or saccharifying enzymes) (see Table 3). The three major types of pectinases are as follows.

### 1.6.2.1. Pectinesterases (PE)

Pectinesterases also known as pectin-methyl hydrolase, catalyzes deesterification of the methoxyl group of pectin forming pectic acid. The enzyme acts preferentially on a methyl ester group of galacturonate unit next to a non-esterified galacturonate unit (Kashyap *et al.*, 2001).

These enzymes provide the first step in complex pectic substances depolymerization, and they are forerunner for the depolymerizing enzymes (Micheli, 2001).

These enzymes are very important for pectin metabolism in microorganisms and may be a quantitatively significant source for methanol generation in nature because of the known activity of pectin methylesterase on methoxyl esters of galacturonic acid (Schink *et Zeikus*, 1980).

### 1.6.2.2. Depolymerizing enzymes

These are the enzymes principally involved in pectin metabolism and depolymerization because they attack the backbone solubilizing the pectic substances. They can be divided in two groups by their pattern of action in:

- a) Hydrolyzing glycosidic linkages that catalyze the hydrolytic cleavage of  $\alpha$ -1,4-glycosidic bonds.
- b) Cleaving  $\alpha$ -1,4-glycosidic linkages by trans-elimination, which results in galacturonide with an unsaturated bond between C4 and C5 at the non-reducing end of the galacturonic acid formed.

These classes of enzymes represent the most abundant and heterogeneous ones because they comprise pectinases that recognize almost any kind of modification of the backbone of the pectic substances and provides its complete depolymerization to most simple compounds and monomers (Kashyap *et al.*, 2001).

Many kind of depolymerizing enzymes have been described in fungi and in bacteria. These classes of enzymes, moreover, comprise most important enzymes involved in retting process of natural fibers (Akin *et al.*, 2000). In particular, it has been described that retting which results in degradation of pectin around and between fiber bundles, can result strictly from the degradation of the unmethylated or low-esterified homogalacturonan regions which occur throughout the plant stem wall (Evans *et al.*, 2002). Furthermore, Zhang *et al.* (2000), predicted via Fried's Test that retting efficiency and the ability to degrade these cell wall components were highly correlated (correlation coefficient=0.99). As the degradation of low-esterified pectin is catalyzed by polygalacturonase and pectate lyase activity (Zhang *et al.*, 2000), a mixture of these enzymes has been reported as able to perform enzymatic retting of same fiber crops (jute, flax and hemp).

### 1.6.2.3. Protopectinase

This enzyme solubilizes protopectin forming highly polymerized soluble pectin. The protopectin is the main constituent of middle lamella between adjacent plant cell. Moreover, its insolubility provide the cementing force that join plant cell in primary cell wall (Mohnen, 2008). This class of enzymes is a heterogeneous group of enzymes that solubilize pectin from the insoluble protopectin in plant tissues by restricted depolymerization. Various protopectinases have been reported with different pectin solubilizing activities depending on the substrate (Nakamura *et al.*, 1995). In literature, many protopectinases have been described and characterized. They are produced both by fungi (Nakamura *et al.*, 1995) and bacteria (Sakai *et al.*, 1989). In particular among bacteria, some strains belonging to the genus *Bacillus*, such as *B. amyloliquefaciens*, *B. cereus*, *B. circulans*, *B. coagulans*, *B. firmus*, *B. licheniformis*, *B. macerans*, and *B. pumilus*, produced a novel kind of protopectinase that don't catalyze the degradation of polygalacturonic acid in protopectin backbone (Sakai *et al.*, 1989).

Enzyme	E.C.no.	Action mechanism	Action pattern	Primary substrate	Product
<b>1. Esterase</b>					
1. Pectin methyl esterase	3.1.1.11	Hydrolysis	Random	Pectin	Pectic acid
<b>Depolymerizing enzymes</b>					
<b>a) Hydrolase</b>					
1. Protopectinase		Hydrolysis	Random	Protopectin	Pectin
2. Endopolygalacturonase	3.2.1.15	Hydrolysis	Random	Pectic acid	Oligogalacturonides
3. Exopolygalacturonase	3.2.1.67	Hydrolysis	Terminal	Pectic acid	Monogalacturonides
4. Exopolygalacturonan-digalacturono hydrolase	3.2.1.82	Hydrolysis	Penultimate bonds	Pectic acid	Digalacturonides
5. Oligogalacturonate hydrolase		Hydrolysis	Terminal	Trigalacturonate	Monogalacturonates
6. $\Delta 4:5$ Unsaturated oligogalacturonate hydrolase		Hydrolysis	Terminal	$\Delta 4:5$ - (Galacturonate)	Unsaturated monogalacturonates & saturated (n-1)
7. Endopolymethyl-galacturonase		Hydrolysis	Random	Highly esterified pectin	Oligomethyl-galacturonates
8. Endopolymethyl-galacturonase		Hydrolysis	Terminal	Gighly esterified pectin	Oligogalacturonates
<b>b) Lyases</b>					
1. Endopolygalacturonase lyase	4.2.2.2	Trans-elimination	Random	Pectic acid	Unsaturated oligogalacturonates
2. Exopolygalacturonase lyase	4.2.2.9	Trans-elimination	Penultimate bond	Pectic acid	Unsaturated digalacturonates
3. Oligo-D-galactosiduronate lyase	4.2.2.6	Trans-elimination	Terminal	Unsaturated digalacturonate	Unsaturated monogalacturonates
4. Endopolymethyl-D-galactosiduronate lyase	4.2.2.10	Trans-elimination	Random	Unsaturated poly-(methyl-D-digalacturonate)	Unsaturated methyl-oligogalacturonates
5. Exopolymethyl-D-galactosiduronate lyase		Trans-elimination	Terminal	poly-(methyl-D-digalacturonate)	Unsaturated methyl-monogalacturonates

**Table 3.** An extensive classification of pectinolytic enzymes (Jayani *et al.*, 2005).

### 1.7. Aims of this study

The sustainable development was stated in order to drive future industrial expansion to more conservative and in order to reduce environmental impact without compromising the development of technology and of economy.

The utilization of natural fibers in new industrial applications met positively all of these constituent parts. Natural fibers are environmental sustainable because they contribute to fix greenhouse gasses in raw materials that are biodegradable. Economically, natural fibers are sustainable because raw material of high quality is employed in manufacturing of high added value, fiber crops cultivation substitute low-income crops. Moreover, socially the production of natural fibers brings benefits not only to Western Countries but also in emerging countries where this production was well widespread and practiced.

In particular, natural fibers of kenaf have more potential of other fiber crops: high yield of biomass with low nutrient requirements; high quality and high tenacity of fibers; low requirements of pesticides and herbicides; rapid growth in much latitude (photosensitivity). These presuppositions stimulate cultivation of kenaf for industrial purposes. The need to meet industrial demand of kenaf fibers encouraged in last decade intensive cultivation of kenaf, but that production must be accomplished by an industrial scale process of extraction not supported by traditional method of fiber crops maceration (water retting and dew retting). Moreover, alternative methods of extraction as mechanical and physical-chemical methods of retting produce low quality fibers suitable only to low income industrial productions.

For these reasons, the major aim of this study was to develop a ribbon retting process of kenaf in bioreactor as a response of industrial demand of natural fibers.

Water retting process was reconsidered and the process was transferred in bioreactor in order to control main parameters of the maceration and to assess a uniform quality of retted fiber. Moreover, retting in bioreactor, by fixing the conditions of maceration, allowed us to better understand the different bacterial populations' dynamics during the process of fibers extraction. Later on the study of main parameters allowed us to better understand their effect on final products and permitted to perform repeatable macerations.

Moreover, the aim of this work was to improve our knowledge about the microflora involved in the water retting process and to isolate strains with high retting activities.

The application of molecular-phylogenetic methods to study natural microbial ecosystems without the traditional requirement for cultivation has resulted in the discovery of many unexpected evolutionary lineages. Although some information is available regarding the nature and activity of microorganisms involved in retting, the purpose of the present study is to report the dominance of bacteria present in the kenaf water retting environment by two complementary methods of analysis: culture-dependent and culture-independent approach.

Eventually, the development of a bacterial *inoculum* will be achieved by analyzing physiologic, metabolic and proteomic characteristics of main strains involved in kenaf ribbon retting in order to improve and standardize the quality of retted fibers.

## **2. Materials and methods**

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## 2.1. Media and solutions

### 2.1.1. Cultural media for bacteria

All cultural media, except when specified otherwise, were arranged by weighting different powdered components and mixing them in appropriated volume of bi-distilled water. pH of solutions obtained where adjusted as specified by producers with solutions (6M) of NaOH or HCl and sterilized by autoclaving at 121°C for 15 minutes at 1 atmosphere of overpressure. Solidified media were obtained by adding Technical Agar n°3 [Oxoid] at 1.2 % before the sterilization step.

#### 2.1.1.1. Nutrient broth

Nutrient broth [Oxoid]	13	g/l
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#### 2.1.1.2. Hemp medium (HM)

Yeast extract [Oxoid]	5	g/l
Tryptone [Oxoid]	10	g/l
Pectone [Oxoid]	5	g/l

Recipe obtained by Tamburini (2003) called rich media for spore-forming high pectinolytic bacteria. In according with the authors, when the media was utilized to grow bacteria in anaerobic environment, filtered solution of glucose (final concentration 2%) and cysteine (final concentration 0.05%) were also added.

#### 2.1.1.3. DMP (Minimal media with Pectin)

Ammonium nitrate ( $\text{NH}_4\text{NO}_3$ ) [Sigma-Aldrich]	3	g/l
Sodium phosphate dibasic ( $\text{Na}_2\text{HPO}_4$ ) [Sigma-Aldrich]	2.2	g/l
Potassium phosphate monobasic ( $\text{KH}_2\text{PO}_4$ ) [Sigma-Aldrich]	0.8	g/l
Pectin from citrus peel [Sigma-Aldrich]	4	g/l

After sterilization in autoclave filter sterilized solution of vitamins and minerals were added:

Wolfe's mineral solution	10	ml/l
Vitamin solution	1	ml/l

Sometimes where specified a percentage of yeast extract was added as an additional source of carbon.

#### 2.1.1.4. DMC (Minimal media with cellulose)

Ammonium nitrate ( $\text{NH}_4\text{NO}_3$ ) [Sigma-Aldrich]	3	g/l
Sodium phosphate dibasic ( $\text{Na}_2\text{HPO}_4$ ) [Sigma-Aldrich]	2.2	g/l
Potassium phosphate monobasic ( $\text{KH}_2\text{PO}_4$ ) [Sigma-Aldrich]	0.8	g/l

Carboxymethylcellulose [Sigma-Aldrich]	4	g/l
After sterilization in autoclave filter sterilized solution of vitamins and minerals were added:		
Wolfe's mineral solution (100X)	10	ml/l
Vitamin solution (1000X)	1	ml/l
Sometimes where specified a percentage of yeast extract was added as an additional source of carbon.		

#### 2.1.1.5. TSB (Tryptic Soy Broth)

Tryptone soy broth [Oxoid]	30	g/l
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#### 2.1.1.6. LB medium (Luria Bertani)

Tryptone [Oxoid]	10	g/l
Yeast extract [Oxoid]	5	g/l
Sodium chloride (NaCl) [Sigma-Aldrich]	10	g/l

#### 2.1.1.7. SOC medium

Tryptone [Oxoid]	20	g/l
Yeast extract [Oxoid]	5	g/l
Sodium chloride (NaCl) [Sigma-Aldrich]	0.5	g/l
250 mM Potassium chloride (KCl) solution	10	ml/l
Bi-distilled water	970	ml

The solution obtained was sterilized by autoclaving, afterword the following of filtered-sterilized ingredients were added:

1 M D(+)-Glucose solution	25	ml
1 M Magnesium chloride (MgCl <sub>2</sub> ) solution	5	ml

### 2.1.2. Media for biochemical and proteomic assays

#### 2.1.2.1. Peh plates

Polygalacturonic acid [Sigma-Aldrich]	10	g/l
Yeast extract [Oxoid]	10	g/l
0,5 M EDTA solution	4.4	ml/l
1 M Sodium acetate buffer pH 5.50	110	ml/l
Technical agar n°3 [Oxoid]	15	g/l

#### 2.1.2.2. Pel plates

Polygalacturonic acid [Sigma-Aldrich]	10	g/l
Yeast extract [Oxoid]	10	g/l

1 mM Calcium chloride ( $\text{CaCl}_2$ ) solution	380	$\mu\text{l/l}$
1 M Tris-HCl buffer pH 8.50	100	$\text{ml/l}$
Technical agar n°3 [Oxoid]	15	$\text{g/l}$

### 2.1.2.3. Cel plates

Carboxymethylcellulose [Sigma-Aldrich]	1	$\text{g/l}$
1 M Buffer phosphate pH 7.00	25	$\text{ml/l}$
Technical agar n°3 [Oxoid]	15	$\text{g/l}$

### 2.1.3. Solutions

#### 2.1.3.1. Wolfe mineral solution (100X)

Nitrilotriacetic acid [Sigma-Aldrich]	1.6	$\text{g/l}$
Magnesium sulfate ( $\text{MgSO}_4 \cdot \text{H}_2\text{O}$ ) [Sigma-Aldrich]	3.5	$\text{g/l}$
Sodium chloride ( $\text{NaCl}$ ) [Sigma-Aldrich]	1	$\text{g/l}$
Iron sulfate ( $\text{Fe}_2(\text{SO}_4)_3$ ) [Sigma-Aldrich]	0.076	$\text{g/l}$
Cobalt sulfate ( $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$ ) [Sigma-Aldrich]	0.13	$\text{g/l}$
Calcium chloride ( $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ ) [Sigma-Aldrich]	0.13	$\text{g/l}$
Zinc sulfate ( $\text{ZnSO}_4 \cdot 2\text{H}_2\text{O}$ ) [Sigma-Aldrich]	0.11	$\text{g/l}$
Ingredients were mixed together and then filter-sterilized with 0,2 $\mu\text{m}$ nitrocellulose filter.		

#### 2.1.3.2. Vitamin solution (1000X)

Biotine [Sigma-Aldrich]	2	$\text{mg/l}$
Folic acid [Sigma-Aldrich]	2	$\text{mg/l}$
Chlorohydrate thiamine [Sigma-Aldrich]	5	$\text{mg/l}$
Riboflavin [Sigma-Aldrich]	5	$\text{mg/l}$
Chlorohydrate pyridoxine [Sigma-Aldrich]	5	$\text{mg/l}$
Cyanocobalamin [Sigma-Aldrich]	10	$\text{mg/l}$
Nicotininc acid [Sigma-Aldrich]	0.1	$\text{mg/l}$
Calcium pantothenate [Sigma-Aldrich]	5	$\text{mg/l}$
Lipoic acid [Sigma-Aldrich]	5	$\text{mg/l}$
Ingredients were mixed together and then filter-sterilized with 0.2 $\mu\text{m}$ nitrocellulose filters.		

#### 2.1.3.3. Carbone-tartrate solution (Folin-Ciocalteau assay)

Sodium carbonate ( $\text{Na}_2\text{CO}_3$ ) [Sigma-Aldrich]	200	$\text{g}$
Sodium tartrate ( $\text{Na}_2\text{C}_4\text{H}_4\text{O}_6 \cdot 2\text{H}_2\text{O}$ ) [Sigma-Aldrich]	12	$\text{g}$
Ingredient were mixed in 750 ml of boiling water, then cooled and took to final volume of 1 liter and stored at 20°C.		

**2.1.3.4. DNS reagent**

The reagent was prepared by placing all solid components in a container and dissolving them simultaneously by stirring with the required volume of sodium hydroxide solution. The reagent contained approximately:

Dinitrosalicilic acid [Sigma-Aldrich]	1	%
Phenol [Carlo Erba]	0.2	%
Sodium sulfite ( $\text{Na}_2\text{SO}_3$ ) [Sigma-Aldrich]	0.05	%
Sodium hydroxide (NaOH) [Sigma-Aldrich]	1	%

**2.1.3.5. Tris-HCl buffer pH 6.8 (4X)**

Trizma base [Sigma-Aldrich]	121.1 g
Sodium dodecylphosphate (SDS) [Sigma-Aldrich]	100 g
Bidistilled water up to 1 liter of final volume. The trizma base solution was made at pH 6.8 with concentrated (37%) HCl [Sigma-Aldrich] prior to adjust the final volume.	

**2.1.3.6. Tris HCl buffer pH 8.8 (4X)**

Trizma base [Sigma-Aldrich]	121.1 g
Sodium dodecylphosphate (SDS) [Sigma-Aldrich]	100 g
Bidistilled water up to 1 liter of final volume. The trizma base solution was made at pH 8.8 with concentrated (37%) HCl [Sigma-Aldrich] prior to adjust the final volume.	

**2.1.3.7. SDS Loading Buffer (5X)**

250 mM Tris-HCl Buffer pH 6.8	12.5 ml
Sodium dodecylphosphate (SDS) [Sigma-Aldrich]	10 g
30% Glycerol solution [Sigma-Aldrich]	30 ml
5% $\beta$ -mercaptoethanol [Sigma-Aldrich]	5 ml
0.04% Bromophenol blue solution [Sigma-Aldrich]	52 ml
Bidistilled water up to 100 ml of final volume. The final solution turned to yellow color if pH value increased up to 3, small amount of KOH 6N solution was added to adjust pH value and to restore blue color.	

**2.1.3.8. SDS-PAGE Running Buffer (10X)**

Trizma base [Sigma-Aldrich]	303 g
Glycine [Sigma-Aldrich]	1440 g
Sodium dodecylphosphate (SDS) [Sigma-Aldrich]	100 g
Bidistilled water up to 10 liters of final volume. No need to adjust pH.	

## 2.2. Ribbon retting in bioreactor

### 2.2.1. Pilot plan bioreactor

Two varieties of kenaf, Tainung 2 and Everglade were grown at Dosolo (MN), Italy by K.E.F.I. S.p.A. during spring-summer seasons in 2007 and 2008. Plants harvested were used for stripping out green ribbons mechanically by ribboner (Figure 11), later on they were air dried in order to be stored for experimental purposes.



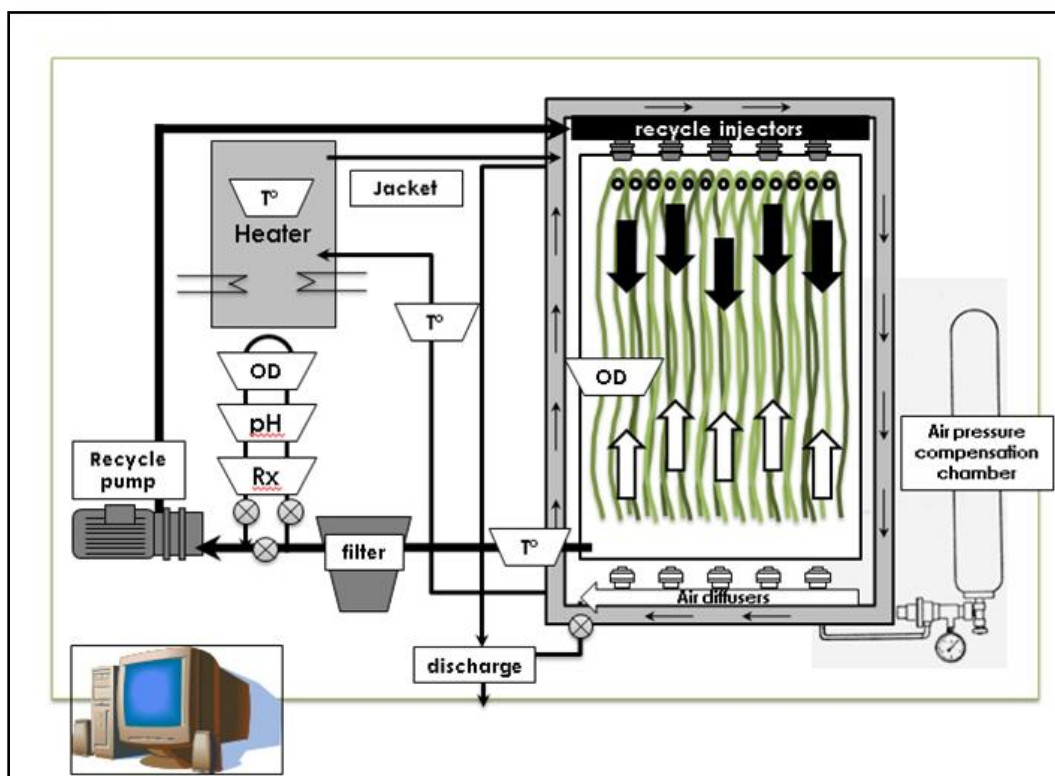
**Figure 11.** Harvested kenaf stalks and ribboner utilized to obtain kenaf ribbons, grooved rolls broke inner core that fell under the machine, while kenaf ribbons passed on the back.

Pilot plant (Figure 12) consists of a tank with a volume of 1 m<sup>3</sup>, surrounded by a warm water-jacket connected to a boiler in order to control the temperature.

Bioreactor was provided also by a system that recycling inner water that pulls liquor from the bottom and injected it to the top. Moreover, at the bottom of the tank an air injection system provided maintain aerated the system.

Several maceration trials were performed in pilot plant bioreactor with two different kind of loading preparations: 6 and 12 kg of dry weight of kenaf ribbons in 700 liters of tap warm waters (30-33°C), air insufflation was applied in order to maintain sub-acid pH (7-5) of retting liquor. During maceration, main parameters (temperature, pH, RedOx and oxygen content) of pilot plant bioreactor were collected by a series of sensors cabled to a PLC system that stored and controlled main parameters.

All sensors were purchased at Endress + Hauser Company, in the specific Ceragel CPS71D sensor registered pH value, Ceragel CPS72D sensor monitored Redox value, ceraliquid CPS41D sensor was applied as a second sensor of pH value and sensor Oxyman COS21D registered oxygen content in retting liquor inside the reactor. All sensors were connected with respective transmitter station Liquiline M CM42, and all transmitters were connected to PLC system.



**Figure 12.** Schematic flow chart of pilot plant bioreactor. Bold black arrows indicates the flow of retting liquor, light black arrows indicates the flow of water used to control the bioreactor's temperature in the jacket. White arrows show air direction in the tank. Green lines shows ribbons of kenaf tidy disposed in the removable array. All components of pilot plant bioreactor are connected to a PLC system represented by a computer.

### 2.2.2. Test macerations

The ribbon retting of kenaf in pilot plant was tested with different kind of loading of kenaf ribbons and with diverse variety of kenaf reassumed in the following table:

Test	Loading	Variety	Pretreatment
K1	6 kg	Everglade	Air dried
K2	6 kg	Everglade	Air dried
K3	12 kg	Everglade	Air dried
K4	25 kg	Tainung	None (fresh/green ribbons)
K5	25 kg	Tainung	None (fresh/green ribbons)
K6	2 kg	Everglade	Air dried
	2 kg	Tainung	Woven dried
	2 kg	Tainung	Air dried

**Table 4.** schematic representation of different loading and kind of kenaf ribbons employed in tested macerations.

### **2.2.3. Fiber quality assessment**

Every 24 hours retted fibers of kenaf were collected for visible analysis of retting progress, arranged samples of 100 g were washed twice in warm water in order to remove rough debris, later on they were treated with compressed air and dried by heater.

#### **2.2.3.1. Organoleptic properties**

Organoleptic properties that distinguish high quality natural fibers are difficult to establish in quantifiable measures, even if they are simply valuable by expert operator using only their hands and eyes. For this reason, different indirect analyses were conducted - in according with previous studies present in literature – in order to define some quantifiable characteristics of retted fibers obtained by controlled maceration ion bioreactor.

#### **2.2.3.2. Fibers' Yield**

The loss of weight between retted fibers and ribbons was registered by measuring dry weight with Mettler Toledo HB43-S instrument. The difference in dry weight indirectly indicates the grade of retting that occurred in the maceration. As reported in literature, a loss of weight of 50% in retted fibers are a signal of a sufficient level of maceration, in according with the composition of the stem of bast fiber crops (Pallesen, 1996).

#### **2.2.3.3. Fibers' tenacity and elongation**

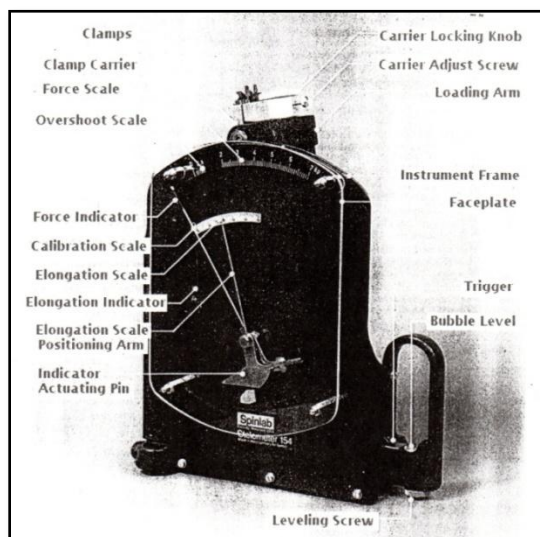
The tenacity of the kenaf fibers was evaluated with the stelometer (Figure 13) STELOLAB code 231B [MESDANLAB].

The stelometer consists of a heavy cast frame on which is mounted a pivoted loading arm assembly which moves on a sealed-bearing pivot, an adjustable silicone-damped dashpot assembly which controls the pendulum's rate of fall, a trigger mechanism which releases the pendulum, a bubble level, a leveling screw, and a covered face plate on which is mounted a force scale and an overshoot scale. Mounted on the loading arm assembly is a pendulum which moves on a sealed-bearing pivot, a pair of removable fiber clamps for holding the fiber bundle, a clamp carrier for holding, adjusting and locking the clamps, and an indicator actuating pin.

Mounted co-axially with the loading arm pivot and visible over the faceplate is an indicator assembly consisting of two arms, the elongation scale positioning arm and the force indicator arm upon which is articulated the elongation pointer. The fiber clamps are standard Pressley-type clamps, widely used in textile industry with many types of strength testers. One of the clamps is held in slots in the pendulum and one in the carrier. The carrier is locked to the loading arm by tightening the carrier locking knob. The position of the carrier may be adjusted with the carrier adjustment screw. The clamps are supplied with a removable 1/8" spacer, allowing the user to test tenacity at 1/8" gage spacing or at zero gage spacing.



This stelometer is an instrument which breaks a flat bundle of textile fiber and indicates, on graduated scales, the force required to break the bundle and the elongation of the fiber moment of breaking.



**Figure 13.** Stelometer description and components identification.

Breaking force may be converted to breaking strength (tenacity) by means of simple calculation when the length of the fibers in the bundle and the weight of fibers are known, by following equation:

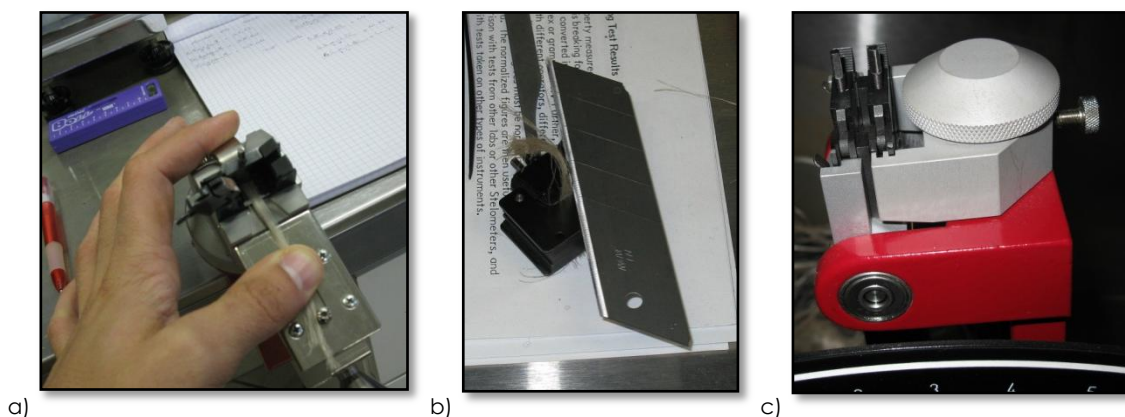
$$\text{tenacity (g/tex)} = \frac{\text{breaking force (kp)} \times \text{specimen length (mm)}}{\text{specimen mass (mg)}}.$$

The stelometer is designed to be used in conjunction with a precision balance to provide precise, repeatable determinations of both tenacity and elongation at 1/8" gage spacing and of tenacity at zero gage spacing (Figure 14).



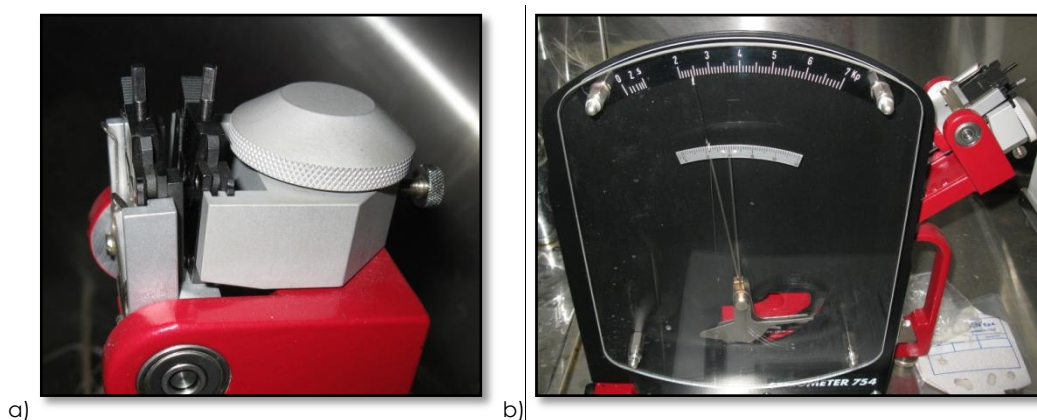
**Figure 14.** Stelometer and accessories (precision balance, vise, wrench, comb, clip, knife and tweezers).

Tenacity and elongation are two physical properties which are of great importance in evaluating the quality of raw fiber and suitability of fiber for yarn making. Knowledge of these properties is essential to the yarn spinner and to researcher who wish to develop natural or man-made fibers with specific or improved characteristics (Figure 15).



**Figure 15.** Some steps necessary for specimen preparation (a and b) and for specimen loading on stelometer (c).

Since the test results will vary with different operators, different instruments, different clamps, different environments, and many other factors, the determinations must be repeated many times and the derived tenacity must be normalized to a known standard (Figure 16). Determinations was normalized with standard tenacity of cotton fibers (as manufacturer instructions in according with Definitions of Terms Relating to Textile Materials ASTM D 1445-60T tentative method of test) and compared to retted flax fibers.



**Figure 16.** Some steps of tenacity and elongation determinations by stelometer: a) breaking of the specimen by pivoting arm, b) determination of breaking force by pointer on faceplate scale.

#### 2.2.3.4. FT-IR analysis

The changing composition of retted and unretted fibers of kenaf were analyzed by FT-IR (Fourier Transform Infrared Spectroscopy) analyses in according with Ouajai and Shanks (2005) in order to evaluate lignin, pectin and cellulose contents. The measurements were

performed using Nicolet Magna 760 spectrometer. A total of 64 scans were taken for each sample with a resolution of 2 cm<sup>-1</sup>. The retted and unretted fibers were arranged in a cutting mill and sieved to provide a size range between 45-120 µm. A mixture of 6.0 mg of dried fibers was pressed in a 300 mg of KBr wafer for FT-IR analyses.

#### 2.2.4. Folin-Ciocalteu assay

Folin-Ciocalteu reagent is a mix of phosphor-molibdate and phosphotungstate used for the determination of phenolic and polyphenolic compounds. The reagent measures the quantity of tested solution necessary to inhibit the oxidation of reactive compounds. Oxidizing reagents adsorb at the specific wavelength of 700 nm.

The determination of tannins present in a sample therefore is made indirectly by measuring phenolic and polyphenolic compounds (principal constituents of tannins) presence, expressed in tannic acid equivalent (TAE) in according with Standard and Methods.

The protocol consists in few passages: 1) mix 3 ml of sample with 100 µl of Folin phenol reagent [Sigma-Aldrich] and 1 ml of Carbone –tartrate solution; 2) incubate for at least 30 minutes at 20°C in order to obtain color formation; 3) then, measure the absorption at 700 nm at spectrophotometer.

For the determination of TAE was necessary to calculate a calibration curves with increasing concentrations of tannic acid (Figure 17).

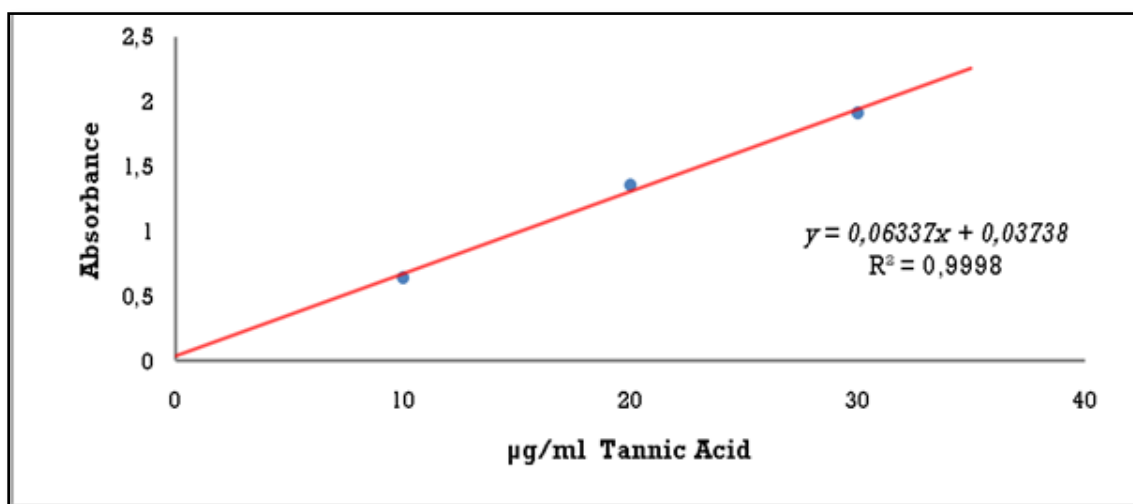


Figure 17. Calibration curve obtained with different tannic acid concentrations

### 2.3. Characterization of bacterial communities

#### 2.3.1. Microbial enumeration

Retting water and retted fibers samples were collected from pilot plant bioreactor every 6-12 hours till the end of the maceration. The water retting liquor collected was divided in

two aliquots: 10 ml were stored at 4°C for within-a-day microbiological analysis and 50 ml were stored at -20°C for later molecular analysis.

Microbial growth in retting liquor and retted fibers were registered by spread onto different media appropriate dilutions in two different growth conditions (aerobiosis and anaerobiosis) and with and without heat treatment (80°C for 10 minutes) in order to recovery spore-forming bacteria.

The same procedures were applied for retted fibers every 24 hours, microbial contamination/colonization was registered into resulting liquor by treatment of 5 g (wet weight) samples of ribbons by STOMACHER 400 [Seward] in 45 ml of 9% NaCl sterile solution.

### **2.3.2. Isolation of pectinolytic bacteria**

Retting liquor was diluted serially ( $10^1$ - $10^7$ -fold dilution) and spread on DMP medium with and without early heat treatment. The resulting plates were incubated in aerobic and anaerobic environment (Anaerogen kit [Oxoid]). The colonies representing different morphologies were picked randomly and purified by re-streaking on the same medium plates till obtaining axenic cultures of isolates.

The pectin-degrading bacteria were also tested for their esopectinolytic enzyme production by CTAB plate assay.

### **2.3.3. Amplified Ribosomal DNA Restriction Analysis (A.R.D.R.A.)**

Pectinolytic isolates were screened by A.R.D.R.A. analyses (De Baere *et al.*, 2002) in order to divide them in OTUs (operational taxonomic units).

The procedure can be reassumed in these following steps:

#### **2.3.3.1. DNA extraction**

Bacterial DNA was extracted from axenic culture by a glass beads method. Briefly, bacterial cells from 2 ml of over-night liquid culture was harvested and resuspended in 0,3 ml of extraction buffer (containing 1% SDS, 2% Triton X-100, 0,1 M NaCl and 1 mM EDTA), added with 0,3 ml of phenol:chloroform:isoamyl alcohol (25:24:1) and 0,3 g of sterilized glass beads (0,5-1 mm of diameter). The mixture obtained was vortexed for 2 minutes at maximum speed, then watery fraction, containing the nucleic acids, was collected and washed early with 1:1 volume of isopropanol and then with 70% ethanol solution. The resulting purified nucleic acids were resuspended in deionized sterile water and stored at -20°C for later analyses.

#### **2.3.3.2. PCR (polymerase chain reaction) of 16S rDNA**

The 16S rDNA (ribosomal DNA) gene was amplified with Eubacterial-specific primers:

- F8 (5'-GAGTTTGATCCTGGCTCAG-3');
- R11 (5'-ACGGCTACCTGTTACGACT-3');

corresponding to 1541 bp fragment of *E.coli* 16S rRNA (Weisburg *et al.*, 1991).

Briefly the PCR mixture contained 10-30 ng of template, 20 pmol of each primer, 5 mmol of each deoxynucleotide triphosphate and 5 µl of 5x GoTaq buffer with 1,5 Unit of GoTaq enzyme [Promega] in a final volume of 25 µl.

PCR program was conducted using a denaturing step of 2 min at 95°C, followed by 30 cycles of 45 sec at 95°C, 45 sec at 43°C and 2 min at 72°C, with a final step extension of 5 min at 72°C.

#### **2.3.3.3. 16S rDNA restriction and OTUs formation**

Approximately 1 µg of amplified 16S rDNA was completely digested according to the manufacturer's specifications for 3 h with 3 units of the restriction endonucleases AluI, RsaI, HhaI and HaeIII [Promega]. The restriction products were electrophoresed on a 1.5% (w/v) agarose gel in TBE buffer containing 0.5 µg/ml of ethidium bromide. On the base of differences among electrophoretic profiles, pectinolytic isolates were grouped in different OTUs and for each OTU a representative strain was chosen.

#### **2.3.3.4. Sequencing of 16S rDNA**

A fragment of 16S rDNA gene (corresponding to position 1-1541 in *E. coli* numbering system) was sequenced for one representative isolate for each OTU by PRIMM Biotech Custom Service (Milan, Italy). The 16S rDNA sequences were compared with GenBank by BLASTn program (Altschul *et al.*, 1990) for identification. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4 (Tamura *et al.*, 2007).

### **2.3.4. Metabolic assays**

#### **2.3.4.1. Pectinolytic activity assay**

The pectin-degrading bacteria were also tested for their esopectinolytic enzyme production by a rapid qualitative CTAB plate assay (Hankin *et al.*, 1971). Isolates were inoculated as a spot on DMP (2.1.1.3.) plates and pectinolytic activity was determined after growth of the organisms by flooding the plate with cetyltrimethylammoniumbromide (CTAB) solution (1%) which precipitates intact polysaccharides. If the organism has degraded the pectin, a clear zone is seen around the colony against an otherwise opaque medium. This technique appeared to offer certain advantages over other methods (Hankin *et al.*, 1971).

#### **2.3.4.2. Cellulolytic activity assay**

In the same way, pectinolytic strains were test for their ability to growth and to degrade cellulose. Isolates were spotted on DMC (2.1.1.4.) plates and after growth the detection of halos were detected by flooding plates with red Congo (Tamburini *et al.*, 2003).

### **2.3.4.3. Radial diffusion assay with tannic acid**

The radial diffusion assay does not quantify the total amount of tannins, but their ability to inhibit growth of bacteria, and therefore addresses only this aspect of the biological significance of these compounds.

The assays tested all pectinolytic isolates by adding increasing concentrations (0,5, 1, 2, and 5 mg/ml) of tannic acid in 6 mm well made in agar plates of different media (DMP, DMP + 0,5% YE and Nutrient) inoculated with bacteria. Briefly, each isolates were inoculated on Petri dishes containing different agar media, then were practiced wells (6 mm of diameter) on surface and increasing concentrations of tannic acid were applied in wells. Later on, Petri dishes were incubated at 37°C for a day and measures of area of inhibition halos were plotted in a graph.

The determination of the area of growth inhibition was obtained by measuring the diameter of the ring twice at right angles and was calculated the surface area from the average diameter. A plot of ring area vs. amount of tannic acid in well (area of well were subtracted) should give a linear relationship (Graça et Barlocher, 2005). The assay was tested in triplicate for each strain on different media. The slopes obtained indicated the physiologic response of tested bacteria in comparison to tannic acid presence.

### **2.3.5. Physiologic assays**

Isolates that showed high pectinolytic activity were tested in growth curves by enumeration of CFU (Colony Forming Unit) during growth in 100 ml of liquid media at 37°C. Growth curves were obtained by inoculating a proper volume of a 4 ml pre-inoculum of tested bacteria sufficient to register an initial OD600 of 0.01. Pectinolytic isolates were tested in DMP and TSB in order to detect different growth rate in presence of different source of carbon.

At pre-established intervals opportune dilutions were plated on Nutrient agar and after incubation at 37°C, colony forming units (CFU) were enumerated.

Moreover, pectinolytic isolates were tested for growing at different pH conditions. The growths were monitored by measuring OD600 with the UNICAM UV/VIS spectrometer UV2.

## **2.4. Molecular analyses**

### **2.4.1. DNA extraction and purification from retting liquor**

Total DNA was extracted from 10 ml samples of retting liquor by FastDNA® Instrument [MPBiomedicals, LCC.] in according with FastDNA® SPIN Kit for Soil protocol.

DNA extraction was checked by electrophoresis on 1% (w/v) agarose gel in TAE buffer containing 0.5 µg/ml of ethidium bromide.



### 2.4.2. Amplification by PCR

Polymerase chain reaction (PCR) amplification of total 16S rDNA gene was carried out using the universal Eubacterial-specific primers with the same conditions described before (2.3.3.2. *PCR (polymerase chain reaction) of 16S rDNA*).

*Bacillus*-specific PCR amplification was also carried out with the primer:

- K-B1/F (5'-TCACCAAGGCRACGATGCG-3');
- K-B1/R (5'-CGTATTCACCGCGGCATG-3');

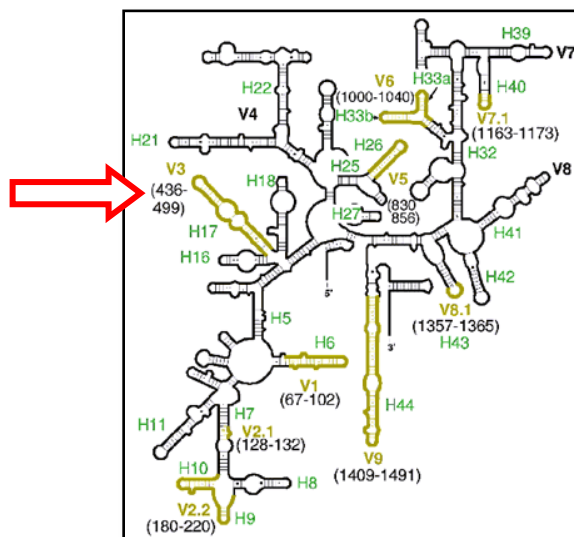
in order to amplify 1114 bp fragment of 16S rDNA belonging to *Bacillus* species (Wu *et al.*, 2006).

Briefly the PCR mixture contained 10-30 ng of template, 20 pmol of each primer, 5 mmol of each deoxynucleotide triphosphate and 5 µl of 5x GoTaq buffer with 1,5 Unit of GoTaq enzyme [Promega] in a final volume of 25 µl.

PCR program was conducted using a denaturing step of 3 min at 94°C, followed by 25 cycles of 30 sec at 94°C, 30 sec at 63°C and 2 min at 72°C, with a final step extension of 10 min at 72°C.

### 2.4.3. Denaturing gradient gel electrophoresis (DGGE)

Denaturing gradient gel electrophoresis (DGGE) is a molecular fingerprinting method that separates polymerase chain reaction (PCR) generated DNA products.



**Figure 18.** Secondary structure of 16S rRNA of *E. coli*, red arrows indicates among hypervariable regions (yellow area) the V3 portion utilized to perform DGGE analysis.



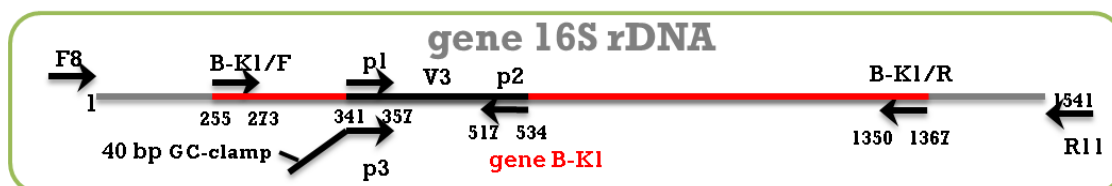
The polymerase chain reaction of environmental DNA can generate templates of differing DNA sequence that represent many of the dominant microbial organisms. However, since PCR products from a given reaction are of similar size (bp), conventional separation by agarose gel electrophoresis results only in a single DNA band that is largely non-descriptive. DGGE can overcome this limitation by separating PCR products based on sequence differences that results in differential denaturing characteristics of the DNA. During DGGE, PCR products encounter increasingly higher concentrations of chemical denaturant as they migrate through a polyacrylamide gel. Upon reaching a threshold denaturant concentration, the weaker melting domains of the double-stranded PCR product will begin to denature at which time migration slows dramatically. Different sequences of DNA (from different bacteria) will denature at different denaturant concentrations resulting in a pattern of bands. Each band theoretically represents a different bacterial population in the community. PCR-DGGE analysis was carried out on hypervariable V3 region of 16S rDNA (Figure 18) by subsequent amplification on both Eubacterial and *Bacillus*-specific amplicons (figure 19). PCR amplification of V3 region was carried out in accordance with Muyzer *et al* (1993) protocol, using the following primers:

- P3 (5'-CGCCCGCCGCGCGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAGGCAGCAG-3');
- P2 (5'-ATTACCGCGGCTGCTGG-3').

Briefly the PCR mixture contained 10 ng of template, 20 pmol of each primer and 12,5 µl of FastStart PCR Master mix [ROCHE] in a final volume of 25 µl.

PCR program was conducted using a denaturing step of 2 min at 95°C, followed by 30 cycles of 45 sec at 95°C, 30 sec at 57°C and 45 sec at 72°C, with a final step extension of 5 min at 72°C.

DGGE analysis for V3-16S rDNA variable region products (about 200 bp) was performed using 8% polyacrilamide gels containing a gradient of 30 to 60% denaturant. A 100% denaturing solution contains 40% (vol/vol) formamide and 7M urea. Electrophoresis was carried out in Dcode™ Universal Detection System [Bio-Rad] in the following running conditions: 16 h at 50 V in TAE 1x buffer maintained at 65°C (Muyzer *et al.*, 1993).



**Figure 19.** Schematic representation of PCR conducted on 16S rDNA for DGGE analysis. grey line shows 16SrDNA gene with numbering system of *E.coli*, red line indicates B-K1 gene amplified by *Bacillus*-specific primers and black line shows Hypervariable V3 region position.

Gels obtained were stained with ethidium bromide and visualized to reveal band patterns that can be used to determine the similarity of sampled microbial communities.

#### 2.4.4. Cloning and sequencing

Major bands in the DGGE profiles were excised and eluted from polyacrylamide gel. Afterward, DNA extracted from gel bands was re-amplified with forward primer P3 without 40 bp GC-clamp (formerly primer P1), sub-cloned in pGem T easy vector (PROMEGA) following manufacturer specifications.

V3 regions of 16S rDNA sequences obtained were sequenced at PRIMM Biotech Custom service and were compared with GenBank by BLASTn program (Altschul *et al.*, 1990).

Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4 (Tamura *et al.*, 2007).

### 2.5. Ribbon retting in mesocosm: looking for an *inoculum*

Ribbon retting in mesocosms was performed in 1 liter flask by plunging 20 g of kenaf ribbons in 600 ml of sterile bidistilled water. Different trials were performed in order to evaluate best technological activity among pectinolytic isolates.

Mesocosms were incubated at 37°C for 5 days without shaking and without air insufflation. For each remarkable strain was performed a retting in mesocosm, by adding a massive *inoculum* consisting in the biomass developed in one day in 600 ml of DMP (2.1.1.3.) broth. Moreover two control mesocosms were set up: the first contained only kenaf ribbons and bidistilled sterile water (negative control) and the second contained kenaf ribbons and bioreactor retting liquor (positive control). The two control mesocosm were performed to determine the influence of *inoculum* in mesocosm trials. The quality of retted fibers obtained from mesocosms trials and control mesocosms was assayed by measuring loss of weight and by analyzing them with stereomicroscope Leica EZ4D.

### 2.6. Proteomic analyses

#### 2.6.1. Quantitative assay of enzymatic activity

The enzymatic activity of most interesting isolates in mesocosm trials were tested by DNS method (Miller, 1959), a colorimetric assay that allows to detect variations of polysaccharides content in solution. DNS method relies on a redox reaction between reducing ends of sugars and the 3,5-dinitrosalicylic acid (DNS).

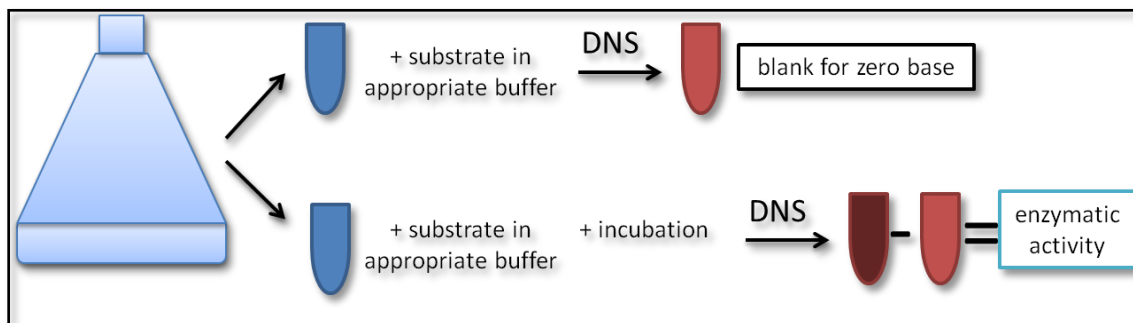
Studied for the determination of sugar content in unknown sample is also suitable for determining enzymatic degradation of sugar by reacting with resulting reducing ends. The chemistry of the dinitrosalicylic acid test for the determination of reducing sugars has been clarified by Miller (1959) at least in part.

The 3,5-dinitrosalicylic acid is reduced to 3-amino-5-nitrosalicylic acid while, in the simplest instances, the aldehyde groups of sugars appear to be oxidized to carboxyl groups (Figure 20). The facts, however, that the equivalence between aminonitrosalicylic acid produced and sugar is not exact and that different sugars yield different amounts of color, suggest that the chemistry of the test may actually be appreciably more complicated.



**Figure 20.** Redox reaction of DNS reagent with reducing sugars that turned yellow solution in red ones.

For these reasons, different polysaccharides required specific adaptation of the protocol by adjusting wavelength during spectrometer determinations and a sugar-specific calibration curves.



**Figure 21.** Schematic representation of analytical procedure applied for DNS method.

After determining the specific wavelength and the calibration curves the color tests were made with 3ml aliquots of DNS reagent (2.1.3.3.) added to 3 ml aliquots of sample in glass tubes. The mixtures were heated for 5 minutes in a boiling water bath and then cooled under running tap water adjusted to ambient temperature.

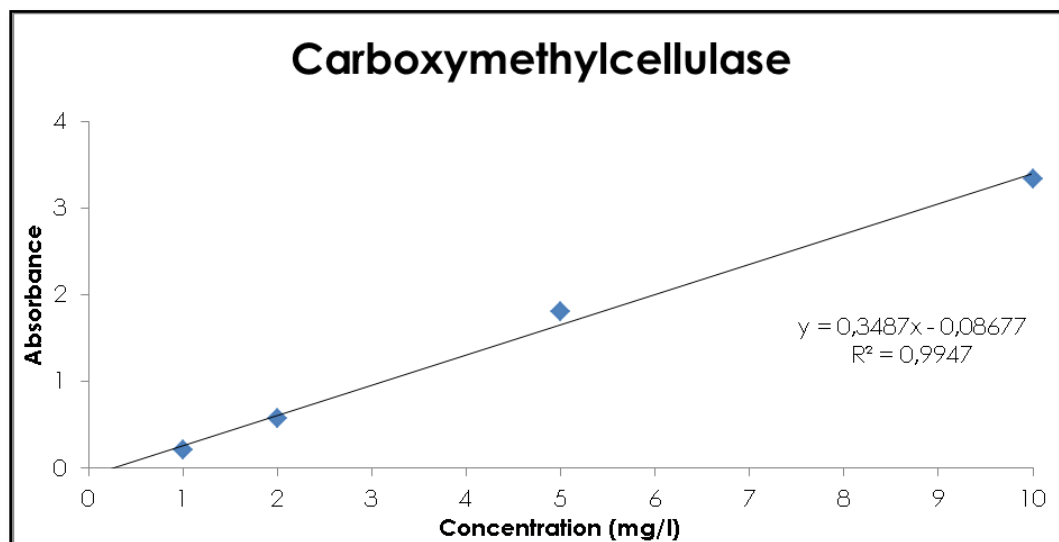
To stabilize the color under these conditions, 1 ml of a 40% solution of the Rochelle salt (Potassium sodium tartrate tetrahydrate [Sigma-Aldrich]) was added to the mixture of reactants subsequent to the development of the color and prior to cooling (Miller, 1959).

The color intensities were measured with UNICAM UV/VIS spectrometer UV2 at specific wavelength for assayed sugars in 1 cm wave path plastic cuvettes.

The composition chosen for the DNS reagent, in according with Miller (1959) was based on the result of preliminary tests which indicated that reagent was optimal and would serve best as the basis of reference for testing effects of variation in sugars content of retting liquor. In order to avoid alteration of the test by unknown substances present in resulting retting liquor, two samples were analyzed by DNS method by adding appropriate sugar as substrate and specific buffer (Figure 21).

Briefly, the first sample operated as blank for zero base lectures and the second sample - after incubation time at sugar specific temperature - served to quantify the enzymatic activity later on the addition of appropriate specific buffer. As substrates of different enzymatic activities consisted of polysaccharides, a master solution of substrate was added to reaction and the determination of calibration curves were performed by adding standard quantities of reducing ends as monomers of the substrate.

### 2.6.1.1. Carboxymethylcellulase



**Figure 22.** Calibration curve obtained by adding standard amount of reducing ends of monomer glucose to a fixed quantity of substrate (CMC 0,2% solution).

Carboxymethyl cellulose (CMC) is a cellulose derivative with carboxymethyl groups bound to some of the hydroxyl groups of the glucopyranose monomers that make up the cellulose backbone. In particular in cellulose backbone CMC provides linkage between crystalline cellulose microfibrils and the carbohydrate matrix.

The carboxymethylcellulase activity was assayed in 0,1 M buffer Acetate pH 5.50 for 45 minutes at 45°C adding a concentrated solution of CMC [Sigma-Aldrich] as a substrate to a final concentration of 0,2%. Calibration curve (Figure 22) was obtained by adding to reaction mixture a concentrated solution of CMC to a final concentration of 0,2% and increasing concentrations of glucose [Sigma-Aldrich] in order to establish a precise amount of reducing ends as produced by enzymatic activity. Color formation was monitored at 640 nm by spectrometer.

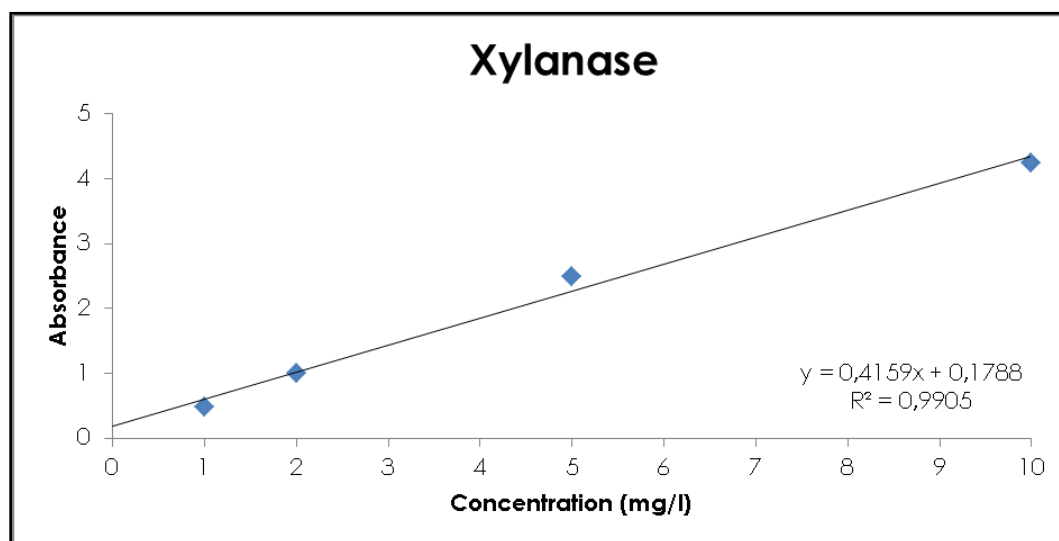
### 2.6.1.2. Xylanase

Xylans are heteropolymers consisting principally of D-xylose as its monomeric unit and traces of L-arabinose (Bastawde, 1992). In plants, xylans or the hemicelluloses are situated between the lignin and the collection of cellulose fibers. The xylan layer with its covalent linkage to lignin and its non-covalent interaction with cellulose may be important in maintaining the integrity of the cellulose in situ and in helping protect the fibers against degradation to cellulases (Gilbert *et al.* Hazlewood, 1993).

The xylanase activity was assayed in 0,1 M buffer Acetate pH 5.00 for 45 minutes at 45°C adding a concentrated solution of xylan [Sigma-Aldrich] as a substrate to a final concentration of 0,2%.

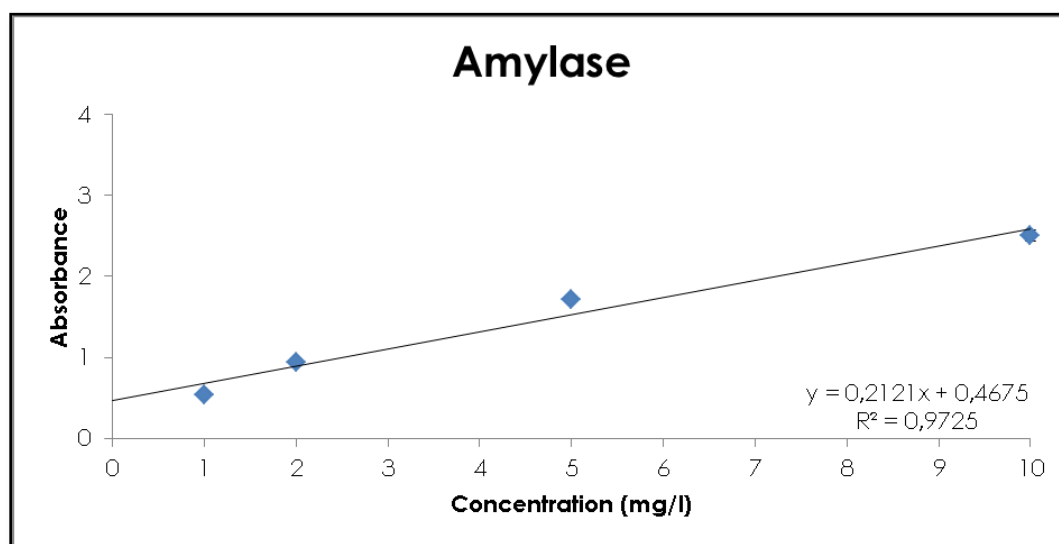
Calibration curve (Figure 23) was obtained by adding to reaction mixture a concentrated solution of xylan to a final concentration of 0,2% and increasing concentrations of xylose

[Sigma-Aldrich] in order to establish a precise amount of reducing ends as produced by enzymatic activity. Color formation was monitored at 575 nm by spectrometer.



**Figure 23.** Calibration curve obtained by adding standard amount of reducing ends of monomer xylose to a fixed quantity of substrate (xylan 0,2% solution).

### 2.6.1.3. Amylase



**Figure 24.** Calibration curve obtained by adding standard amount of reducing ends of monomer glucose to a fixed quantity of substrate (soluble starch 0,2% solution).

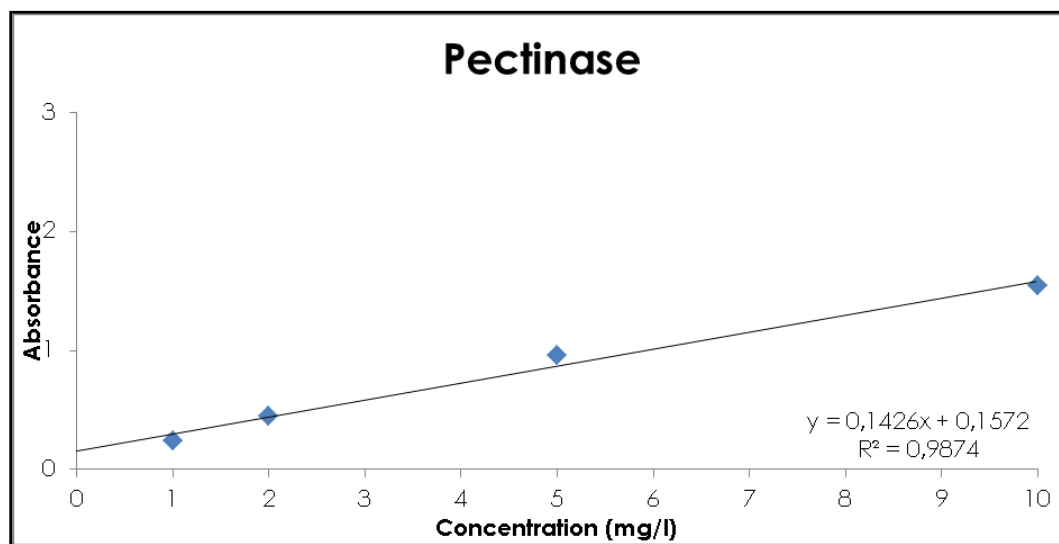
Starch or amylum is a polysaccharide carbohydrate consisting of a large number of glucose units joined together by glycosidic bonds. It consists of two types of molecules: the linear and helical amylose and the branched amylopectin. Depending on the plant, starch

generally contains 20 to 25% amylose and 75 to 80% amylopectin. Since starch is a reserve sugar for the plant, glucose molecules are bound in starch by the easily hydrolyzed alpha bonds.

The amylase activity was assayed in 0,1 M buffer Tris-HCl pH 7.00 for 45 minutes at 45°C adding a concentrated solution of soluble starch [Sigma-Aldrich] as a substrate to a final concentration of 0,2%. Calibration curve (Figure 24) was obtained by adding to reaction mixture a concentrated solution of soluble starch to a final concentration of 0,2% and increasing concentrations of glucose [Sigma-Aldrich] in order to establish a precise amount of reducing ends as produced by enzymatic activity. Color formation was monitored at 640 nm by spectrometer.

#### 2.6.1.4. Pectinase and polygalacturonase

Pectin composition and role in plant structure were exhaustively explained in paragraph 1.6.2. The pectinases (polygalacturonase and pectate lyase) activities towards PGA and pectin were assayed in the same 0,1 M buffer Phosphate pH 7.00 for 45 minutes at 45°C adding a concentrated solution of polygalacturonic acid (PGA) and pectin [Sigma-Aldrich] as substrates to a final concentration of 0,2%. Calibration curve (Figure 25) was obtained by adding to reaction mixture a concentrated solution of PGA to a final concentration of 0,2% and increasing concentrations of galacturonic acid [Sigma-Aldrich] in order to establish a precise amount of reducing ends as produced by enzymatic activity. Color formation was monitored at 520 nm by spectrometer. Moreover, pectinolytic activities towards PGA and pectin were assayed in different pH condition by altering pH value of buffer Phosphate.



**Figure 25.** Calibration curve obtained by adding standard amount of reducing ends of monomer galacturonic acid to a fixed quantity of substrate (PGA 0,2% solution).

### 2.6.2. Protein extraction and purification

The protein samples were obtained from supernatant of crude liquid cultures of K2H1 isolate and pectinolytic bacteria used as reference strains: *Bacillus subtilis* subsp. *subtilis* and *Dickeya dadantii*.

The procedure consisted in growing bacteria in 100 ml of DMP and Nutrient media, later on bacterial cells were removed by centrifugation.

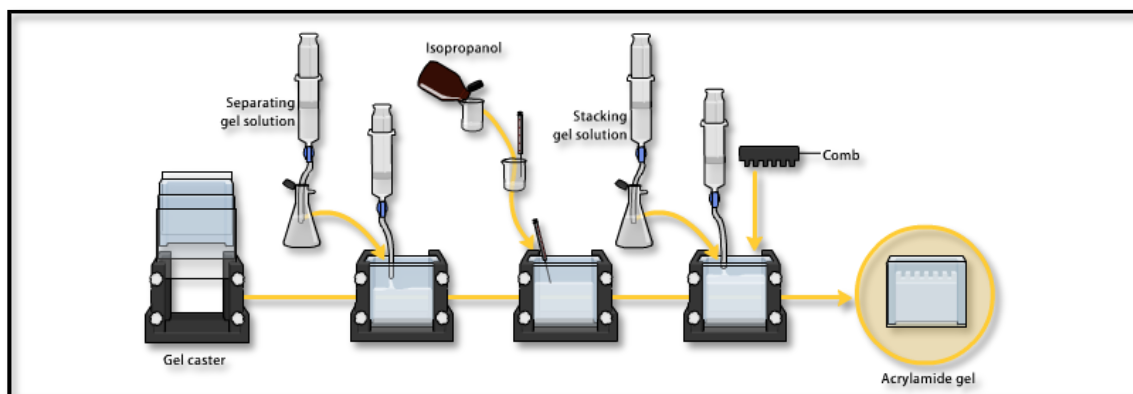
The supernatant containing esoenzymes was collected and treated with inhibitor of proteases Complete MINI EDTA Free [Roche]. Then supernatant was filtered using a vacuum pump with cellulose acetate filters with 0,45  $\mu\text{m}$  exclusion size in order to remove remaining cells. A second filtration with syringe filter of nylon with 0,2  $\mu\text{m}$  exclusion size was applied to remove completely cell debris. Later on, esoenzymes in resulting liquid were concentrated and partially purified by Vivaspins with cut-off of 5 kDa.

Proteins contained in samples were quantified by Bradford method (Bradford, 1976), later on esoenzymes were suspended in an appropriate volume of buffer 0.1 M HEPES buffer (pH 7.5) in order to obtain a final concentration of at least 2  $\mu\text{g}/\mu\text{l}$ .

Protein samples were dispensed in small aliquots containing 30  $\mu\text{g}$  of protein (quantity necessary for 3 runs on SDS-PAGE) and stored at  $-20^{\circ}\text{C}$  after freezing with liquid nitrogen (about  $-80^{\circ}\text{C}$ ).

### 2.6.3. SDS-PAGE analysis (Sodium Dodecyl Sulphate PolyAcrylamide Gel Electrophoresis)

SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis, is a technique widely used to separate proteins according to their electrophoretic mobility (a function of length of polypeptide chain or molecular weight).



**Figure 26.** Schematic representation of procedure necessary to prepare polyacrylamide gel (PAG).

Polyacrylamide gels consist of acrylamide, bisacrylamide, SDS and Tris-HCl buffer with adjusted pH. The gel is formed because the acrylamide solution contains a small quantity of bisacrylamide which can form cross-links between two polyacrylamide molecules. The acrylamide concentration of the gel can also be varied, generally in the range from 5% to



25%. Lower percentage gels are better for resolving very high molecular weight proteins, while much higher percentages are needed to resolve smaller proteins. In fact, the pore size of a gel is determined by two factors, the total amount of acrylamide present (%T) (T = Total acrylamide-bisacrylamide monomer concentration) and the amount of cross-linker (%C) (C = Cross-linker concentration). Pore size decreases with increasing %T; with cross-linking, 5%C gives the smallest pore size. Any increase or decrease in %C increases the pore size, as pore size with respect to %C is a parabolic function with vertex as 5%C. This appears to be because of nonhomogeneous bundling of strands in the gel.

The gel solution is degassed before the polymerization step to prevent air bubbles formation. Usually, polyacrylamide gel (PAG) consisted of the combination of two different gels: stacking gel and resolving gel. The resolving gel is usually more basic and has higher polyacrylamide content than the stacking gel. The gels are polymerized in a gel caster (Figure 26): first the resolving gel is poured and allowed to polymerize. Later on a thin layer of isopropanol is added, causing to the top of the resolving gel to form a smooth surface. Next, the stacking gel is poured and a comb is placed to create the wells. After stacking gel is polymerized the comb is removed and gel is ready for electrophoresis. The stacking gel is a large pore PAG (4%T). This gel is prepared with Tris/HCl buffer pH 6.8 of about 2 pH units lower than that of electrophoresis buffer (Tris/Glycine). These conditions provide an environment for Kohlrausch reactions determining molar conductivity, as a result, SDS-coated proteins are concentrated to several folds and a thin starting zone of the order of 19  $\mu\text{m}$  is achieved in a few minutes. This gel is cast over the resolving gel. The height of the stacking gel region is always maintained more than double the height and the volume of the sample to be applied. This is based on isotachopheresis. The polymerization of gels is obtained by adding N,N,N',N'-Tetramethylethylenediamine (TEMED) [Sigma-Aldrich] and ammonium persulfate [Sigma-Aldrich].

<b>Calibrated Molecular Weights of Kaleidoscope Standards*</b>		
Protein	Color	Prestained Standards
Myosin	Blue	216,000
beta-galactosidase	Magenta	132,000
BSA	Green	78,000
Carbonic anhydrase	Violet	45,700
Soybean trypsin inhibitor	Orange	32,500
Lysozyme	Red	18,400
Aprotinin	Blue	7,600
Insulin	Blue	-

**Figure 27. Calibrated molecular weights contained in the Kaleidoscope Standards size marker [BioRad<sup>2</sup>].**

Following electrophoresis, the gel is stained with Coomassie Brilliant Blue R-250 [BioRad], allowing visualization of the separated proteins. After staining, different proteins will appear as distinct bands within the gel. The weight of separated proteins is determined by resolving

<sup>2</sup> \* Molecular weights are of representative lots; actual weights may vary. The lot-specific molecular weights are included in each vial (BioRad specification).

the prestained size marker Calibrated Molecular Weight of Kaleidoscope Standards [BioRad] in a separate lane in gel (Figure 27). In the specific, the protein sample of pectinolytic bacteria were analyzed by SDS-PAGE consisting of a 4%T stacking gel and a 12%T resolving gel (Table 5).

Stacking gel 4%T		Resolving gel 12%T	
<b>Acrylamide 40%T 2,5%C</b>	0.5 ml	<b>Acrylamide 40%T 2,5%C</b>	3 ml
<b>4X Tris HCl buffer pH 6.8</b>	1.25 ml	<b>4X Tris HCl buffer pH 6.8</b>	2.5 ml
<b>10% SDS solution</b>	0,1 ml	<b>10% SDS solution</b>	0.1 ml
<b>H<sub>2</sub>O</b>	3.15 ml	<b>H<sub>2</sub>O</b>	4.4 ml
<b>APS</b>	20 µl	<b>APS</b>	20 µl
<b>TEMED</b>	10 µl	<b>TEMED</b>	10 µl
<b>Final volume</b>	10 ml	<b>Final volume</b>	10 ml

Table 5. Composition of polyacrylamide gel utilized in SDS-PAGE of protein samples.

Protein samples were mixed with appropriate volume of 5X SDS Loading Buffer (2.1.3.6.) in order to load about 10 µg of protein. Samples obtained were boiled for 5 minutes in water bath in order to denature and to bind SDS to protein molecules. Electrophoresis was carried out in 1X SDS-PAGE Running Buffer (2.1.3.7.) in BioRad equipment (figure 28) at the following running conditions: 10 mA of current for first 30 minutes till loaded samples reached the interface between stacking and resolving gels. Later on current was increased to 20 mA for 2 hours till the end.

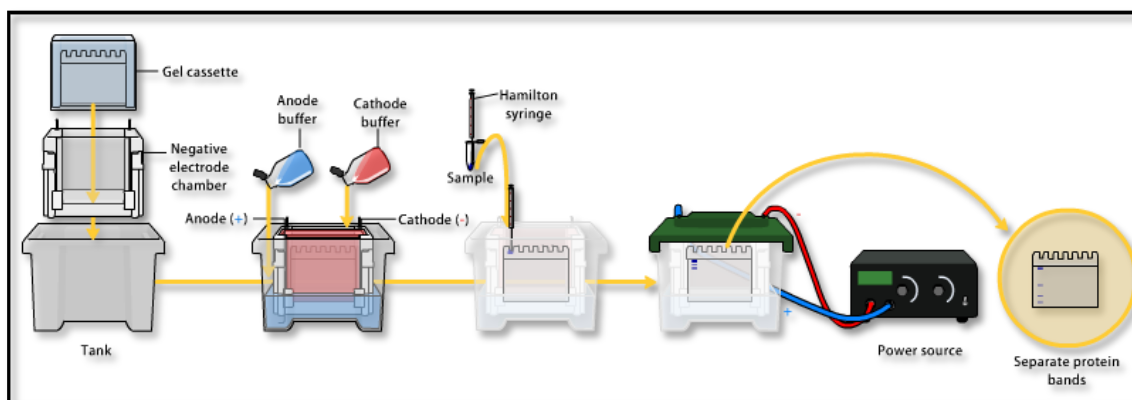


Figure 28. BioRad equipment for SDS-PAGE analysis.

### 2.6.4. Zymogram

Zymography is an electrophoretic technique, based on SDS-PAGE that includes a substrate copolymerized with the polyacrylamide gel, for the detection of enzyme activity. There are two other modification protocols called:

- Reverse zymography copolymerizes both the substrate and the enzyme with the acrylamide, and is useful for the demonstration of enzyme inhibitor activity. Following staining, areas of inhibition are visualized as dark bands against a clear (or lightly stained) background.
- Imprint technique: the enzyme is separated by native gel electrophoresis and the gel is laid on top of a substrate treated agarose.

In according with Kluskens (2004), an imprint technique was adopted to detect pectinolytic protein. Protein samples were boiled 2.5 min before applying onto a 12% SDS-PAGE for gel utilized for zymogram (the boiling time was reduced to increase the efficiency of refolding into an active enzyme). Denaturing agent SDS was removed by washing gels overnight in shaking bath with 0.1 M HEPES buffer (pH 7.5), 1% (w/v) Triton X-100, 1 mM DTT (Kluskens, 2004). Subsequently, gels were rinsed and deposited on Peh plates (2.1.2.1.) and incubated in 0.1 M HEPES buffer (pH 7.5), 1 mM  $\text{CaCl}_2$ , at 37°C overnight. Pectinolytic activity was revealed by removing PAG and flooding Peh plates with 1% CTAB solution. PAG was stained with Coomassie Brilliant Blue R-250 [BioRad] and halos on Peh plate were conducted to respective bands on PAG.

### 2.6.5. Protein sequencing and identification

Spots of bands that showed activity in zymogram and main bands present on gels were carefully cut out and subjected to in-gel trypsin digestion. Peptides from 8  $\mu\text{L}$  of each sample were then separated by reversed phase nano-HPLC-Chip technology (Agilent Technologies, Palo Alto, CA, USA) online-coupled with a 3D ion trap mass spectrometer (model Esquire 6000, Bruker Daltonics, Bremen, Germany). The chip was composed of a Zorbax 300SB-C18 (150mm $\times$ 75 $\mu\text{m}$ , with a 5 $\mu\text{m}$  particle size) analytical column and a Zorbax 300SB-C18 (40 nL, 5 $\mu\text{m}$ ) enrichment column. The complete system was fully controlled by ChemStation (Agilent Technologies) and EsquireControl (Bruker Daltonics) softwares. The scan range used was from 300 to 1800 m/z. For tandem MS experiments, the system was operated with automatic switching between MS and MS/MS modes. The three most abundant peptides of each m/z were selected to be further isolated and fragmented. The MS/MS scanning was performed in the normal resolution mode at a scan rate of 13.000 m/z per second. A total of five scans were averaged to obtain an MS/MS spectrum. Database searches were conducted using the MS/MS ion search of Mascot against all entries of the non-redundant NCBI database with the following parameters: specific trypsin digestion, up to one missed cleavage; fixed and variable modifications: propionamide (Cys) and oxidation (Met), respectively; peptide and fragment tolerances:  $\pm 0.9$  Da and  $\pm 0.9$  Da, respectively, and peptide charges: +1, +2 and +3. For positive identification, the score of the result of  $[-10 \times \text{Log}(P)]$  had to be over the significance threshold level ( $p < 0.01$ ) and at least 2 different peptides ( $p < 0.05$ ) had to be assigned.

Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4 (Tamura et al., 2007).

### **3. Results and discussion**

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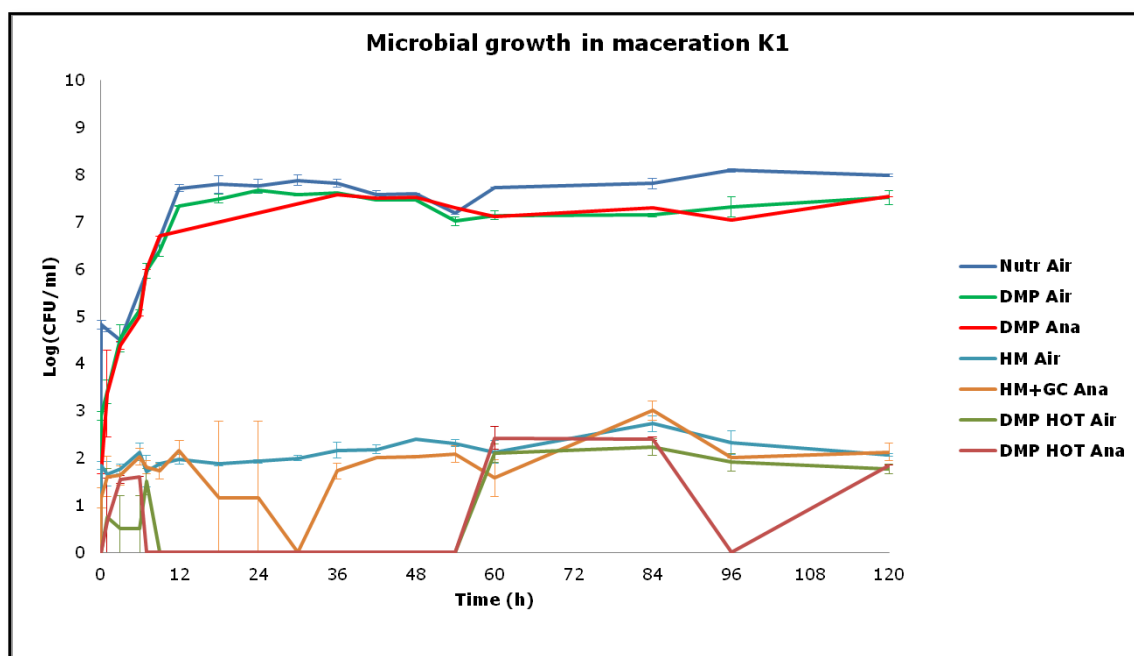
### 3.1. Retting in the bioreactor

Ribbon retting trials in pilot plant bioreactor were performed in Dosolo (MN), in the production center of K.E.F.I. S.p.A.. The experimental procedure was established in according with Dr. Marco Errani, responsible of retting of kenaf at K.E.F.I. S.p.A..

On the basis of experience matured on water retting of hemp of Dr. Errani, the ribbon retting (dried material except when otherwise specified) was conducted in warm water with slow air insufflation.

#### 3.1.1. Test K1 (trial stage)

In particular the first maceration process (maceration K1) tested the pilot plant, the PLC system and all sensors. In fact during this first trial a lot of problems were encountered and solved (black out, variation in air pressure, interferences between sensors). For these reasons data on main parameters are not available. The bioreactor was loaded with 6 kilograms of kenaf ribbons in 700 liters of water; the maceration was thermo-regulated at 33°C. In spite of all those problems, the conditions applied resulted in very high quality retted fibers.



**Figure 29.** Microbial growth registered on different media and in different culture conditions during the first maceration in pilot plant bioreactor. Nutr = Nutrient agar; HM = Hemp medium; DMP = Minimal media with pectin as unique source of carbon; Air = aerobiosis; Ana = anaerobiosis; GC glucose and cysteine additions; HOT = thermic treatment of retting liquor (80°C for 10 minutes).

Figure 29 summarizes microbial counts registered in retting liquor on different media in different culture conditions (aerobiosis vs. anaerobiosis and heat treatment). In first instance, the curves are divided in two groups: the top curves represent total heterotrophic

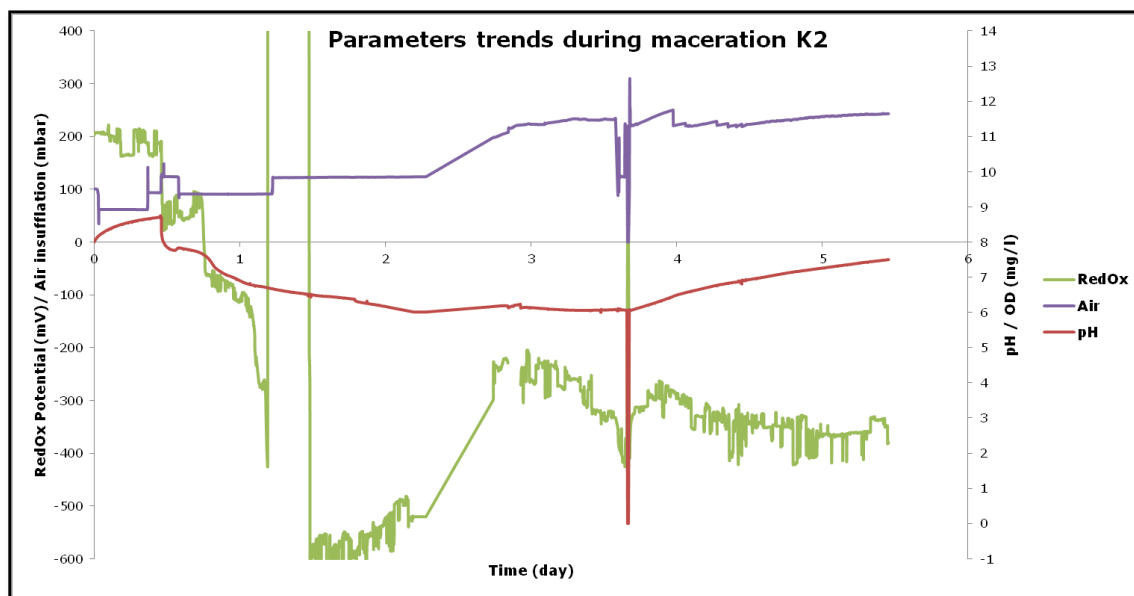
bacteria (Nut Air) and pectinolytic bacteria (DMP Air and Ana), while at the bottom there are curves of spore-forming bacteria represented by HM Air and Ana curves (total spore-formers) and by DMP Air and Ana (pectinolytic spore-formers). In the second instance, it is clearly noticeable that there are no differences in bacterial growth on the same media incubated in different condition (aerobic vs. anaerobic environment). The unique remarkable difference was registered between HM Air and HM+GC Ana at 30 hours from the beginning of maceration. Perhaps curve before and after this point was characterized by high standard deviation.

In the specific it is interesting to notice the contamination of water utilized in bioreactor (about  $10^5$  CFU/ml at zero time) which didn't affect the pectinolytic bacteria development in the liquor that within first 12 hours reached the level of total heterotrophic bacteria present in the liquor.

Finally, the microbial population dynamics seemed to develop quickly in bioreactor and to stabilize during the rest of the process in applied conditions.

### 3.1.2. Test K2

The second maceration, represent a repetition of the first "test" maceration. The main parameters trends were available even if two black out events (caused by summer thunderstorm) interrupted the registration of data during second and third day of maceration (Figure 30). For this reason the pilot plant was provided till the third maceration with an emergency generator.



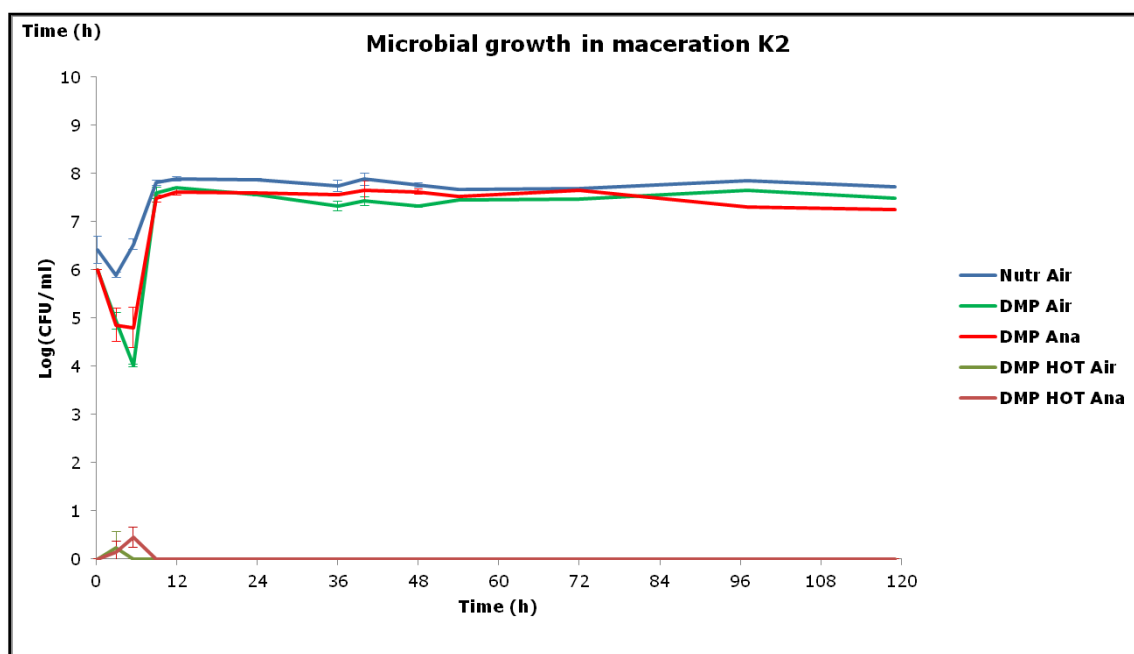
**Figure 30.** Main parameter trends registered during the second maceration. Redox potential curves shows problem of signalling due to electric interference.

On the other hand, the graph shows the condition applied during maceration: the aeration was minimized in the first part (less than 100 mbar of pressure) and in



correspondence of decreasing of pH value it was increased in order to fix acidity of retting liquor in sub-acidic condition. The redox potential quickly decreased to negative value and partially recovered after air insufflation. Finally, the main parameter trends were quite stabilized by air insufflation during the maceration.

The analysis of microbial population trends (Figure 31) on different media reflected more or less the instances noticed in the first maceration (K1): two groups of curves that divided sporeformers from total and pectinolytic bacteria; no differences on the same media in different culture condition; the rapid development of pectinolytic bacteria within first 12 hours of the test; and the stabilization of contamination of retting liquor in applied conditions.

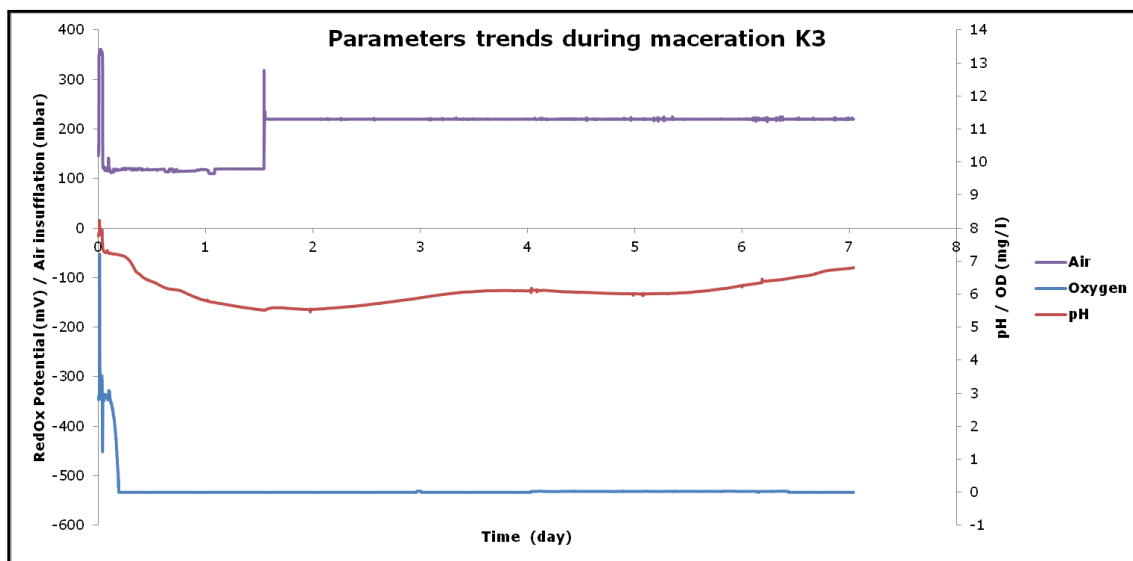


**Figure 31.** Microbial growth curves registered during the second maceration on different media in different growth condition: Nutr = Nutrient agar; DMP = Minimal media with pectin as unique source of carbon; Air = aerobiosis; Ana = anaerobiosis; HOT = thermic treatment of retting liquor (80°C for 10 minutes).

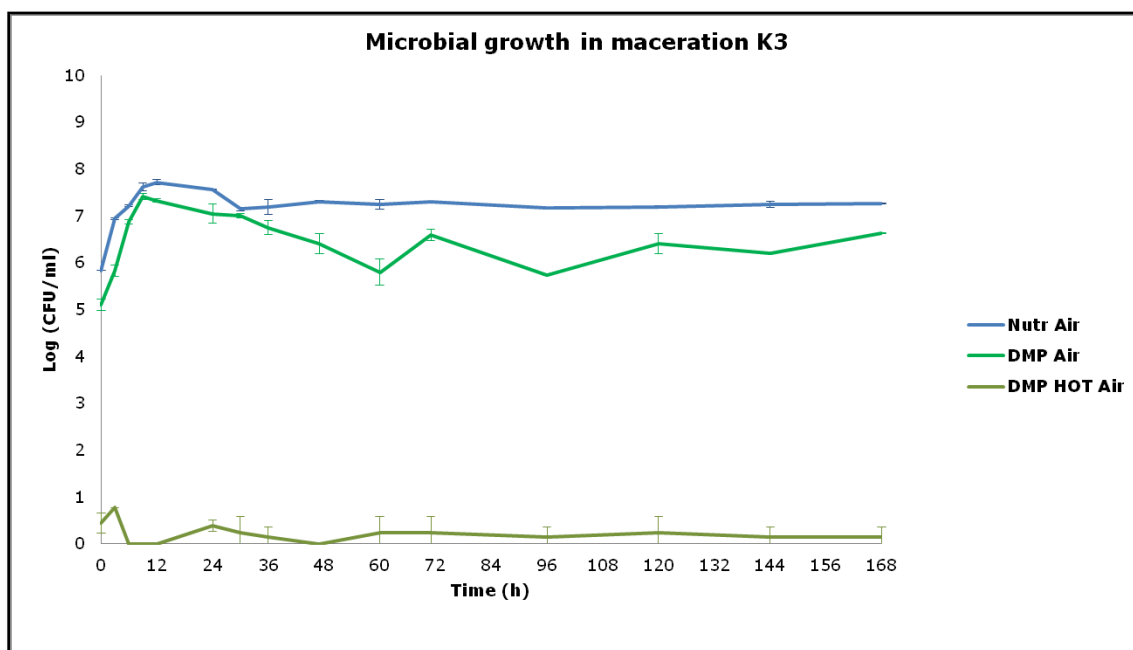
### 3.1.3. Test K3 (overloaded)

The third maceration was performed with the doubled loading of kenaf ribbons in the same volume of water, in order to reduce ribbons/water ratio. The main parameter trends (Figure 32) indicated that even bioreactor's loading was doubled the conditions were stabilized by air insufflation. The unique difference registered in respect of the K1 and K2 trial was an extension of retting time from 5 to 7 days before obtaining the same grade of retting. In fact, during this third maceration was necessary to improve more drastically air insufflation (from 120 to 220 mbar). On the other hand, pH value of retting liquor remained in sub acidic condition as previous stated.

During K3 trial, microbial population dynamics showed the same trends registered in the early macerations (Figure 33). Anaerobic bacteria's growth curves were not registered.



**Figure 32.** Main parameter trends registered during third maceration. Redox potential was not reported.



**Figure 33.** Microbial growth curves registered during the third maceration on different media: Nutr = Nutrient agar; DMP = Minimal media with pectin as unique source of carbon; Air = aerobiosis; HOT = thermic treatment of retting liquor (80°C for 10 minutes).

Reassuming, the doubled loading affected time of retting but not the microbial population dynamics. Moreover, the pilot plant bioreactor, as was designed, was suitable to control maceration process also at low ribbons/water ratio. The objective of this test was to detect

the limit of the retting in bioreactor, and then the unique limit of bioreactor management was the quantity of ribbons.

However, in order to better understand and control the ribbon retting in bioreactor successive maceration were performed with 6 kilograms of loading.

#### 3.1.4. Test K4 and K5 ('green' ribbon retting)

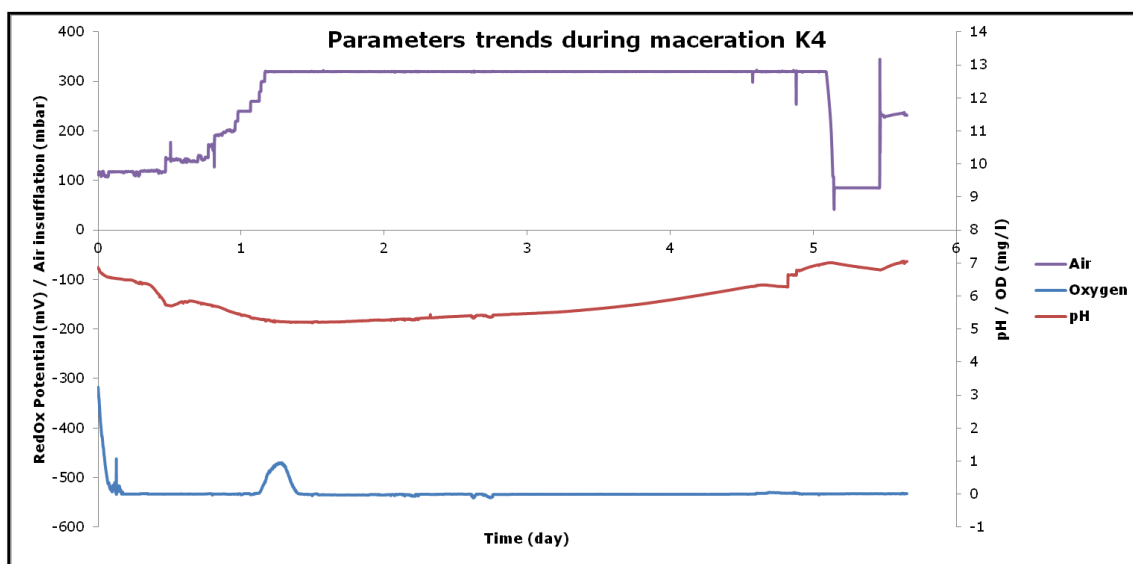


Figure 34. Main parameter trends registered during fourth maceration. Redox potential was not reported.

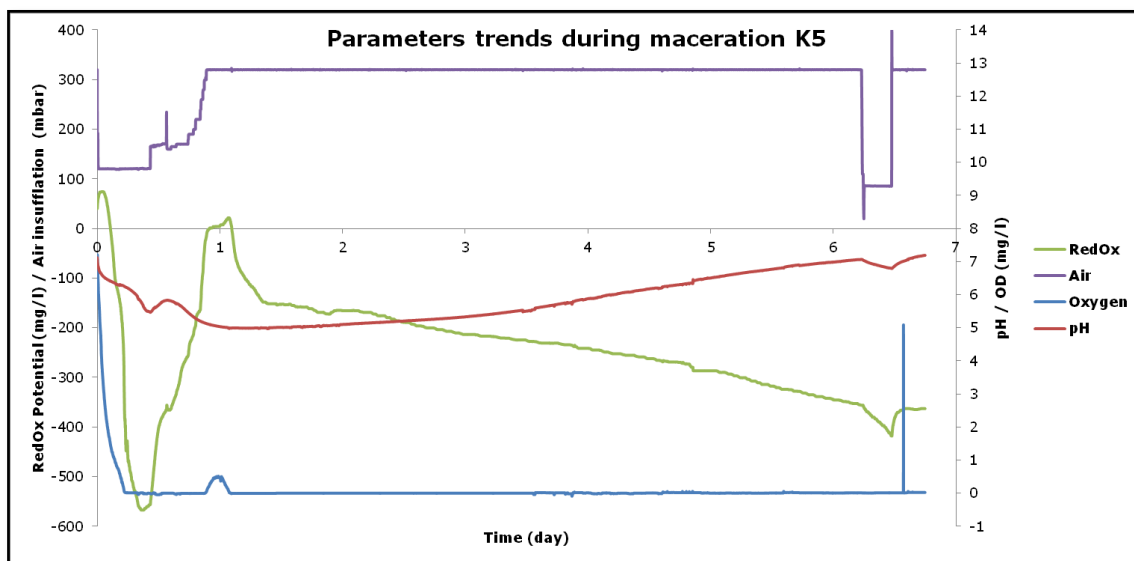


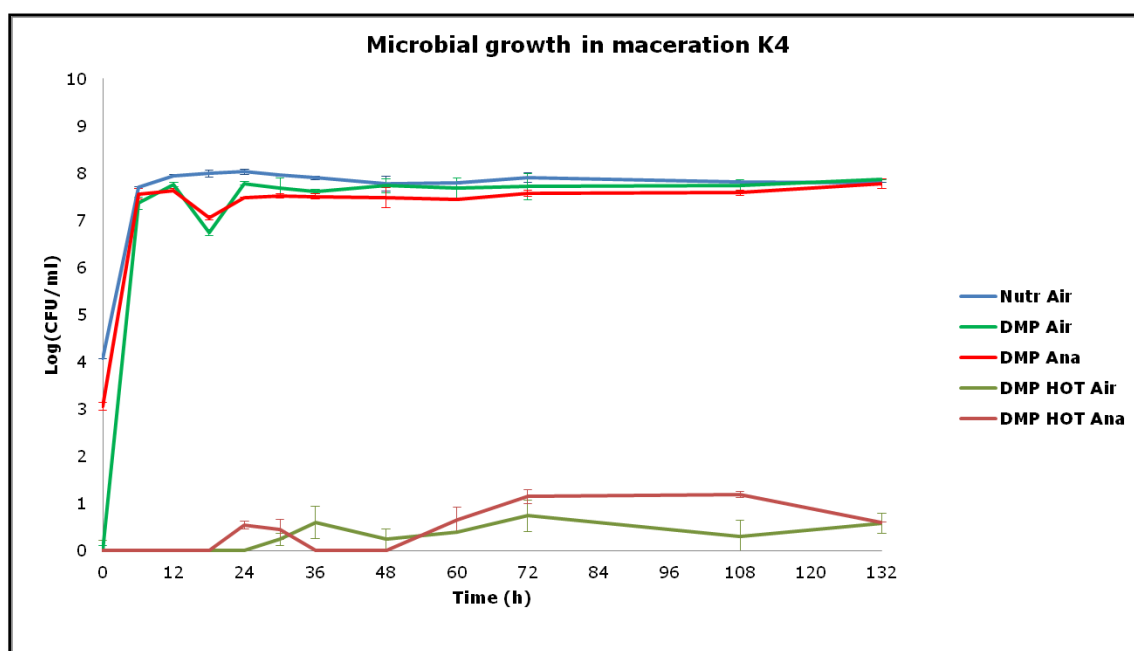
Figure 35. Main parameter trends registered during fifth maceration.

The fourth and the fifth test of maceration in pilot plant were set up on green ribbons of kenaf. In these two tests of maceration the process of retting was completed in six days. The green ribbons/water ratio was changed because of high weight of green ribbons if

compare with the dried ones. In fact 24-25 kilograms of green ribbons were processed in 700 liters of water. The green macerations were characterized by an improvement of air insufflation (from 120 to 320 mbar) but also in this case the bioreactor management succeeded to control and to stabilize the process (Figure 34).

In particular, the air insufflation was so high to restore Redox potential to positive values, however it rapidly decreased at negative values (Figure 35). The retting time was extended of one day (from 5 to 6 days) in order to obtain the same grade of retting. Retted fiber resulted more soft at touch with a green reflex in their white color. No differences had been noticed between fourth and fifth maceration.

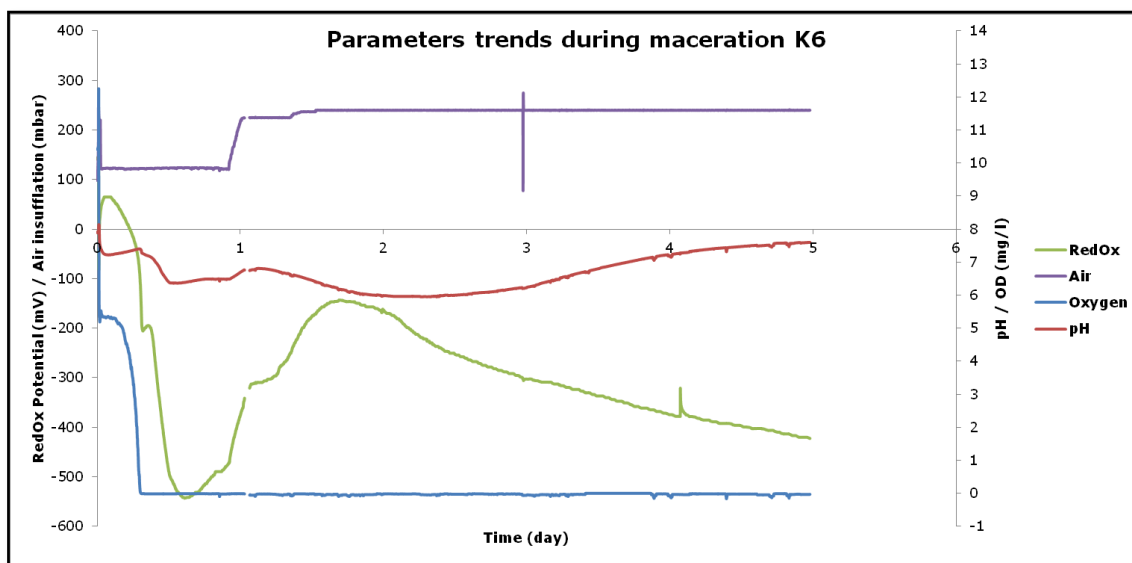
Microbial growth curves registered during fourth maceration show no significant differences from those obtained in the other maceration (Figure 36). Only pectinolytic bacteria (DMP Air and Ana curves) showed a decrease within 24 hours but recovered after the increasing of air insufflation.



**Figure 36.** Microbial growth registered on different media and in different culture conditions during the fifth maceration in pilot plant bioreactor. Nutr = Nutrient agar; DMP = Minimal media with pectin as unique source of carbon; Air = aerobiosis; Ana = anaerobiosis; HOT = thermic treatment of retting liquor (80°C for 10 minutes).

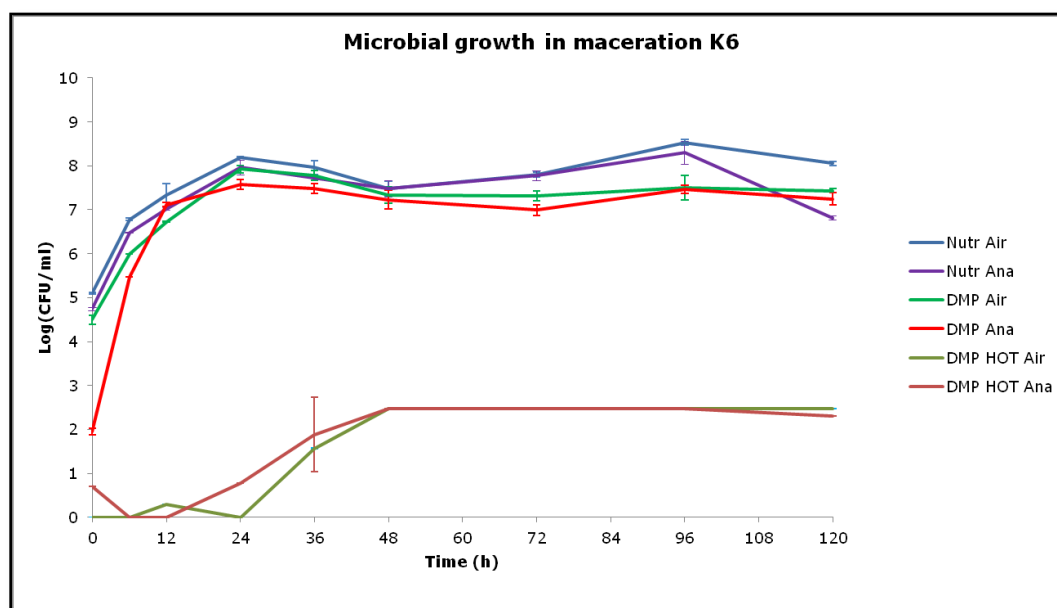
### 3.1.5. Test K6

The sixth maceration was characterized by the loading of three different kinds of kenaf ribbons. The bioreactor was loaded with 6 kilograms of ribbons divided in two kilograms of each kind of kenaf ribbons. Different kinds of kenaf consisted in kenaf ribbons of cultivar Everglade harvested in 2007 and air dried before to store and kenaf ribbons of cultivar Tainung harvested in 2008 and in part air dried and part dried in a woven at 120°C for about 2 minutes. This maceration trial had the objective to detect differences among the different kinds of ribbons and to control if forced drying of ribbons altered retted fibers.



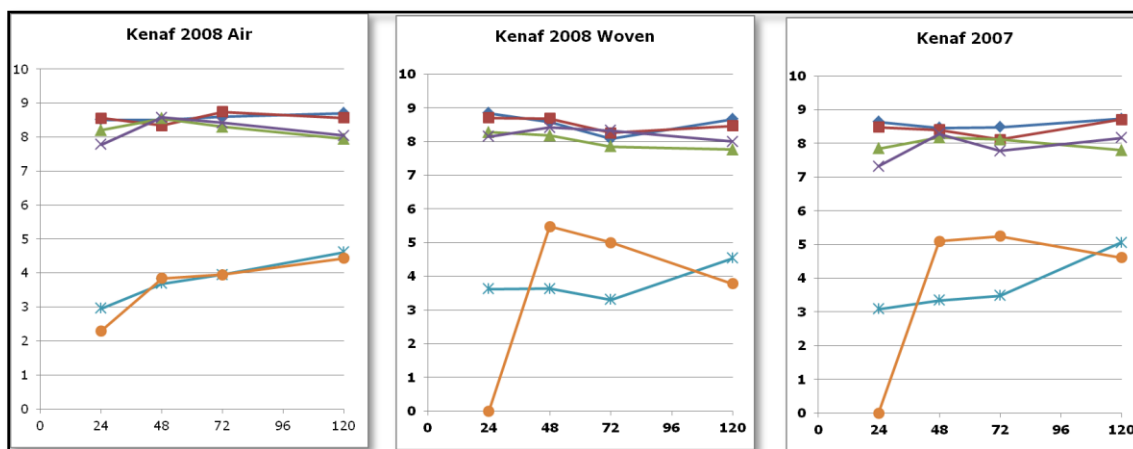
**Figure 37.** Main parameter trends registered during sixth maceration.

Also in this test, bioreactor's main parameter trends were stabilized as in previous macerations (Figure 37).



**Figure 38.** Microbial growth registered on different media and in different culture conditions during the sixth maceration in pilot plant bioreactor sampling retting liquor. Nutr = Nutrient agar; DMP = Minimal media with pectin as unique source of carbon; Air = aerobiosis; Ana = anaerobiosis; HOT = thermic treatment of retting liquor (80°C for 10 minutes).

In the same way, microbial growth curves on different media in diverse culture conditions reflected the trends registered in the previous macerations (Figure 38). Only the presence of spore-forming bacteria seemed higher at the end of the process.



**Figure 39.** Microbial growth registered on different media and in different culture conditions during the first maceration in pilot plant bioreactor sampling the retted fibers. Nutr Air = blue, Nutr Ana = violet, DMP Air = green, DMP Ana = red, DMP HOT Air = light blue, DMP HOT Ana = orange. Nutr = Nutrient agar; DMP = Minimal media with pectin as unique source of carbon; Air = aerobiosis; Ana = anaerobiosis; HOT = thermic treatment of retting liquor (80°C for 10 minutes).

During K6 test the microbial dynamics on retted fiber besides in retting liquor were also monitored (Figure 39). The microbial cells were detached from fibers by processing them with a stomacher, and bacteria were enumerated on different kind of ribbons.

The first difference observed was the highest contamination of spore-forming bacteria in respect to the value obtained for retting liquor. These data indicated that spore-forming bacteria preferentially colonized ribbons suggesting their relevant importance in the maceration process. In the specific no relevant differences were obtained among different kind of kenaf ribbons.

### 3.1.6. Phenolic compounds content in the retting liquor

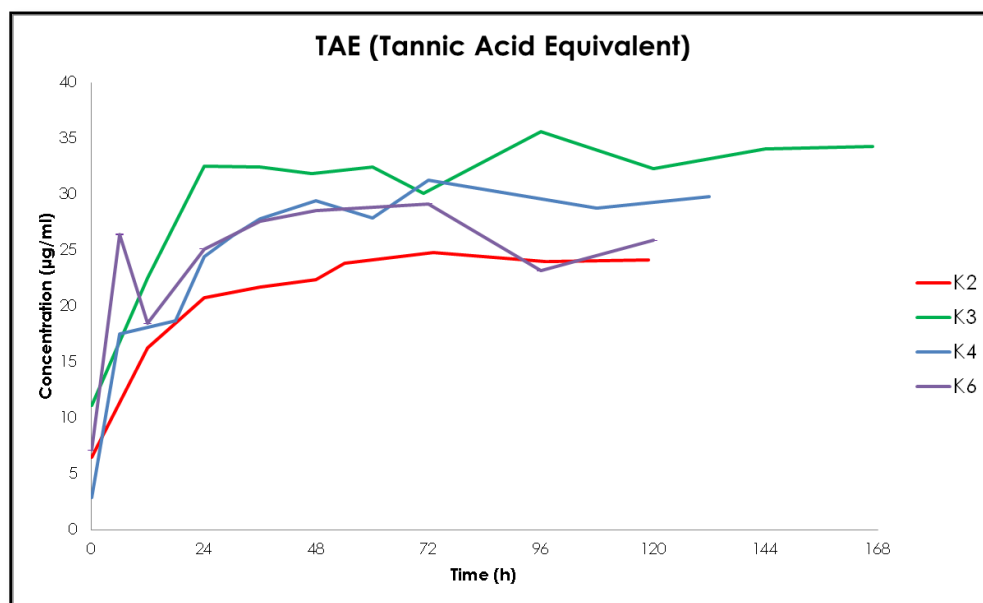
The determination of phenolic compounds as Tannic acid equivalent (TAE) was monitored during all trial macerations in the retting liquor. In the figure 40 there are reported the most significant results obtained from trials of maceration with different kind of kenaf ribbons (K2 air dried Everglade kenaf, K3 double loaded Everglade kenaf ribbons, K4 Tainung green ribbons, K6 Everglade and Tainung kenaf ribbons with different kind of drying).

Content of phenolic compounds increased up during the maceration of kenaf ribbons indicating a release of phenolic compounds probably originating by the depolymerization of lignin presents in plant tissues (Figure 40).

Significantly, the phenolic compounds content stabilized within first 24-48 hours in spite of a linear increment suggesting a contemporary degradation or transformation of phenolic compounds.

In particular, different amount of kenaf ribbons and different variety of kenaf caused different phenolic compounds release. This hypothesis was confirmed by maceration K3 (12 kg of Everglade kenaf ribbons) which showed an increase in the phenolic compound less than maceration K2 (6 kg of Everglade kenaf ribbons), in spite of a doubled level.

Moreover, green ribbons of Tainung variety (maceration K4) registered approximately the same TAE values of dried kenaf ribbons (maceration K6 with 6 kg of Everglade and Tainung varieties), suggesting that drying step didn't altered the composition of kenaf stalks. Actually only the kenaf variety employed influenced TAE level registered. This data suggested a major content of lignin or more degradable lignin in Tainung variety of kenaf. In literature studies determined lignin content of lignin and phenolic compounds without correlating data obtained to different varieties of kenaf (Seca *et al.*, 2001a; Seca *et al.*, 2001b).



**Figure 40.** Tannic acid equivalent registered by Folin-Ciocalteu assay on retting liquor for most important macerations.

### 3.1.7. Evaluation of retted fibers quality

To understand the grade of retting of natural fibers is sufficient a simple organoleptic analysis by well-practice operator (Pallesen, 1996).

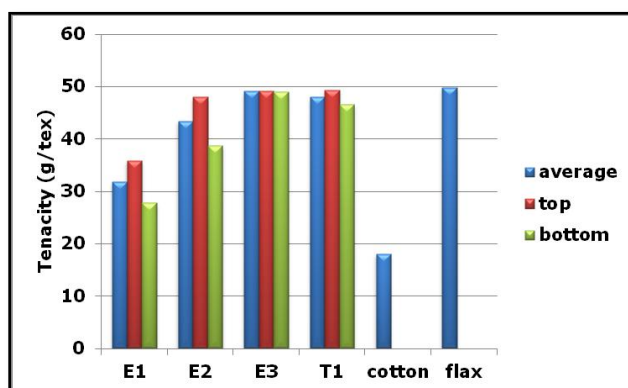
In according with evaluations of natural fiber quality present in literature, the determinations of tenacity were performed at the top and at the bottom part of stalks in order to normalize the expected differences occurring in plant fibers (Pallesen, 1996). Data obtained from retted fibers were presented in order to evidence not only the maceration test, but also stressing the difference of cultivar o kenaf. Briefly, E1, E2 and E3 belong to the first three macerations conducted on Everglade cultivar (K1, K2, and K3 macerations), while T1 indicated maceration of Tainung variety (K6 maceration).

As an alternative, in this study the evaluation of retted fibers was achieved by combining different approach: the evaluation of tenacity by stelometer, bioreactor's yield and retted fibers' composition by infrared spectroscopy (FT-IR).

In order to better understand the quality of retted fibers, data obtained were compared with data present in literature and with data obtained from some natural fibers as

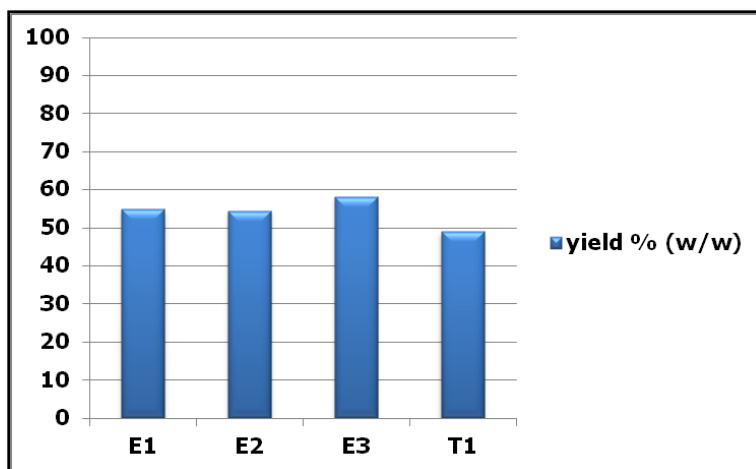


references (cotton and flax) (Ouajai *et al.* Shanks, 2005). Tenacity, as previously described (2.2.2.3), is an important property of textile fibers, especially in yarn making.



**Figure 41.** Evaluation of tenacity of retted fibers and some reference fibers. E1 = Everglade maceration K1; E2 = Everglade maceration K2; E3 = Everglade maceration K3; T1 = Tainung maceration K6.

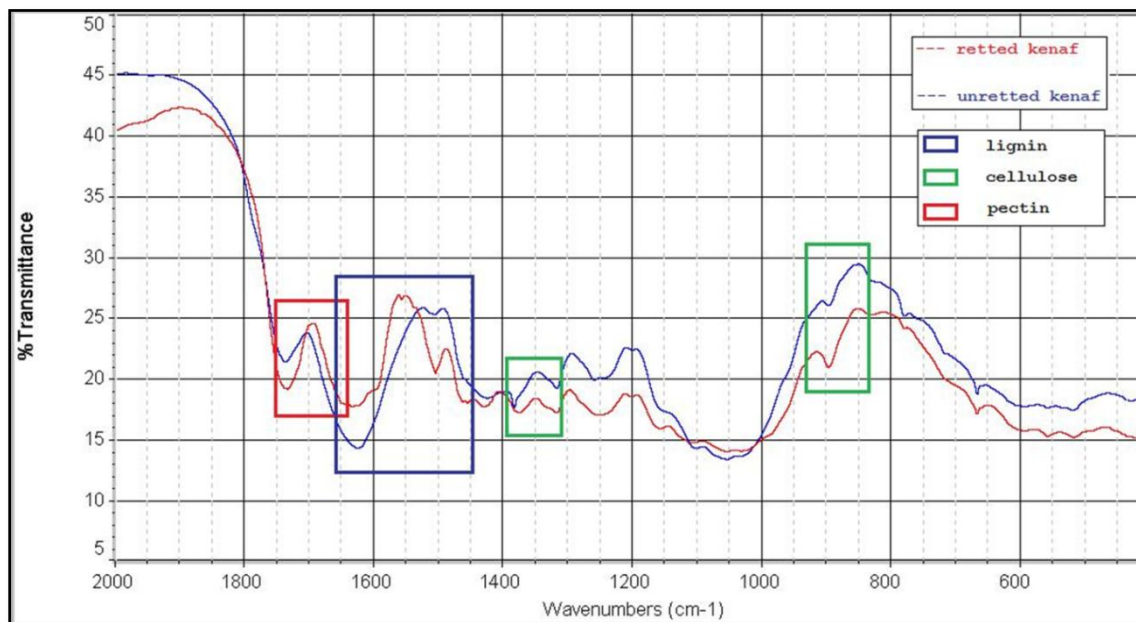
Natural fibers from kenaf obtained in bioreactor as demonstrated by tenacity determinations evidenced a good value similar to retted flax (Figure 41). Natural fibers obtained from flax were traditionally employed in high income apparel productions, suggesting a future expansion in this field even for kenaf.



**Figure 42.** Percentual of yield in retted fibers calculated in dry weight.

Data obtained analyzing retted fibers' yield (Figure 42) are in accordance with literature which stated that average content of cellulose in the bark of kenaf was about the 50% (Amaducci *et al.*, 2000). Indirectly, bioreactor's yield data confirmed the good grade of maceration of retted fibers. Results indirectly evinced by bioreactor yield were confirmed by the analysis of composition of retted fiber vs. unretted ones with infrared spectroscopy. Infrared spectra of unretted and retted kenaf fibers are shown in figure 43. In general, the spectrum of retted fibers is similar to that registered for the raw kenaf fibers. However, the spectra had two intense bands at  $1733\text{ cm}^{-1}$  assigned to stretching C=O vibration of methyl

and carboxylic acid in pectin and hemicellulose and  $1637\text{ cm}^{-1}$  attributed to bending of water  $\delta(\text{H}_2\text{O})$  overlapped with the asymmetric stretching vibration of carboxylate anion  $\text{vas}(\text{COO}^-)$  respectively (Copikova *et al.*, 2001). Pectin contains both esterified and carboxylic acid groups in the structure. The variation of 1637 to 1733 ratio indicated that after retting the pectin still existed in fibers but with a high degree of methyl ester content (Ouajai *et Shanks*, 2005). These data suggested that during the maceration the structure of pectin was preferentially attacked on no esterified fraction. The band at  $1372\text{ cm}^{-1}$  was attributed to a C-H vibration in the cellulose, and the band at  $897\text{ cm}^{-1}$  corresponds to  $\beta$ -D-glycosides. The decreasing of pectin-specific peaks and the increasing of the cellulose-specific peaks indicated that cellulose content in kenaf fibers did not exhibit significant changes during the retting process. The bands at  $1626\text{ cm}^{-1}$  and  $1514\text{ cm}^{-1}$  were assigned as aromatic vibration, indicating the existence of lignin in the unretted and retted hemp fibers (Zhang *et al.*, 2008). In particular between these two regions the spectra register major changing in shape suggesting a lignin modification during aerated ribbon retting.



**Figure 43.** Infrared spectra obtained by analyzing retted and unretted fibers.

Concluding, FT-IR spectroscopy for the determination of quality of retted fibers is complicated, but could give information about the single constituent of fibers, in the specific, it's important to understand if alteration of cellulose occurred during maceration (over-retting).

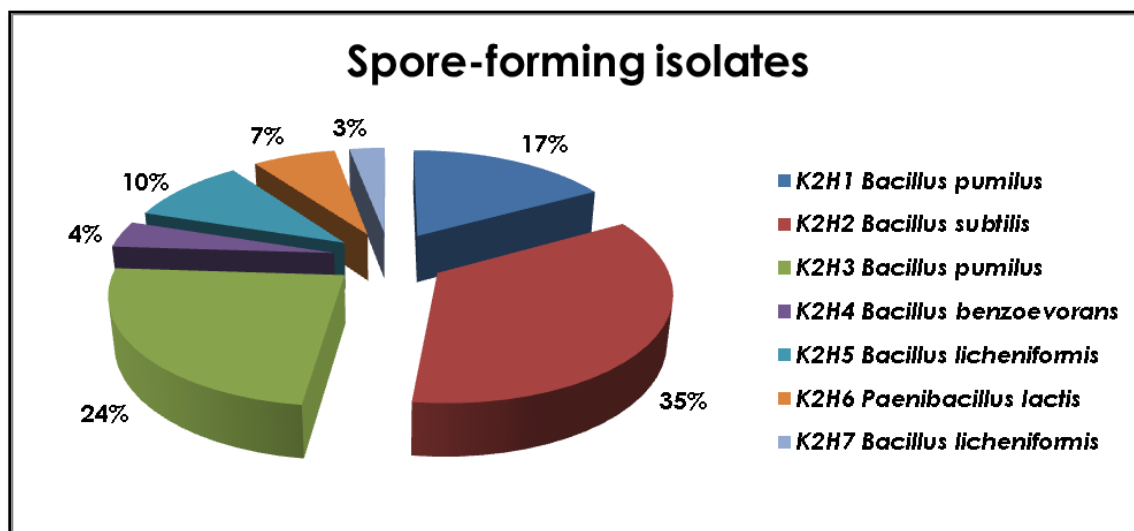
## 3.2. Retting bacteria

### 3.2.1. Isolation and characterization

Microbial populations in bioreactor were enumerated by culturing on different growth media and in different condition. However, culture dependent approaches underestimate the real presence of bacteria in a complex matrix such as the retting liquor. For these reasons two complementary but different approaches were adopted to study and characterize the diachronic evolution of microbial community during the retting process in bioreactor.

#### 3.2.1.1. Culture dependent approach

Culture dependent approach allowed isolating from retting liquor some cultivable species of bacteria recurring in maceration in bioreactor. Isolates able to growth on pectin as unique source of carbon were obtained from retting liquor of K2, K3, K4 and K6 maceration, screened by A.R.D.R.A. technique. In particular, pectinolytic bacteria - grouped for their ability to produce spore - were isolated on DMP media in different growth conditions (aerobiosis and anaerobiosis). Unexpectedly, bacterial strains obtained in anaerobic growth conditions coincided with those isolated in the aerobic conditions, suggesting that pectinolytic bacteria tolerate absence of oxygen.



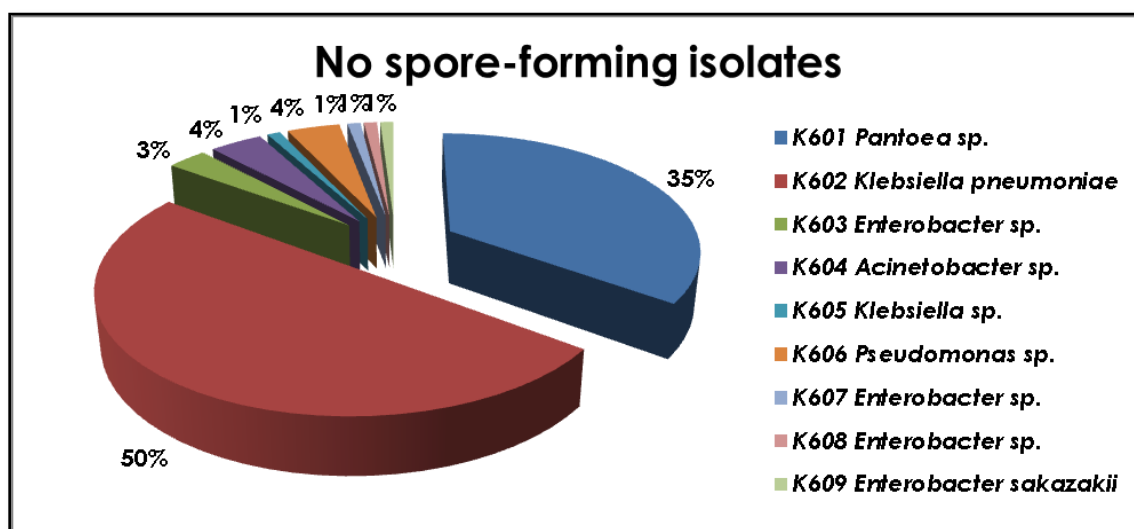
**Figure 44.** Schematic representation of spore-forming isolates and their relative abundance among total isolates.

Among spore-forming bacteria screened through A.R.D.R.A. technique – seven strains belonging to five species of *Bacilli*<sup>3</sup> - were identified. In the specific, two species (*B. subtilis*

<sup>3</sup> In appendix (chapter 6.2.) are reported the tables indicating the homology, the taxonomic identification and principal reference sequences founded in alignment on NCBI database.

and, *B. pumilus*) represented more than 70 % of all isolates obtained (Figure 44). About, 96 isolates belonging to maceration K2 and K3 were analyzed. *Bacillus subtilis*, *Bacillus pumilus* are bacterial species well-known in literature for their ability to produce exo-pectinases. On the other hand, *Bacillus licheniformis* is a multi-purpose bacteria involved in numerous industrial fermentations. Gene *Pel* similar to that in *Bacillus subtilis* species was identified into the genome of these strains.

No spore-forming bacteria (about 72 isolates) were obtained in maceration K6. A.R.D.R.A. analysis evidenced the presence of 9 different strains belonging to five species of *Proteobacteria* (Figure 45). Even in this case, two isolates (*Pantoea* sp. and *Klebsiella pneumonia*) represent more than 80% of the all isolates obtained.



**Figure 45.** Schematic representation of no spore-forming isolates and their relative abundance among total isolates.

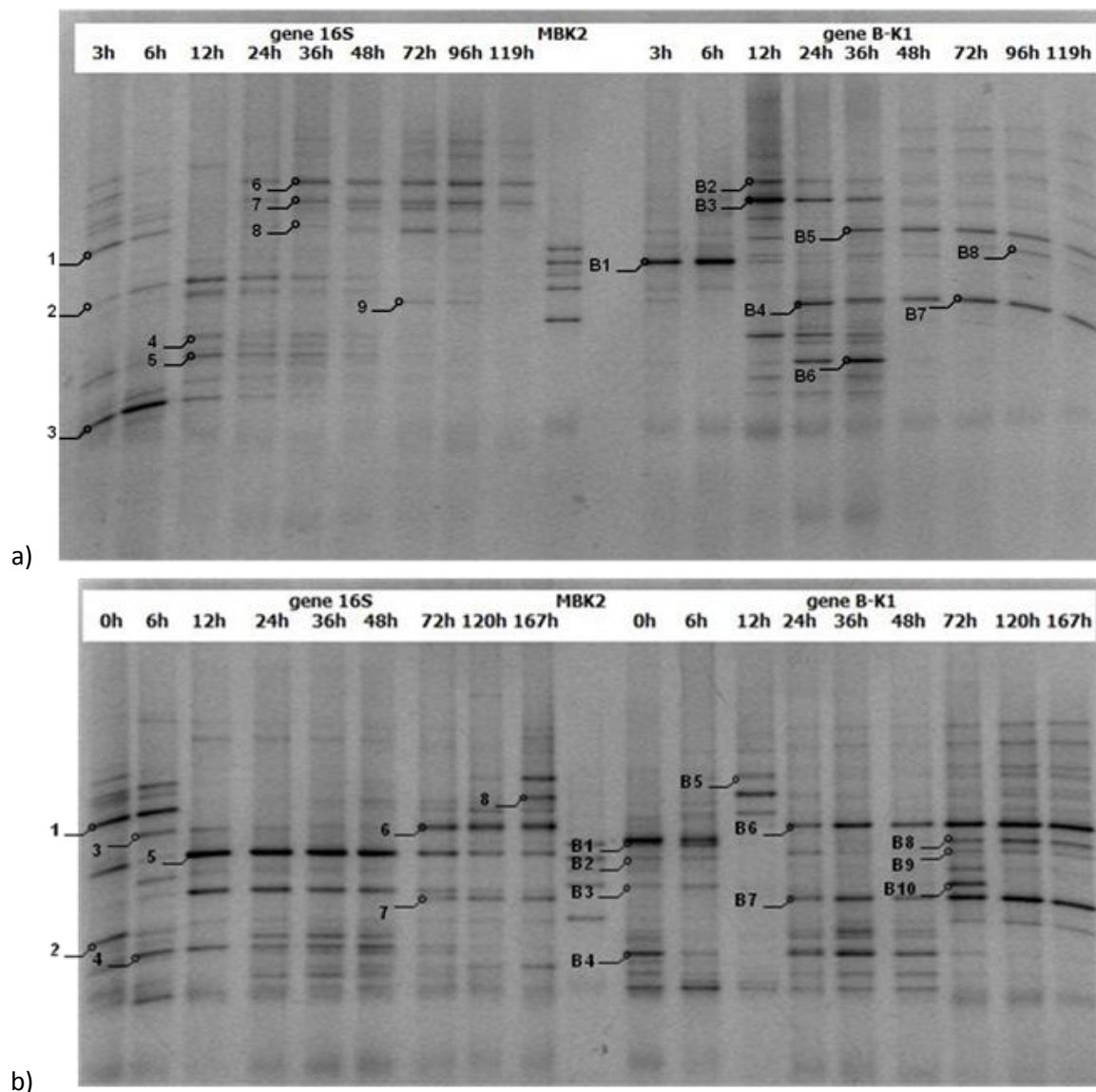
All strains isolated belonged to *Gamma Proteobacteria*. In particular the order most represented is *Enterobacteriales*. *Enterobacteriaceae* families were isolated in paper and pulp mill effluent with no occurrence of fecal contamination (Gauthier *et al.* Archibald, 2001). They were also isolates in association with plant and soil rhizosphere. (Gauthier *et al.* Archibald, 2001; Suihko *et al.* Skytta, 2009).

Previous studies on water retting of fiber crops as flax (Donaghy *et al.*, 1990), hemp (Tamburini *et al.*, 2003) and jute (Ali, 1958) (Ahmed *et al.* Akhter, 2001) (Banik *et al.*, 2003) documented the presence of spore formers in retting liquor and indicated two main population *Bacillus* and *Clostridium* as the main responsible of the maceration process. In fact, in traditional water retting two distinct phases accompanied the process: a first aerobic phase dominated by *Bacillus* species that began retting process and consumed oxygen, on the other hand in the second anaerobic phase prevailed the *Clostridium* species.

Furthermore, few works reported the presence of *gamma-Proteobacteria* in retting liquor and their presence could be considered as opportunistic because they were detected in paper and pulp mill wastewaters which are very similar in term of content of nutrient deriving from plant tissues (lignin, cellulose, pectin and hemicellulose).

### 3.2.1.2. Culture independent approach

Culture independent analysis like DGGE has the advantage to discover quite all bacteria present in a complex matrix relying on the presence of nucleic acid, not on cultivability of microorganisms (Muyzer *et al.*, 1993). Usually DGGE profiles of complex matrix are quite complex to appreciate because detected many bands for each line. However, the advantage of molecular techniques that rely on PCR is the introduction of selective primers that allowed filtering the complexity of information.



**Figure 46.** DGGE profile of retting liquor lines in each profile show, from left to right, the diachronic evolution of retting microflora at different time: on the left *Eubacterial*-specific profiles; MBK2 marker in the middle; and on the right *Bacillus*-specific profiles. a) Maceration K2 (6 kg loading of kenaf ribbons); b) maceration K3 (12 kg loading of kenaf ribbons). Main bands excised and sequenced are indicated by numbers and letters.

Previous studies evidenced the importance of spore-forming bacteria in retting process of other bast fibers like flax (Donaghy *et al.*, 1990), hemp (Tamburini *et al.*, 2003) and jute (Ahmed *et al.*, 2001; Banik *et al.*, 2003; Ali, 1958). For these reason, classic DGGE profile of total Eubacteria species present in retting liquor, was compared with DGGE profile obtained with *Bacillus*-specific primers.

DGGE profiles obtained analyzing retting liquor of maceration K2 and K3 (Figure 46) reflected the main parameters trends registered in the bioreactor and the composition of microbial community was in agreement of results obtained in culture dependent approach reported before. In fact, the diachronic evolution of different population of bacteria stabilized within first 24 hours and slightly changed during the raising of air insufflation rate. Afterword, low differences in main bands reflected the modification of bioreactor environment by air insufflation, after 48 hours.

The analysis of sequences of main bands revealed the recurring presence of the same species isolated with the culture dependent approach prevalently belonging to *Bacillus* and *Proteobacteria* species (Figure 47). In the specific the MBK2 markers in the middle were prepared using five isolates that registered highest pectinolytic activity (in order from the top: K2H1B. *pumilus*, K2H2 *B. subtilis*, K2H7 *B. licheniformis*, K2H3 *B. pumilus*, K3H5 *B. licheniformis*).

*Acinetobacter*, *Klebsiella* and *Achromobacter* isolates are known to be associated to plant stem and rhizosphere. *Exiguobacterium* isolates from paper and pulp effluents. *Bacteroidetes* isolates in anaerobic bioreactors. *Clostridium* sp. isolates producing cellulosomes. *Bacillus silvestris*, *Bacillus koreensis*, *Bacillus licheniformis*, *Bacillus nealsonii* associated to plant. *Bacillus nealsonii* isolates from herbaceous fibers extraction. *Bacillus licheniformis*, *Bacillus oleronius*, *Clostridium* sp. isolates from paper and pulp mill effluents.

Despite its *Bacillus* specificity, B-K1 primers amplified a *Clostridium* band that characterized early phase of the process. In particular air insufflation during retting seemed to control over retting risk due to cellulolytic activity by limiting development of *Clostridia* species.

In the specific, the comparison of DGGE profile of both macerations (K2 and K3) revealed that the main bands evidenced the presence of some recurring species.

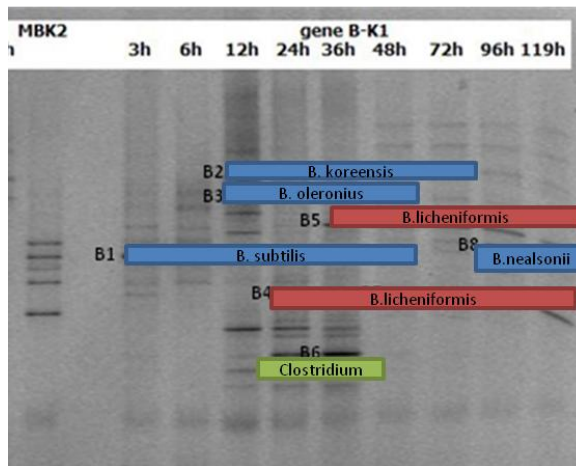
For example in DGGE profile regarding total Eubacteria some bands belonging to *Proteobacteria* (*Acinetobacter* sp., *Klebsiella* sp.) and *Clostridia* branch were revealed in the early phase. Afterwards, *B. licheniformis* took place in the late phase of the process.

In the same way, the BK-1 DGGE profiles of both macerations revealed the presence of *Bacillus* spp. during all the aerobic retting processes. In particular, as already registered in DGGE profiles on V3 region, the BK-1 profiles evidenced the presence of two distinct phases of the process.

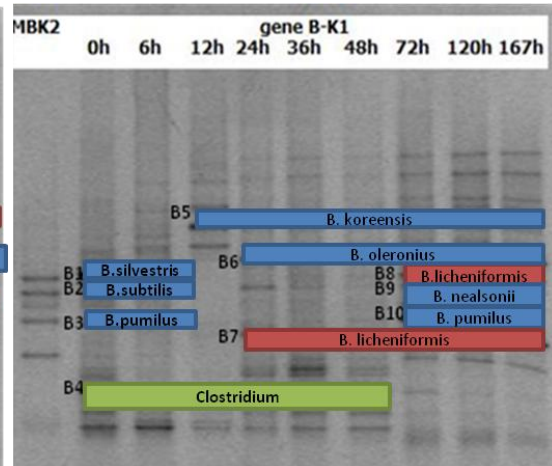
*B. koreensis*, *B. oleronius* and *B. subtilis* dominated the early phase, whereas *B. licheniformis* and *B. nealsonii* are present in the late phase.



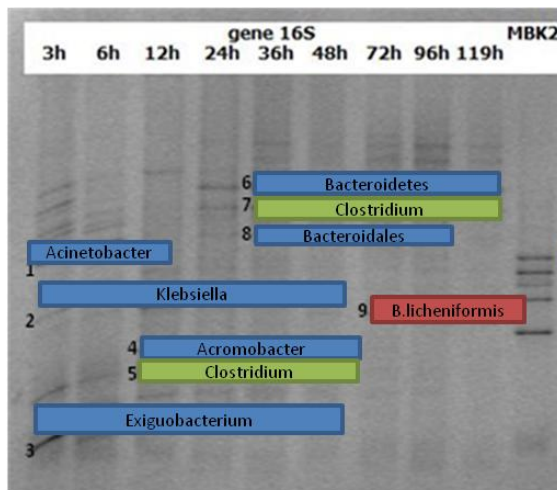
A)



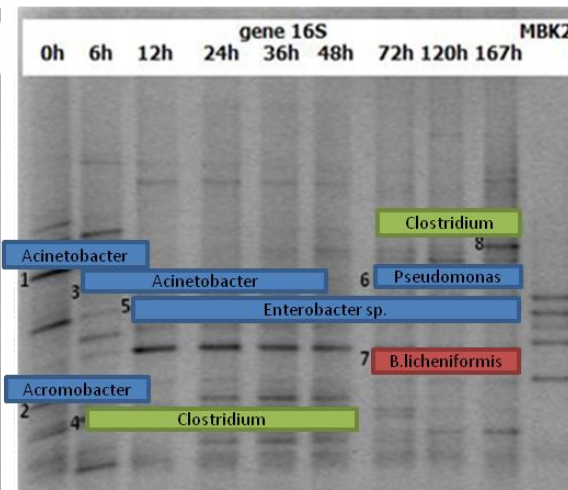
Maceration K2



Maceration K3

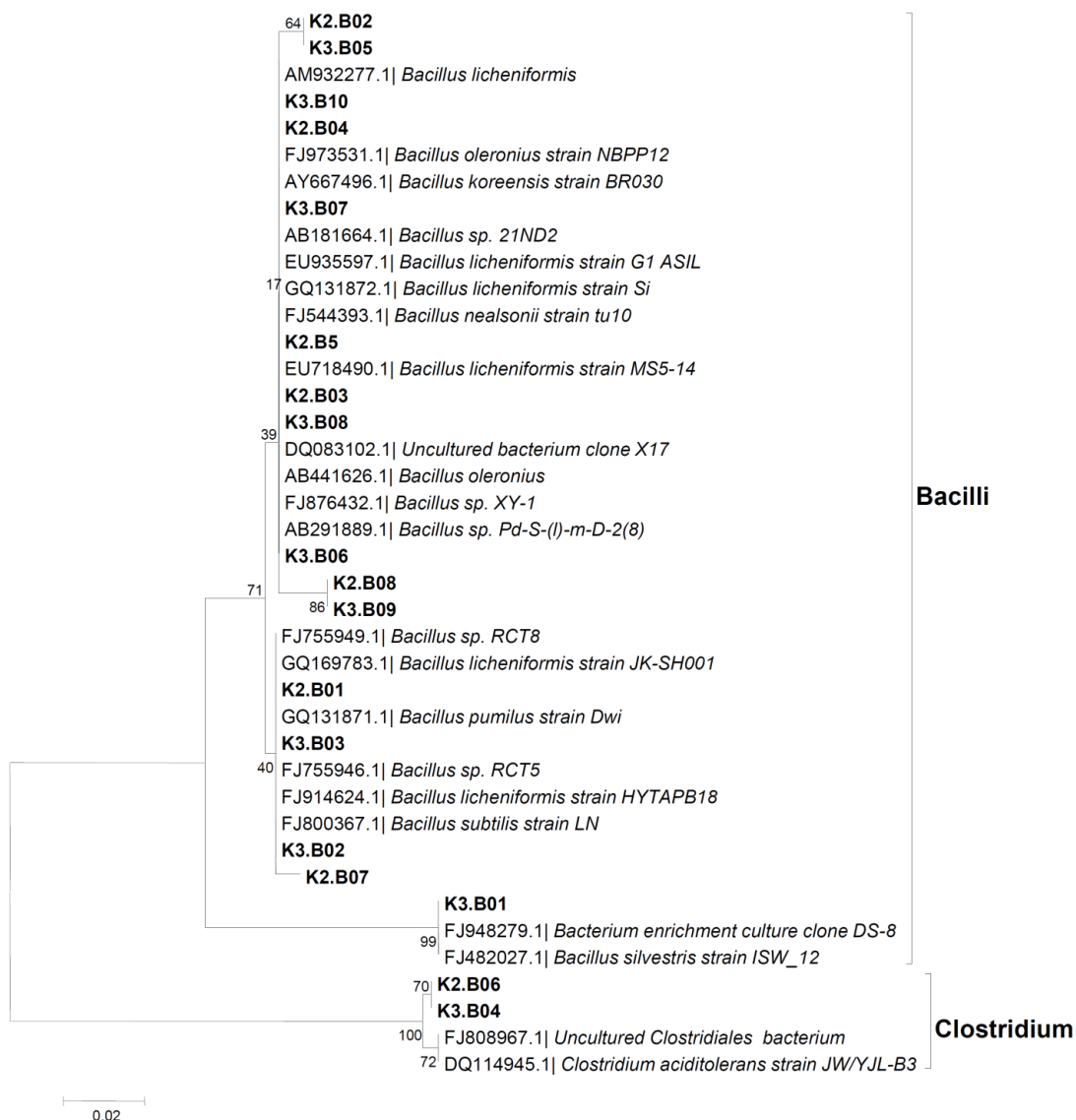


Maceration K2

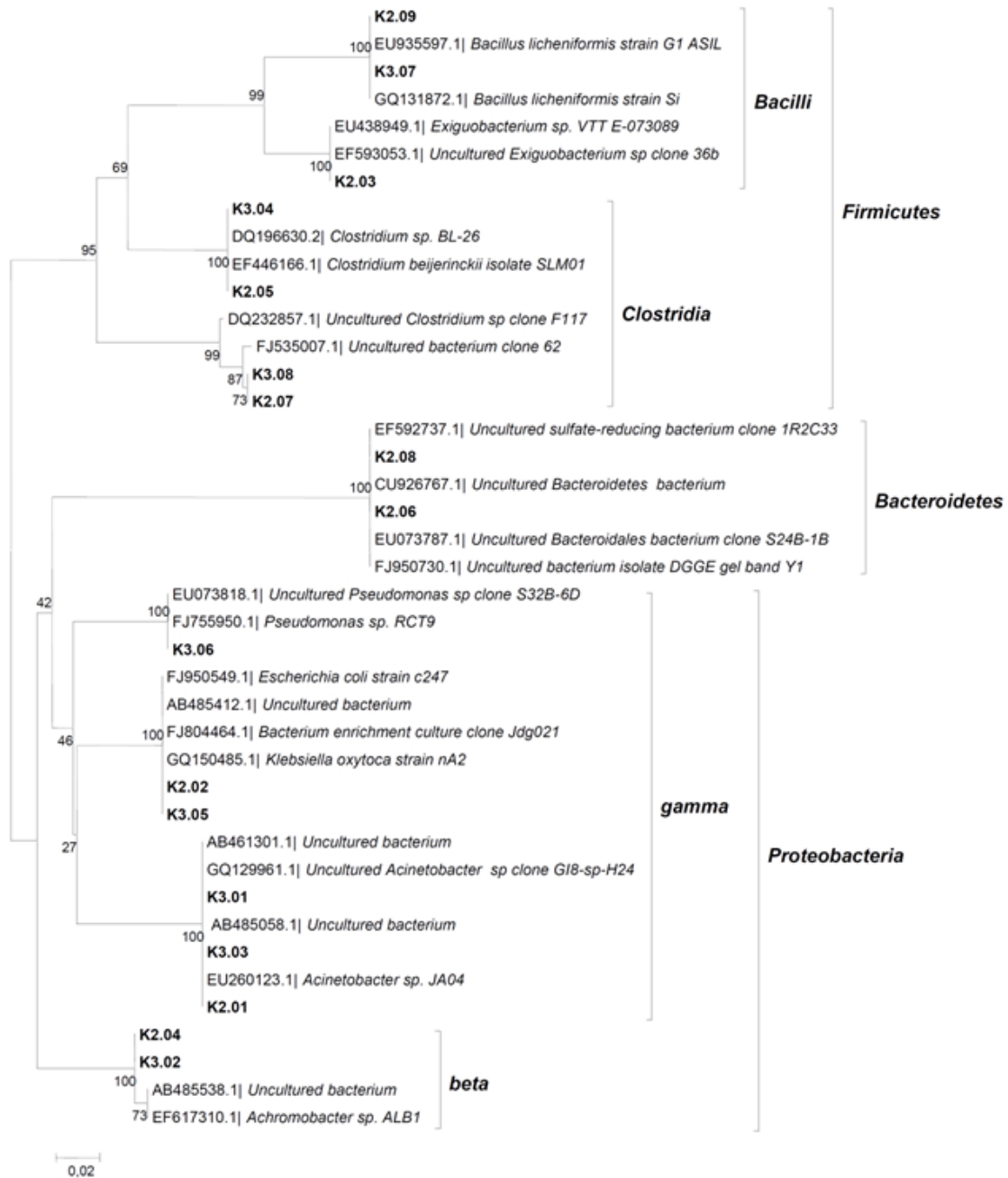


Maceration K3

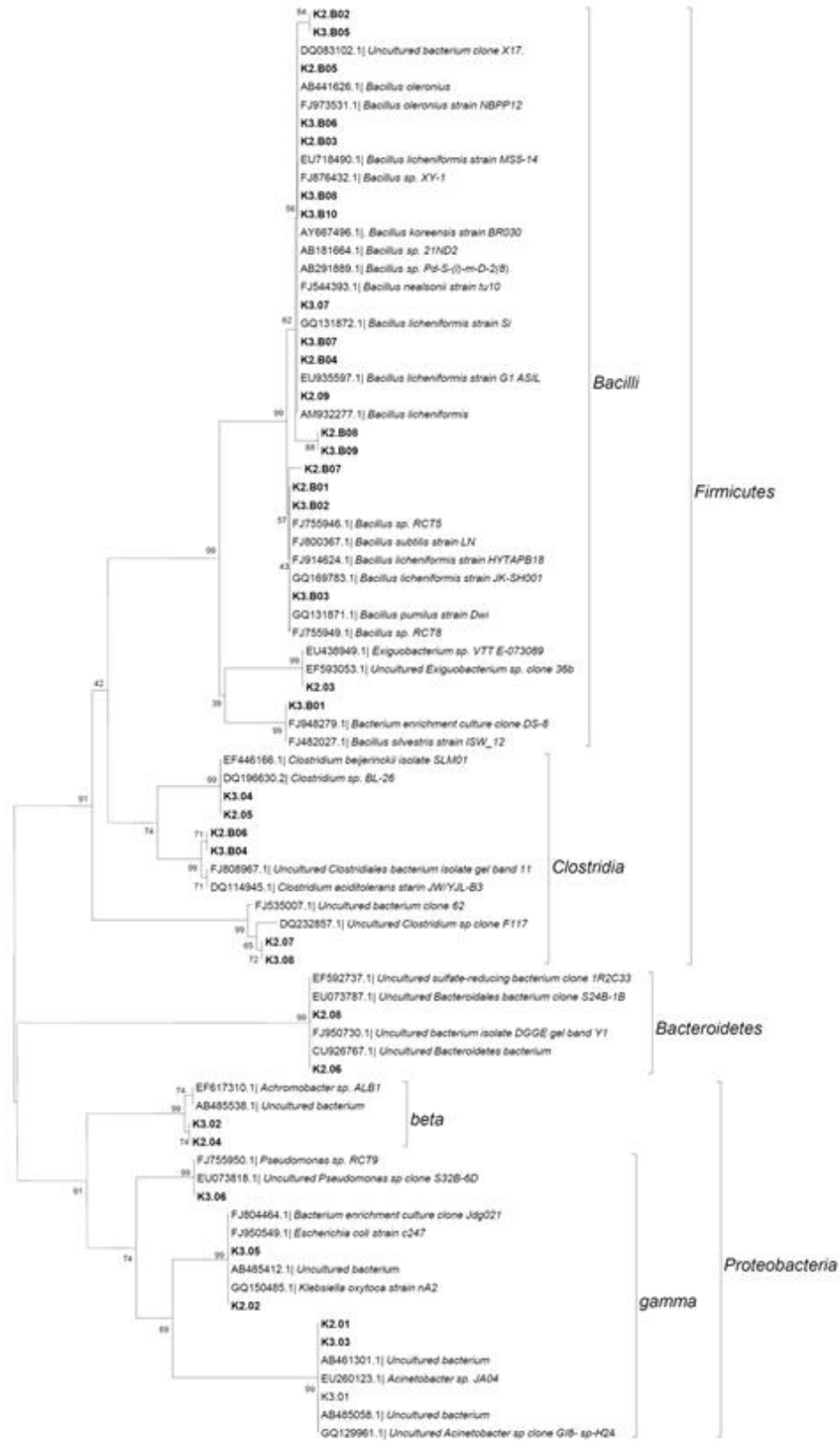




A)



B)



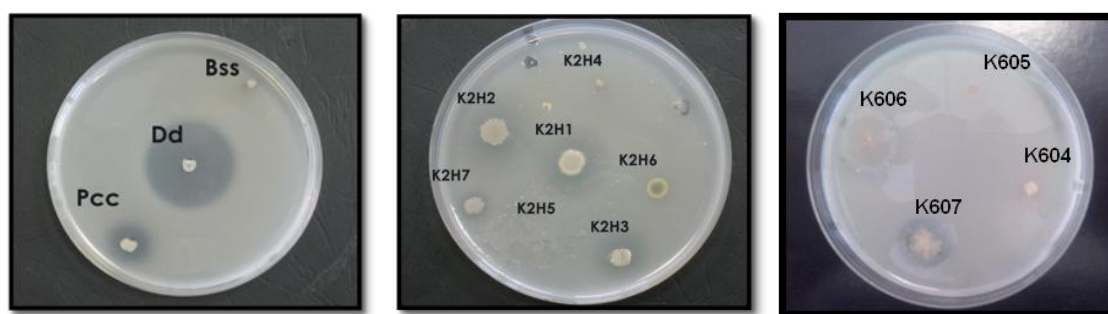
c)

**Figure 47.** Neighbor-joining phylogenetic tree constructed by using bacterial hypervariable V3 region of 16S rDNA sequences of the main bands retrieved from DGGE profile of the retting liquor samples. Accession number followed by the name of the bacteria identified 16S rDNA sequences of main homologues retrieved in GenBank by BLASTn program (Altschul *et al.*, 1990). Names of the major DGGE profile bands are shown in boldface. Numbers at nodes represent the percentages of occurrence of nodes in 1000 bootstrap trials. The scale bar represents the expected number of substitutions per nucleotide position. In previous page: A) DGGE profiles of K2 and K3 macerations with results of identification of main bands; B) Phylogenetic tree obtained from sequences of main *Bacillus*-specific bands of both macerations; C) Phylogenetic tree obtained from sequences of main Eubacterial bands of both macerations; D) Phylogenetic tree with all main bands from both macerations. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4 (Tamura *et al.*, 2007).

### 3.2.2. Metabolic and physiologic screening of retting bacteria

#### 3.2.2.1. Pectinolytic and cellulolytic activity

The pectinolytic activity and cellulolytic activity of all isolates were tested qualitatively on DMP media in order to detect strains with high esoenzymes production. The growth of isolates on minimal growth media containing only specific polysaccharide as source of carbon (DMP and DMC) elicited the production of specific esoenzymes around the colony of bacteria. Then, after a period which allowed diffusion and action of esoenzymes the addition of CTAB solution for pectinolytic enzymes and Red Congo solution for cellulolytic enzymes revealed not colored halos around positives strains. In particular this assay tested also same reference strains: *Bacillus subtilis* subsp. *subtilis*, *Dickeya dadantii* and *Pectobacterium carotovorum* subsp. *carotovorum*. The first was a reference of high pectinolytic producer strain employed in industrial production of much kind of esoenzymes, and the others two strains are the first bacteria studied for their ability to produce pectinolytic enzymes (Collmer *et al.*, 1988). They are also involved in many rotting case of vegetable (Hugouvieux-Cotte-Pattat *et al.*, 1996; Zaidi-Yahiaoui *et al.*, 2008).



**Figure 48.** CTAB assay plates showed high pectinolytic activity and eso-pectinase production by forming a halo around positive strains. K2H1 *B. pumilus*, K2H2 *B. subtilis*, K2H3 *B. pumilus*, K2H4 *B. licheniformis*, and K607 *Enterobacter* sp.. Reference strains: Bss *Bacillus subtilis* subsp. *subtilis*, Dd *Dickeya dadantii*, Pcc *Pectobacterium carotovorum* subsp. *carotovorum*.

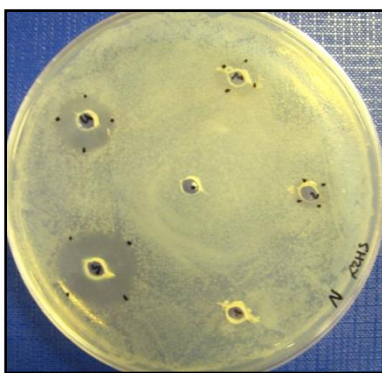
Qualitative assays (pectinolytic and cellulolytic assays) revealed that in all strains the ability to use a polysaccharide as unique source of carbon didn't correspond with a distinctive ability to produce esoenzymes. In fact, no cellulolytic activity were detected for all isolates and reference strains, instead only few isolates showed high pectinolytic activity production as reference strains (Figure 48).

In particular, *Dickeya dadantii* and *Pectobacterium carotovorum* subsp. *carotovorum* showed the highest esoenzymes production. On the other hand, spore-forming isolates: K2H1 *B. pumilus*, K2H2 *B. subtilis*, K2H3 *B. pumilus*, K2H7 *B. licheniformis* registered a higher esoenzymes production than observed for *Bacillus subtilis* subsp. *subtilis* (reference of the genus). At last, among the no spore-forming isolates only K607 *Enterobacter* sp. showed esoenzymes production.

This data underlined that *Bacillus* species played a central role in degumming fibers and justified their relevant presence on retted fibers in bioreactor.

### 3.2.2.2. Radial diffusion assay with tannic acid

The radial diffusion assay with tannic acid registered the inhibition of growth of isolates in case due by the release of tannins in retting liquor during the maceration.



**Figure 49.** Example of RDA with tannic acid of K2H5 *B. licheniformis* on Nutrient agar plates where increasing concentrations of tannic acid were applied in numbered wells. In the center the control well filled with same amount of water.

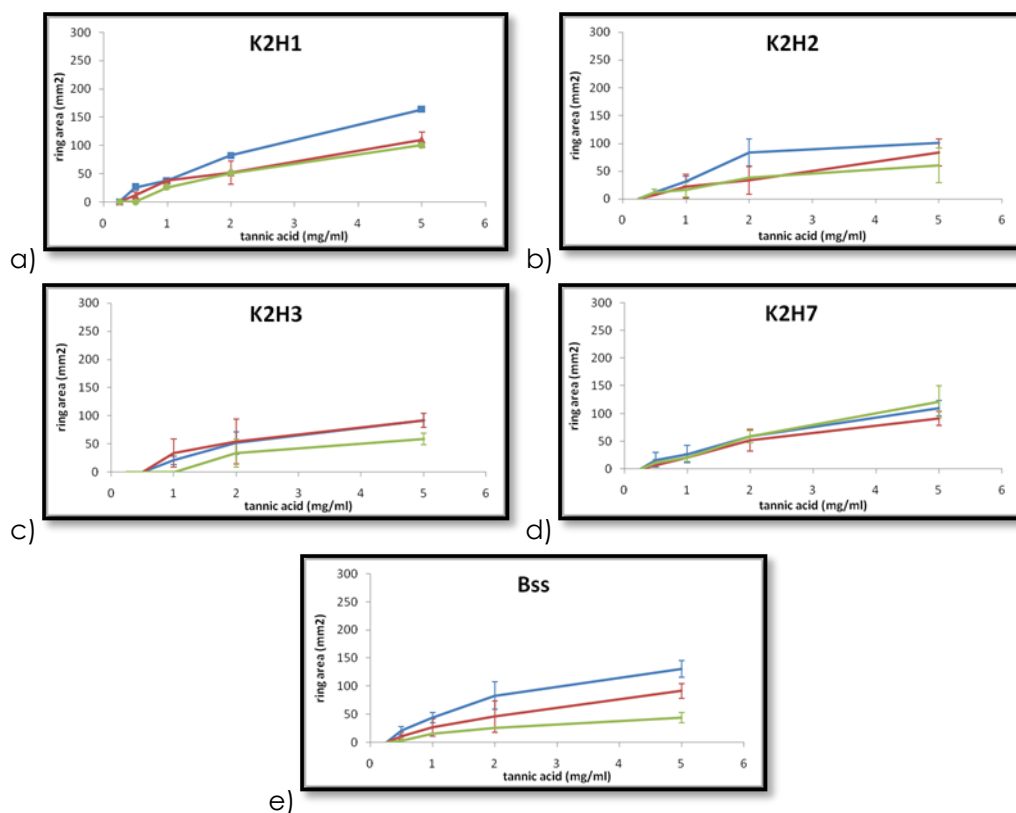
The inhibition of growth was registered for all isolates on different media and only spore formers strains showed the formation of halos (Figure 49).

In the graph were plotted the ring area representing the inhibition of growth of most important isolates with *Bacillus* reference strain. In particular, only the reference strain *Bacillus subtilis* subsp. *subtilis* showed a decreasing inhibition with the increasing of the complexity of culture media utilized: DMP>DMP+YE>Nutrient, as shown in figure 49 d). These evidences are in agreement with metabolic properties of bacteria which in general better tolerate the presence of toxic substances in presence of a rich font of nutrients.

In figure 50 graphs obtained only for highest pectinolytic strains with spore forming reference *Bacillus subtilis* subsp. *subtilis* are represented. Spore-forming strains with high pectinolytic activity showed a resistance towards tannic acid. At last, a linear relationship between tannic acid concentration and growth inhibition was expected. On the contrary, in same lines the inhibition decreased in correspondence of high tannic acid concentrations. These data suggested that isolates activated a stress response to high concentration of toxic substance.

On the other hand, no spore-forming isolates belonging to *Proteobacteria*, didn't show halo formation in presence of the tested tannic acid concentrations, suggesting that these

species were not inhibited by tannins released during maceration, indicating their probable implication in retting during late phase of the process.



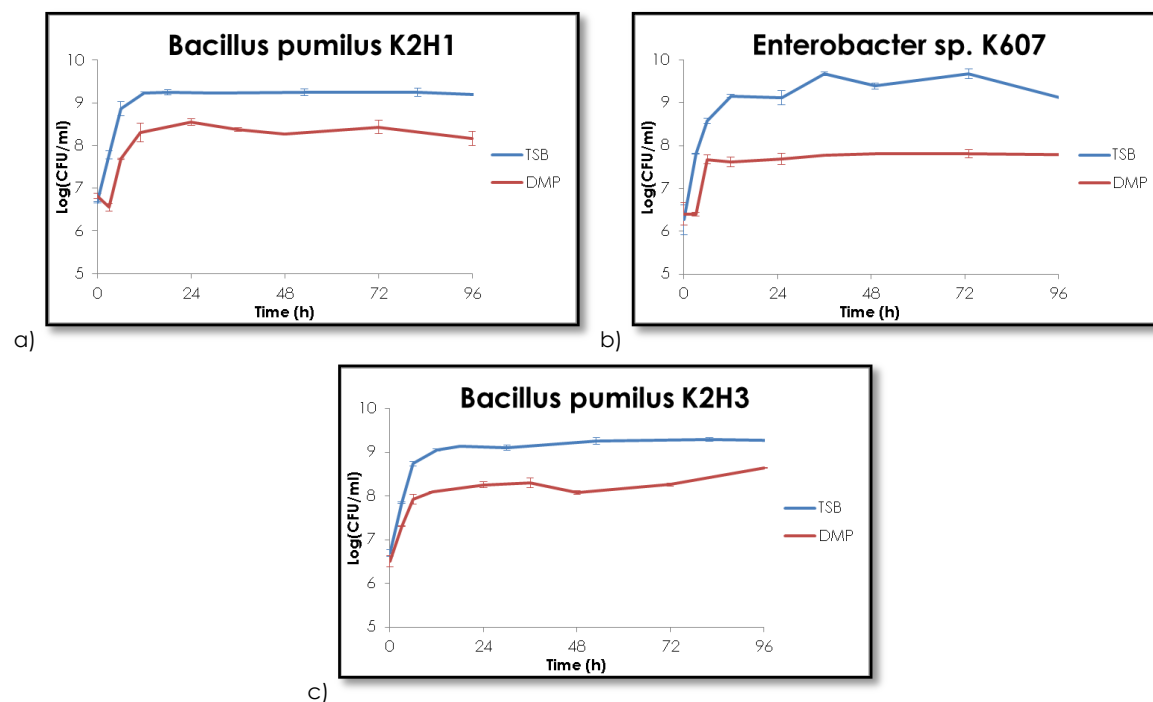
**Figure 50.** Inhibition of growth calculated on area of halo registered by RDA with tannic acid on different media. K2H1 *B. pumilus*, K2H2 *B. subtilis*, K2H3 *B. pumilus*, K2H7 *B. licheniformis*, and Bss: reference strain *Bacillus subtilis* subsp. *subtilis*. On graphs blue line = DMP medium, red line = DMP medium with 0,5 % Yeast extract and green line = Nutrient agar.

### 3.2.2.3. Growth curves

The most important isolates which showed highest pectinolytic activity (K2H1 *B. pumilus*, K2H2 *B. subtilis*, K2H3 *B. pumilus*, K2H7 *B. licheniformis*, and K607 *Enterobacter* sp.) were grown in minimal DMP and rich TSB media in order to compare each other for their ability to growth on pectin as unique source of carbon. All isolates showed a limited growth on pectin as unique source of carbon, suggesting less efficiency of growth on pectin due to high energy request to metabolism.

In particular, isolates response to pectin can be grouped in three main dynamics reassumed in figure 51. Isolates K2H1 and K2H2 showed an initial lag phase before the exponential growth may be due to the induction of pectin's metabolic pathway, on the other hand K2H3 and K2H7 seemed to constitutively express pectinolytic metabolic pathway. Moreover, isolate K607 showed an initial lag phase but also a lower biomass production on pectin (about 2 orders of magnitude) if compare with that registered with a rich culture medium (highest growth about  $10^{10}$  CFU/ml).

Particularly interesting resulted the difference in constitutively expression of pectinolytic pathway among isolates K2H1 and K2H3 belonging to the same specie: *B. pumilus*.



**Figure 51.** Examples of growth curves obtained from most important isolates with high pectinolytic activity. Isolates were grown on minimal DMP and rich TSB media.

### 3.2.3 Retting process in mesocosm trials

Isolates with highest pectinolytic activity and tannic acid resistance (K2H1 *B. pumilus*, K2H2 *B. subtilis*, K2H3 *B. pumilus*, K2H7 *B. licheniformis*, and K607 *Enterobacter* sp.) were tested in mesocosms trial. They were added as massive *inoculum* singularly in order to evaluate their specific contribution to retting process and eventually to find the best *inoculum* putatively exploited as starter.

Experimental mesocosm didn't represent exactly the bioreactor conditions (no aeration, no liquor recycling) but could be considered as worst conditions that could happen during maceration.

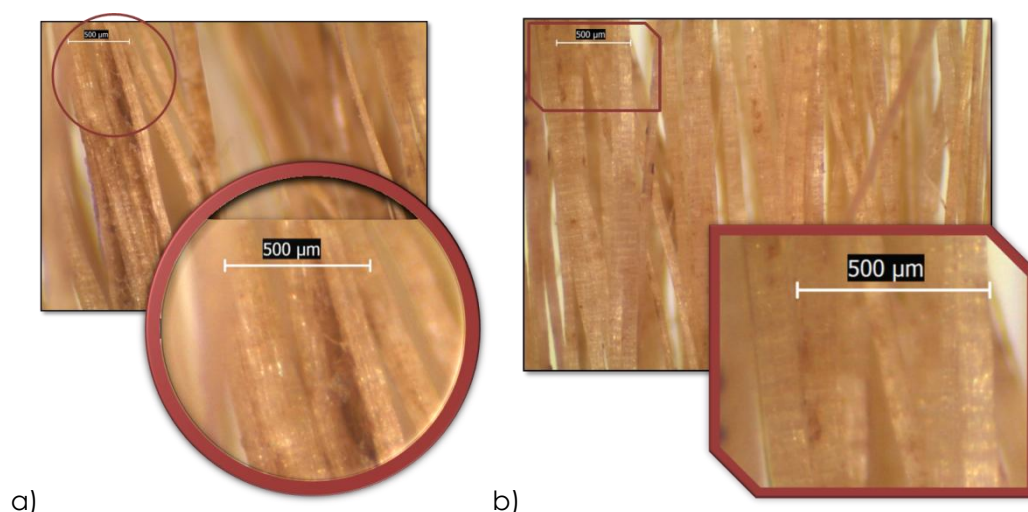
Maceration tests in mesocosm were repeated in triplicate for all tested isolates and compared with two control mesocosms (positive control and negative control).

Data obtained revealed that two mesocosms inoculated with K2H3 and K607 respectively didn't produced retted fibers in the tested time of retting (6 days) as the negative mesocosm containing non-inoculated bi-distilled water and kenaf ribbons. In particular K607 *inoculum* didn't survive in tested conditions and K2H3 needed more time to perform a good maceration.

On the other hand, other mesocosms performed a good maceration of kenaf ribbons similar or even better than that registered for the positive control containing the retting



liquor of bioreactor. Furthermore, these interesting retting results were confirmed by analyzing retted fibers on stereomicroscope as shown in figure 52.



**Figure 52.** Images obtained at stereomicroscope of retted fibers. a) unretted fibers still grouped in bundles from negative control, in evidence the presence of brown encrusting material among fibers. b) retted fibers from positive control with free single fibers.

Data obtained from loss of weight and stereomicroscope analysis indicate that K2H1 *B. pumilus* isolate performed the best maceration as the positive control and could be employed as starter *inoculum*. Data obtained for all mesocosm tests were reassumed in table 6.

Mesocosm	Encrusting material	Separation bundles of fibers	Separation of single fiber	Loss of weight (dry weight)	Final result
#MN	No	Yes	Yes	45%	Good maceration
#H2O	Yes	Partially	No	32%	Incomplete maceration
K2H1	No	Yes	Yes	47%	Good maceration
K2H2	Yes	Yes	Partially	38%	Partial maceration
K2H3	Yes	No	No	20%	Incomplete maceration
K2H7	Yes	Yes	Partially	30%	Partial maceration
K6O7	Yes	No	No	17%	Incomplete maceration

**Table 6.** Schematic table of results obtained by retting of kenaf ribbons in mesocosms inoculated with pectinolytic isolates and incubated at 37°C for 6 days.

### 3.3. Proteomic analyses

The technological capability of isolate K2H1 *B. pumilus* was investigated by analyzing its enzymatic activity and characterizing its pectinolytic enzymes.

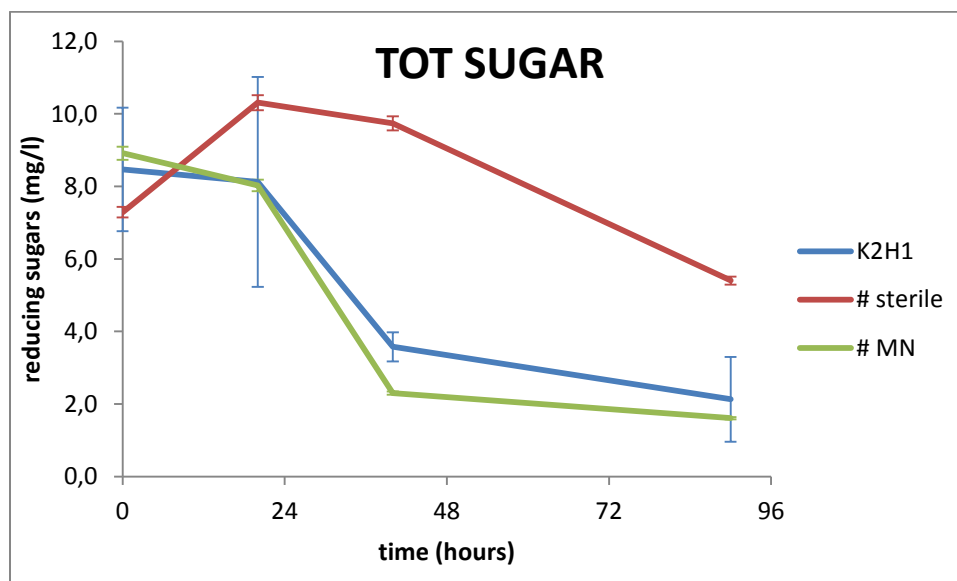
#### 3.3.1. Enzymatic activities in retting liquor

In order to better understand the technological capabilities of strain K2H1, its principal enzymatic activities were monitored during the maceration process in mesocosm.

The DNS method (2.6.1.) quantitatively monitored enzymatic activity towards principal polysaccharides of plant tissue: cellulose, starch, hemicellulose and pectin in order to assess which enzymatic activity took place comparing with the results with positive and negative controls.

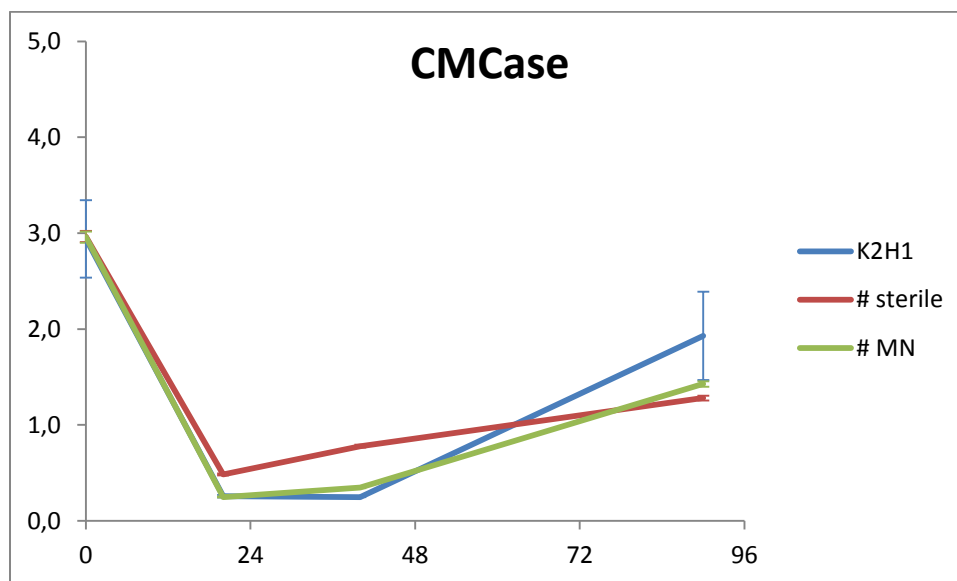
Moreover the total sugar content was monitored in order to understand the available of source of energy for microbial growth.

The figure 53 represents the total sugar content in retting liquor during the maceration. It was evident how in control mesocosm (#MN) and in mesocosm inoculated with K2H1 isolate the total sugar content decreased rapidly in comparing with to negative control mesocosm (#H<sub>2</sub>O). In fact, the initial sugar content (about 8 mg/l) decreased within first 24 hours and stabilized to low values (about 2 mg/l or less). Instead, in negative control the sugar content increased to maximum value (about 10 mg/l), indicating that sugar released by kenaf ribbons were consumed only in late phase when microbial population occurring on kenaf ribbons began to develop in sterile water.



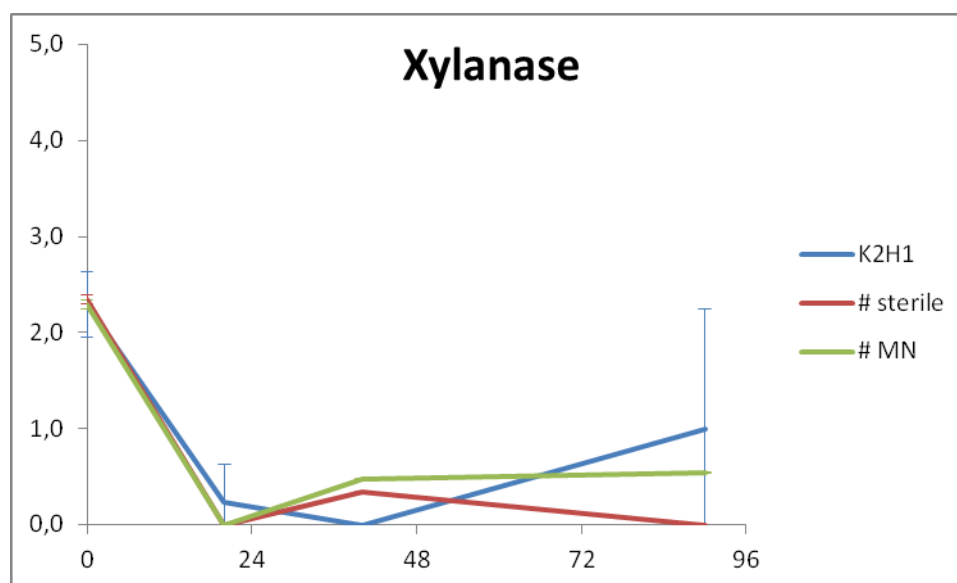
**Figure 53.** Graph of total sugar content calculated by measuring reducing ends by DNS method during maceration in mesocosms.

The carboxymethylcellulase activity monitored during retting in mesocosms showed as this enzymatic activity was similar in all mesocosms and increased in activity during late phase of the process when increased the over-retting risk with consequent damages on cellulose fibers (Figure 54).



**Figure 54.** Graph of carboxymethylcellulase activities monitored by DNS method during maceration in mesocosms.

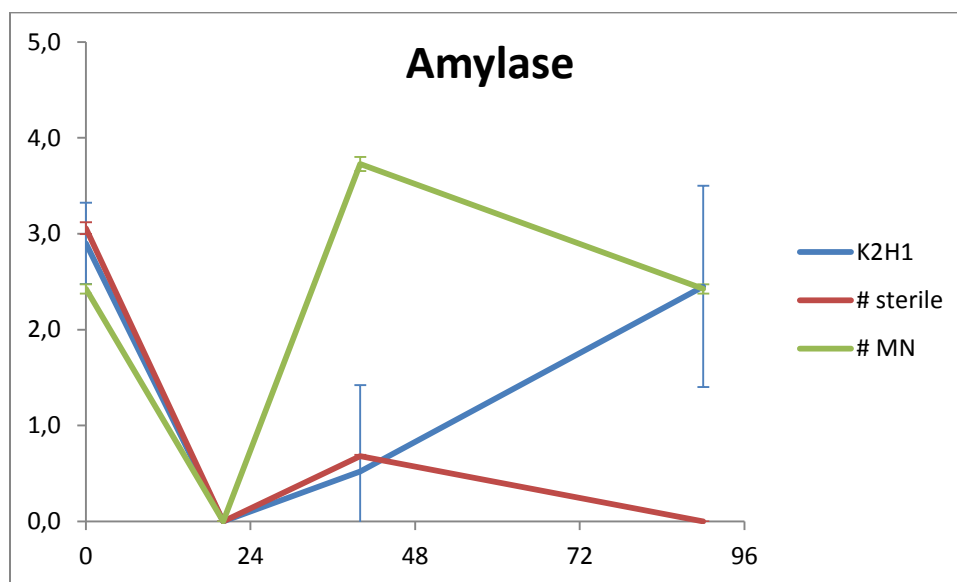
The xylanase activity registered the enzymatic activity towards hemicellulose present in cell wall of plant cell. The activity monitored during retting in mesocosms registered approximately the same values in all three condition tested, indicating a low activity during the process.



**Figure 55.** Graph of xylanase activities monitored by DNS method during maceration in mesocosms.

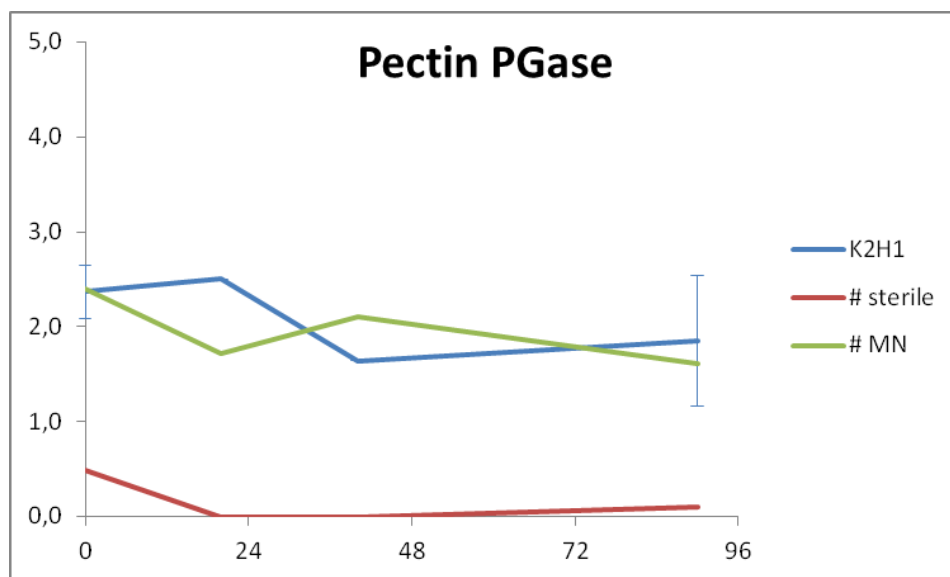
Data suggested a minor role of this enzymatic activity during the retting process in mesocosm, indicating the preservation of hemicellulose layer that protect fibers against cellulolytic activity (Figure 55).

Amylase activity monitored enzymatic activity towards starch present in plant tissue of kenaf ribbons during the maceration. In figure 56, it was evident that microbial populations present in #MN mesocosm (positive control) were more efficient in consume starch present in retting liquor registering a high enzymatic activity. Instead K2H1 isolate registered a high value of activity only during late phase of retting. Data suggested that during positive control maceration the release of starch present in kenaf ribbons began in early phase, instead in K2H1's mesocosm gradually increased during maceration.

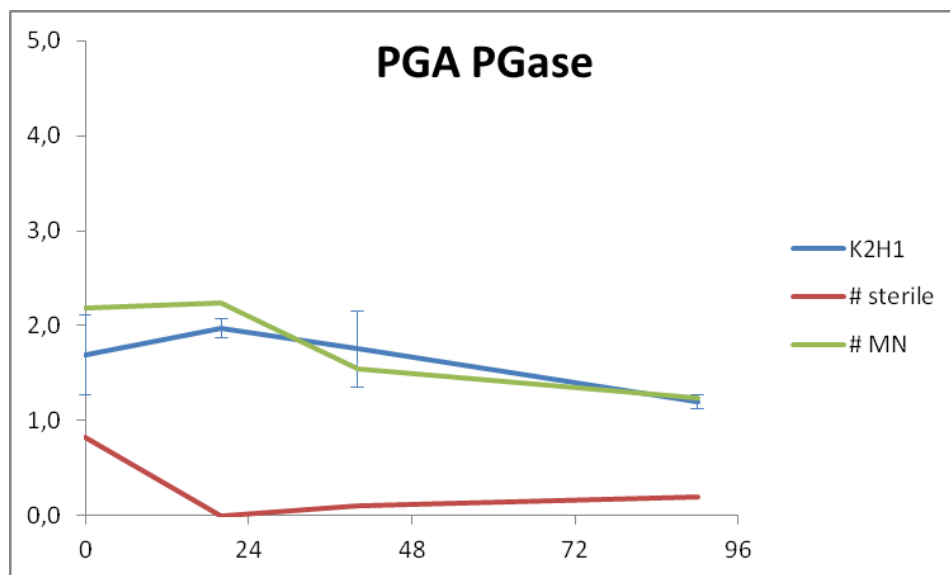


**Figure 56.** Graph of amylase activities monitored by DNS method during maceration in mesocosms.

At last, the pectinolytic activities during retting in mesocosms were monitored against pectin (complex substrate) and PGA (homopolymers of galacturonic acid and backbone of pectin). As shown in figures 57 and 58, pectinolytic activities were high and constant during retting in positive control and in mesocosm inoculated with K2H1, on the other hand the activity was low in negative control. Data confirmed that pectinolytic activity was main enzymatic activity important for retting kenaf ribbon. Moreover no differences were registered between two different substrate utilized in enzymatic assay, suggesting that enzymatic activity acted prevalent on pectin backbone composed prevalently by PGA. Reassuring the rapid decrease of registered activities towards cellulose, hemicellulose and starch in first 24 hours of all curves indicates the presence of constitutive enzyme expression by occurring microorganisms in all mesocosms suggesting that soluble polysaccharides were the primary font of energy for retting bacteria. Later on, the consumption of these rapidly available polysaccharides improved the expression of pectinases and the consequent attack of pectin for tissue maceration.



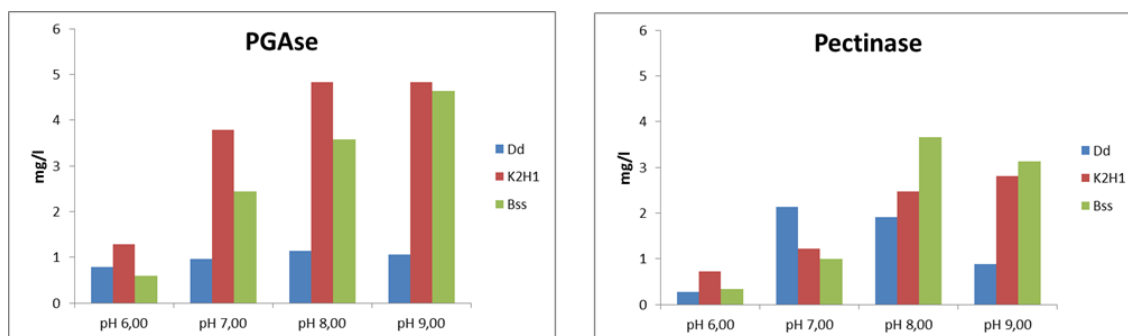
**Figure 57.** Graph of pectinolytic activities towards pectin monitored by DNS method during maceration in mesocosms.



**Figure 58.** Graph of pectinolytic activities towards pectin monitored by DNS method during maceration in mesocosms.

The increasing of cellulose activity in late phase of maceration evidenced the reduction of available pectin on fibers and indicated the beginning of over-retting phase suggesting the end of the retting process.

Moreover, in order to better characterize the pectinolytic activity of K2H1 isolate, its enzymatic activities against pectin and PGA were monitored at different pH conditions in DMP broth media (Figure 59). To better define its pectinolytic activities, data obtained were compared with enzymatic activity of two reference strains: *Dickeya dadantii* (Dd) and *Bacillus subtilis* subsp. *subtilis* (Bss).

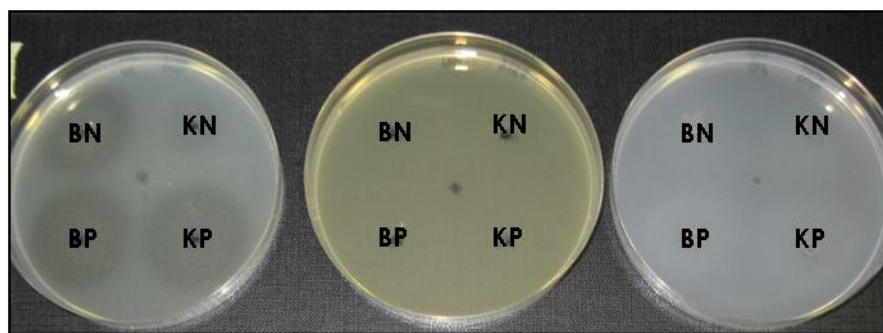


**Figure 59.** Graph of pectinolytic activities towards pectin (Pectinase) and PGA (PGAase) registered by DNS method at different pH conditions for K2H1 isolate and reference strains: Dd: *Dickeya dadantii* and Bss: *Bacillus subtilis* subsp. *subtilis*.

The pectinolytic activities of K2H1 isolate and *Bacillus subtilis* subsp. *subtilis* were higher than *Dickeya dadantii* at basic pH values. In particular, K2H1 registered highest enzymatic activity on PGA than pectin. Data are in according with alkaline pectynolitic enzyme production by *Bacillus* species, suggesting a crucial role to maintain liquor during retting in sub acidic level and consequently underlined the importance of air insufflation worked in bioreactor.

### 3.3.2. SDS-PAGE

The further characterization of enzymes produced by K2H1 and *Bacillus subtilis* subsp. *subtilis* was achieved by analyzing protein in surnatant culture in rich (Nutrient) and DMP media by SDS-PAGE, in order to better understand the differential expression of esoenzymes during growth of bacteria on pectin as unique source of carbon.

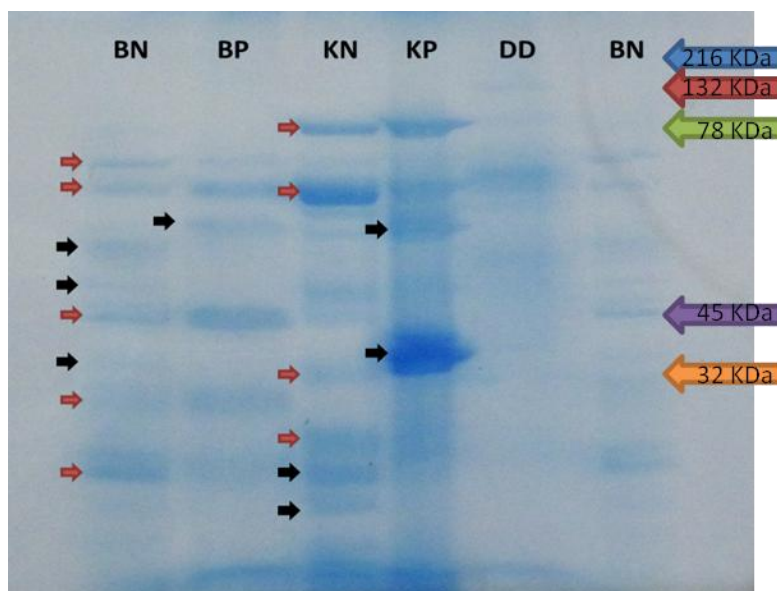


**Figure 60.** Results of plate enzymatic assays. On the left Peh assay (pectate lyase) with halos, in the middle Peh assay (polygalacturonase) and on thr right Cel assay (carboxymethylcellulase). BN: protein extract of *Bacillus subtilis* subsp. *subtilis* grown on Nutrient broth, BP: protein extract of *Bacillus subtilis* subsp. *subtilis* grown on DMP broth, KN: protein extract of K2H1 isolate grown on Nutrient broth, KP: protein extract of K2H1 isolate grown on DMP broth.

After a partial purification and concentration of protein in surnatant cultures on different media, principal enzymatic activities (pectate lyase, polygalacturonase and carboxymethylcellulase) of protein extracts were tested on plate assays (Figure 60).

The plate assays revealed pectinolytic activity on Peh plate assay in both protein extracts of *Bacillus subtilis* subsp. *subtilis* and only on protein extract of K2H1 grown on DMP broth, confirming that K2H1 needed to be induced by substrate to produce pectinolytic enzymes and the constitutive pectinolytic enzymes production of reference strain.

No activity was registered by all protein extracts in Peh and Cel palte assays, indicating the presence of only pectate lyase in protein extarcts.



**Figure 61.** SDS-PAGE of protein extracts with relative molecular weights. Red and black arrows indicate the bands present in different protein extracts. BN: protein extract of *Bacillus subtilis* subsp. *subtilis* grown on Nutrient broth, BP: protein extract of *Bacillus subtilis* subsp. *subtilis* grown on DMP broth, KN: protein extract of K2H1 isolate grown on Nutrient broth, KP: protein extract of K2H1 isolate grown on DMP broth, DD: protein extract of *Dickeya dadanti* grown on DMP broth.

SDS-PAGE analysis evidenced, as expected, how the different growth conditions altered the protein expression of bacteria tested, but, also, how different was the protein profiles among tested bacteria (Figure 61).

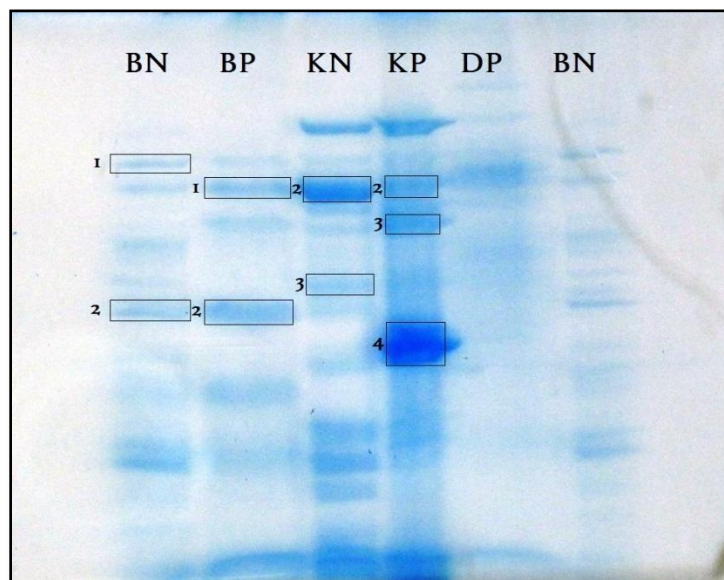
Moreover, K2H1 altered drastically protein profile after induction of pectin in DMP media (KN and KP lines). In particular, in KP line, a high intensity band with mass comprised between 32 and 45 kDa appeared.

### 3.3.3. Zymogram

Bands corresponding to pectinolytic enzymes in SDS-PAGE profiles were detected by zymogram rely on imprint technique. After SDS-PAGE, gels were washed in a detergent solution in order to renature proteins by removing SDS. Later on, gels were overlaid on Pel plate say in order to detect enzymatic activity. The position gels were marked on plates and the position of halo corresponding to enzymatic activity were accompanied to relative bands on SDS-PAGE profiles (Figure 62).



In the specific, in BN and BP lines belonging to protein extract of reference strain the activity was detected in bands numbered 2 (BN2 and BP2). On the other hand, in KP line pectinolytic activity was registered for band numbered 4 (KP4).



**Figure 62.** SDS-PAGE profiles indicating main bands of protein extracts. BN: protein extract of *Bacillus subtilis* subsp. *subtilis* grown on Nutrient broth, BP: protein extract of *Bacillus subtilis* subsp. *subtilis* grown on DMP broth, KN: protein extract of K2H1 isolate grown on Nutrient broth, KP: protein extract of K2H1 isolate grown on DMP broth, DD: protein extract of *Dickeya dadanti* grown on DMP broth.

### 3.3.4. Protein identification and sequencing

Main bands, indicated in figure 62, were spotted from gels and after digestion with trypsin analyzed by mass spectrometer in order to identify proteins present (Table 7).

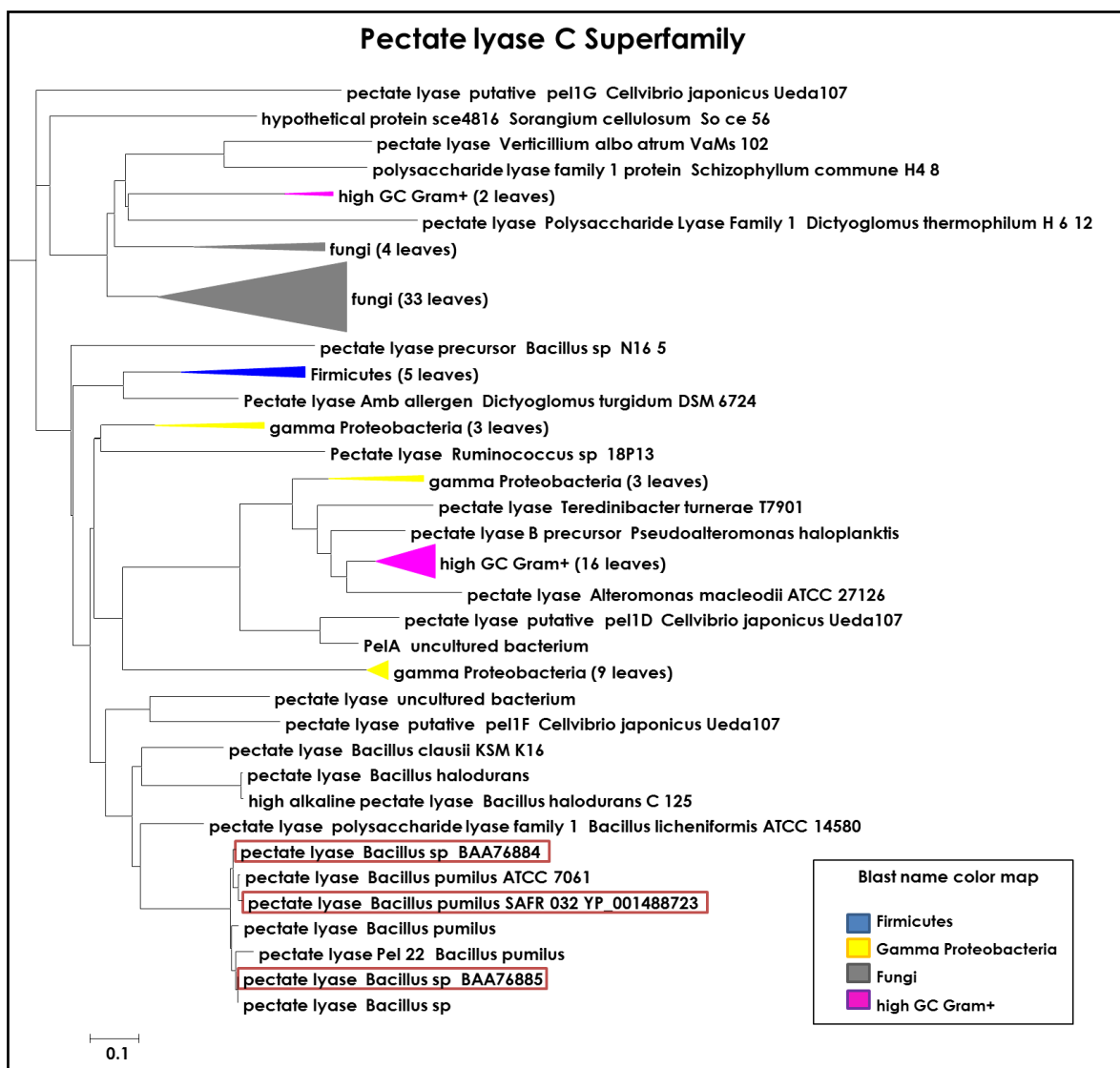
The analysis of spots revealed the presence of pectate lyases in BN2, BP2 and KP4 bands, confirming results of zymogram. Moreover, theoretical mass weights of pectate lyases were approximately in accordance with experimental data. In the specific, tested bacteria produced different pectate lyases. In fact, in spot of KP3 band was detected a pectate lyase very similar to BP2 band (NCBI accession number gi | 4589751), suggesting that both bacteria possess a common pectate lyase gene. But, in spot of KP4 band (the most intensive band of the line) was detected a different pectate lyase (NCBI accession number gi | 4589753) specific of K2H1 isolates.

In particular, the fragment of pectate lyase identified in spot KP4 resulted similar to three different pectate lyases (BAA76885 *Bacillus* sp., #YP\_001488723 *Bacillus pumilus* SAFR-032, #BAA76884 *Bacillus* sp.). Further analysis of the sequences of fragments and of three pectate lyases revealed that all enzymes analyzed belonged to Pectate lyase C superfamily (Figure 63) and were almost identical differing only for a few residues (Figure 64). Briefly, the analysis of sequences indicated that K2H1 produce two pectate lyases: one similar to which produced by *Bacillus subtilis* subsp. *subtilis* of about 33 kDa and a specific pectate

lyase C similar to three different pectate lyase produce by *Bacillus* sp. and *B. pumilus* strains of about 37 kDa.

Protein name	Gene name	Spot (Fig. 61)	NCBI acc.	Mr. (Da)	pI	No. of peptides identified	Mascot score	Sequence Coverage (%)
Alpha-amylase	amy	BN1	gi 6502575	72276	5.66	8	326	16%
Pz-peptidase		BN1	gi 1651216	72355	5.50	3	172	5%
leucyl aminopeptidase	yuiE	BN1	gi 16080258	53624	4.76	3	84	7%
Flagellin	hag	BN2	gi 16080589	32607	4.97	7	317	32%
Spore peptidoglycan hydrolase	yaaH	BN2	gi 16077084	48607	5.72	4	98	11%
Membrane associated protein kinase with beta propeller domain	yxal	BN2	gi 50812309	43850	6.33	4	97	11%
Oligopeptide ABC transporter binding lipoprotein	oppA	BP1	gi 16078208	61453	5.83	12	429	25%
Vegetative catalase	Kat-19+	BP1	gi 142861	54797	6.07	6	224	15%
Flagellin	Hag	BP2	gi 16080589	32607	4.97	7	469	36%
Pectate lyase	pelC	BP2	gi 4589751	33336	8.98	3	106	13%
Dihydrolipoamide dehydrogenase	pdhD	KN2	gi 157692139	49547	4.95	11	621	33%
Oligopeptide-binding protein OppA	oppA	KN2	gi 194014339	61151	7.03	10	358	18%
Catalase		KN2	gi 194014488	56734	5.45	4	144	9%
Oligopeptide ABC transporter substrate-binding protein	appA	KN2	gi 157691844	61655	5.00	2	70	5%
Glycosyl hydrolase	ynfF	KN3	gi 157692517	47471	9.11	4	204	11%
Flagellin	Hag3	KN3	gi 157691932	32200	5.04	3	153	12%
Gamma-glutamyltransferase		KN3	gi 194014918	63244	4.98	3	115	9%
Oligopeptide-binding protein OppA	oppA	KP2	gi 194014339	61151	7.03	13	393	28%
Dihydrolipoamide dehydrogenase	pdhD	KP2	gi 157692139	49547	4.95	8	310	24%
Flagellin	hag	KP2	gi 16080589	32607	4.97	6	176	32%
ABC transporter ATP-binding protein	yticQ	KP3	gi 157693421	56699	5.81	13	592	26%
Pectate lyase	pelC	KP3	gi 4589751	33336	8.98	3	90	13%
Oligopeptide-binding protein OppA		KP3	gi 194014339	61151	7.03	1	61	2%
Pectate lyase	pelC	KP4	gi 4589753	37628	9.18	9	320	26%
Flagellin	hag3	KP4	gi 157691932	32200	5.04	3	168	13%
ABC transporter ATP-binding protein	yticQ	KP4	gi 157693421	56699	5.81	3	104	7%
Dihydrodipicolinate synthase	dapA 2	KP4	gi 157692361	30544	5.69	3	101	11%
ABC transporter		KP4	gi 56421742	47081	5.74	1	63	4%

**Table 7.** Results of sequencing and identification of spot of main bands in SDS-PAGE profile retrieved using the MS/MS ion search of Mascot against all entries of the non-redundant NCBI database.



**Figure 63.** Phylogenetic tree obtained comparing 100 enzymes similar to pectate lyase in spot KP4 retrieved with BLASTp program of NCBI (Altschul *et al.*, 1990) with following parameters: tree method Fast Minimum Evolution; max distance=0.85; distance=Grishin

#BAA76885_Bacillus_sp.	M	L	T	L	K	R	K	V	P	M	L	A	G	V	L	A	V	G	L	V	T	S	L	F	A	P	N	G	E	A	K	A	A	A	K	F	P	E	S	P	[ 40]
#YP_001488723_Bacillus_pumilus_SAFR-032	-	-	-	M	K	K	K	V	P	M	L	A	G	V	L	A	V	G	L	V	T	S	L	F	A	P	N	G	E	A	K	A	A	A	K	Y	P	E	S	P	[ 40]
#BAA76884_Bacillus_sp.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	[ 40]	
#BAA76885_Bacillus_sp.	S	I	M	A	N	F	N	Q	Q	G	F	S	T	L	N	G	G	T	T	G	G	E	G	G	K	T	V	T	V	K	T	G	N	E	L	L	A	A	L	K	[ 80]
#YP_001488723_Bacillus_pumilus_SAFR-032	S	I	M	A	N	F	N	Q	Q	G	F	S	T	L	N	G	G	T	T	G	G	E	G	G	K	T	V	T	V	K	T	G	N	E	L	L	A	A	L	K	[ 80]
#BAA76884_Bacillus_sp.	-	-	-	A	N	F	N	Q	Q	G	F	S	T	L	N	G	G	T	T	G	G	E	G	G	K	T	V	T	V	K	T	G	N	E	L	L	A	A	L	K	[ 80]
#BAA76885_Bacillus_sp.	N	K	G	T	N	E	K	L	K	I	V	V	D	G	T	I	T	P	S	N	T	S	A	N	K	I	D	V	K	D	T	N	N	V	S	I	V	G	K	G	[120]
#YP_001488723_Bacillus_pumilus_SAFR-032	S	K	G	K	N	E	K	L	K	I	V	V	D	G	T	I	T	P	S	N	T	S	A	N	K	I	D	V	K	D	T	N	N	V	S	I	V	G	K	G	[120]
#BAA76884_Bacillus_sp.	S	K	G	T	N	E	K	L	K	I	V	V	D	G	T	I	T	P	S	N	T	S	A	N	K	I	D	V	K	D	T	N	N	V	S	I	V	G	K	G	[120]
#BAA76885_Bacillus_sp.	T	N	G	E	F	N	G	I	G	I	K	V	W	R	A	N	N	I	I	I	R	N	L	K	I	H	H	S	K	I	G	D	K	D	A	I	G	I	E	G	[160]
#YP_001488723_Bacillus_pumilus_SAFR-032	T	K	G	E	F	N	G	I	G	I	K	V	W	R	A	N	N	I	I	I	R	N	L	K	I	H	H	S	K	I	G	D	K	D	A	I	G	I	E	G	[160]
#BAA76884_Bacillus_sp.	T	N	G	E	F	N	G	I	G	I	K	V	W	R	A	N	N	V	I	I	R	N	L	K	I	H	H	S	K	I	G	D	K	D	A	I	G	I	E	G	[160]
#BAA76885_Bacillus_sp.	G	S	K	N	I	W	V	D	H	N	E	L	Y	N	T	L	N	S	G	K	D	D	Y	D	G	L	F	D	V	K	N	D	S	D	Y	I	T	F	S	W	[200]
#YP_001488723_Bacillus_pumilus_SAFR-032	A	S	K	N	V	W	V	D	H	N	E	L	Y	N	T	L	N	S	G	K	D	D	Y	D	G	L	F	D	V	K	N	D	S	D	Y	I	T	F	S	W	[200]
#BAA76884_Bacillus_sp.	A	S	K	N	V	W	V	D	H	N	E	L	Y	N	T	L	N	S	D	K	D	D	Y	D	G	L	F	D	V	K	N	D	S	D	Y	I	T	F	S	W	[200]
#BAA76885_Bacillus_sp.	N	Y	V	H	D	S	W	K	T	M	L	M	G	S	S	D	N	D	N	Y	N	R	K	I	T	F	H	N	N	R	F	E	N	L	N	S	R	V	P	S	[240]
#YP_001488723_Bacillus_pumilus_SAFR-032	N	Y	V	H	D	S	W	K	T	M	L	M	G	S	S	D	N	D	N	Y	N	R	K	I	T	F	H	N	N	R	F	E	N	L	N	S	R	V	P	S	[240]
#BAA76884_Bacillus_sp.	N	Y	V	H	D	S	W	K	T	M	L	M	G	S	S	D	N	D	N	Y	N	R	K	I	T	F	H	N	N	R	F	E	N	L	N	S	R	V	P	S	[240]

#BAA76885_Bacillus_sp.	M	R	F	G	E	G	H	V	Y	N	N	Y	Y	K	G	I	L	T	T	A	I	N	S	R	M	G	A	K	M	R	I	E	H	N	V	F	E	N	T	N	[280]		
#YP_001488723_Bacillus_pumilus_SAFR-032	M	R	F	G	E	G	H	V	Y	N	N	Y	Y	K	G	I	H	T	T	A	I	N	S	R	M	G	A	K	M	R	I	E	H	N	V	F	E	N	T	K	[280]		
#BAA76884_Bacillus_sp.	M	R	F	G	E	G	H	V	Y	N	N	Y	Y	K	N	I	L	T	T	A	I	N	S	R	M	G	A	K	M	R	I	E	H	N	V	F	E	N	T	K	[280]		
#BAA76885_Bacillus_sp.	N	A	I	G	S	W	D	S	S	Q	V	G	T	W	H	V	I	N	N	S	Y	I	N	S	T	G	S	L	P	T	S	S	T	G	T	Y	N	P	P	Y	[320]		
#YP_001488723_Bacillus_pumilus_SAFR-032	N	A	I	G	S	W	D	S	R	Q	V	G	T	W	H	V	I	N	N	S	Y	I	N	S	S	G	S	L	P	T	S	S	T	G	T	Y	N	P	P	Y	[320]		
#BAA76884_Bacillus_sp.	N	A	I	G	S	W	D	S	R	Q	V	G	T	W	H	V	I	N	N	S	Y	I	N	S	T	G	S	L	P	T	S	S	T	G	T	Y	N	P	P	Y	[320]		
#BAA76885_Bacillus_sp.	N	Y	S	L	L	N	V	N	N	V	K	S	E	V	I	S	N	A	G	V	G	K	V	N	P																		[345]
#YP_001488723_Bacillus_pumilus_SAFR-032	N	Y	S	L	L	N	V	N	N	V	K	S	E	V	L	S	N	A	G	V	G	K	V	N	P																		[345]
#BAA76884_Bacillus_sp.	N	Y	S	L	L	N	V	N	N	V	K	S	E	V	V	S	N	A	G	V	G	K	V	N	P																		[345]

**Figure 64.** Sequence alignment of protein obtained by MEGA version 4 (Tamura *et al.*, 2007) with protein fragments (orange squares) obtained in MS/MS analyses and Mascot search in NCBI database. In table red letters indicate conserved sites in protein sequences.



## **4. Conclusions**

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Kenaf (*Hibiscus cannabinus* L.) bast fiber offers the advantage of being biodegradable, renewable, environmentally safe, and have been traditionally used in Africa as a source of fiber for making clothing, rugs, rope and other products. Among bast fiber crops kenaf is the most promising plant for production and extraction of natural fibers due to:

- High biomass production relying on the best photosynthetic apparatus with highest fixation rate of CO<sub>2</sub>;
- Easy cultivation and adaptation to diverse latitude and sun exposition;
- Low pesticide and weed control during cultivation;
- Good quality and quantity of bast fibers production suitable for many industrial purposes.

Studies have been done with various methods of processing in order to extract kenaf bast fibers and these fibers have been evaluated for their chemical or physical properties.

In the recent years, kenaf cultivation has been increased and has been subject of numerous research projects concerning environmental issues and prevalently paper resource in many countries (i.e. U.S.A.). These studies underlined the great value of diversified biodegradable woven and nonwoven fabrics made from kenaf bast fibers.

Moreover, the process of extraction of fibers, a valid principle for all fiber crops not only for kenaf (i.e. hemp, flax, and jute), is of great importance, since the quality as well as the quantity of extracted fibers is strongly influenced by the methods of extraction employed.

Retting of kenaf is a kind of fermentation process in which the cortical and phloem tissues of the bark of the plants containing free strands are decomposed to separate fiber from non-fibrous woody stem. The fermentative microorganisms consume the cementing materials (pectins, hemicelluloses and proteins) with release of galacturonic acid and sugar in retting water. As a consequence pH of retting water drops and Eh reaches a highly negative value. All these observations indicate that traditional water retting is a strictly anaerobic process which causes environmental pollution problems. However, most of the problems of kenaf retting can be minimized through ribbon retting. Ribbon retting reduces:

- the requirement of water;
- the length of retting time and
- the level of environmental pollution to almost one-fourth

in comparison to that of whole plant retting. Besides, ribbon retting assured the production of better quality kenaf fiber in terms of fiber strength, fineness, color, and overall absolutely bark free kenaf fiber.

Moreover, the ribbon retting of kenaf in bioreactor will assure industrial production of kenaf bast fibers necessary to overcome the increasing demand of raw material for woven and nonwoven fabrics registered in last decades.

In this work, in collaboration with K.E.F.I. S.p.A., a pilot plant bioreactor was developed to perform aerated ribbon retting of kenaf in order to assess the main parameters driving the aerobic ribbon retting of kenaf and to decipher the microbial community responsible for the kenaf degumming process. Moreover, bacterial strains with high pectinolytic activity, isolated from retting liquor were studied in order to candidate a possible starter *inoculum* capable of improving the retting process in a bioreactor.

The results obtained can be summarized in the following points:

- The controlled environment provided to establish constant and reproducible conditions during maceration and to obtain an efficient method to extract bast fibers transferable to industrial scale. The optimum retting conditions delineated during this study resulted in attaining high quality bast fibers from different variety of kenaf (Everglade, Tainung) independently from their storing conditions (green ribbon, air dried ribbon and woven dried ribbon).



- The aerated ribbon retting in bioreactor improved and stabilized the development of retting microorganisms preventing the development of anaerobic environment and reducing the over-retting risk. The increasing phenolic content of retting liquor indicated a modification of lignin in bast fibers reducing stiffness and coarseness of retted fibers. The air insufflation, moreover, seemed to reduce abiotic and toxic effects of phenolic compounds on retting microflora.
- The retted fibers obtained after five days of maceration in bioreactor satisfied quality and quantity requirements for industrial production of raw material. Eventually, evaluation of the quality of retted fibers registered good yield and good tenacity. In particular, the analysis of fibers composition revealed the presence of changes in pectin and lignin peaks with constant cellulose composition. These data confirmed that a good retting process had took place in the tested conditions.
- The bioreactor location resulted as a perfect environment in order to assess and decipher the microbial community involved in degumming bast fibers.
- DGGE analysis evidenced the presence of recurring species in both kind of loading conditions. The major bands observed in the V3 DGGE profiles resulted to be related to *Proteobacteria* and *Clostridium* species (early phase of the process) afterwards *Bacillus licheniformis* took place (late retting phase). On the other hand, the major bands in the BK-1 DGGE profiles resulted to be related to *B. koreensis*, *B. oleronius* and *B. subtilis* (early phase) and subsequently to *B. licheniformis* (late phase). Moreover DGGE profiles showed that the presence of *Clostridium* strains was negatively affected by air insufflation but not totally depleted.
- Microbial growth analyses evidenced no differences in the two different culture conditions adopted (aerobiosis and anaerobiosis) suggesting a dominance of facultative anaerobic microflora in all tested media. Moreover, microbial growth kinetics showed a rapid transition of an initial aspecific total microflora into pectinolytic bacteria. Spore-forming bacteria growths were low than that observed for the other populations. Nevertheless these former registered an increase of three orders of magnitude on plant fibers. These data suggested a great importance of spore-forming bacteria in the depectination of cellulose fibers.
- The analysis of the bacteria composition by culture dependent approach evidenced the presence of two distinct populations. On one hand the spore-forming bacteria, which are mainly represented by *Bacillus pumilus*, *Bacillus subtilis* and *Bacillus licheniformis* isolates. On the other hand, no spore-forming bacteria are all related to the *Gamma Proteobacteria*, mainly represented by *Enterobacter* species such as *Pantoea* sp. and *Klebsiella pneumoniae*. These results are in according with those obtained from culture independent approach. Nevertheless *Clostridia* were not detected. Pectinolytic isolates were tested for some metabolic properties: CTAB assay registered esopectinase production and gave positives results for four of the seven *Bacillus* strains tested; meanwhile, only one of the nine *Proteobacteria* isolates gave the same positive results. These evidences underlined once again the enhanced pectinolytic capabilities of *Bacillus* species.
- RDA with acid tannic revealed that phenolic compounds as tannic acid influenced negatively the presence of *Bacillus* species, meanwhile, didn't alter the development of *gamma-Proteobacteria*.
- High pectinolytic isolates were detected and identified and prevalently belonged to spore forming bacteria: K2H1 *B. pumilus*, K2H2 *B. subtilis*, K2H3 *B. pumilus*, K2H7 *B. licheniformis*, and K607 *Enterobacter* sp.. Studies of their physiological and metabolic properties indicated that a starter *inoculum* will provide to reduce the

time of retting. In fact, growth curves showed a lag phase necessary to the adaptation prior the growth of bacteria on pectin. For this reason the introduction of a massive *inoculum* of high pectinolytic bacteria pre-induced with pectin will result in shortening retting time of about one day or more.

- Studies with massive *inocula* of high pectinolytic bacteria in mesocosms identified the strain K2H1 *B. pumilus*, as a good candidate for a starter *inoculum*. The advantage of this strain was to perform by itself a good maceration in 6 days without aeration and shaking. Clearly, the technological capabilities of this strain were underestimated in a mesocosm environment and predictably in bioreactor environment supported by aeration and liquor's recycling will improve its performances.
- The characterization of K2H1 *B. pumilus* enzymatic activities during maceration in relation to positive and negative references underlined and confirmed the importance of pectinolytic activity in retting bast fibers. Moreover, pectinolytic enzymes produced by K2H1 *B. pumilus* were isolate and identified by SDS-PAGE analysis coupled by zymography.
- MS/MS analysis of protein produced by K2H1 *B. pumilus* when grown on pectin as unique source of carbon revealed the production of two kind of pectate lyase: one similar to which produced by *Bacillus subtilis* subsp. *subtilis* of about 33 kDa and a specific pectate lyase C similar to three different pectate lyase produce by *Bacillus* sp. and *B. pumilus* strains of about 37 kDa.
- Pectate lyase attacks preferentially the backbone of pectin the polygalacturonic acid (PGA) resulting in disrupting the complexity of polysaccharides in small soluble units.

Considering the interesting results obtained in this study it is possible to formulate the following future perspectives:

- The technological capabilities of K2H1 *B. pumilus* strains must be confirmed in pilot plant bioreactor. An implementation of aerated ribbon retting will be foreseeable by introducing K2H1 *B. pumilus* as starter *inoculum* in term of shortening the time of retting and maintaining high quality of resulting fibers.
- A complete sequencing and characterization of pectate lyases produced by K2H1 *B. pumilus* must be achieved to better understand their role in depectination of bast fibers. Specific studies on enzymes involved in the maceration process will help to improve base knowledge on pectinolytic enzymes, to discover new kind of microbial pectinolytic enzymes with other industrial application, and - in long term future perspective - to realize enzymatic retting of kenaf.

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## **6. Appendix**

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### 6.1. Appendix of principal phytoextract obtained by kenaf (*Hibiscis cannabinus*)

Position	Molecule	Reference	Position	Molecule	Reference
Leaf	2,4-E-E-hexadienal	(Kobaisy <i>et al.</i> , 2001)	Leaf	geranyl-acetone	(Kobaisy <i>et al.</i> , 2001)
Stem	2,5-dimethyl-3-O-beta-D-glucopyranosyl-naphthol	(Seca <i>et al.</i> , 2001a)	Leaf	globulol	(Kobaisy <i>et al.</i> , 2001)
Leaf	2-acetylfuran	(Kobaisy <i>et al.</i> , 2001)	Stem	glutininol	(Seca <i>et al.</i> , 2000)
Leaf	2-nonanol	(Kobaisy <i>et al.</i> , 2001)	Stem	grossamide	(Seca <i>et al.</i> , 2001a)
Stem	3-OH-3-methoxy-4-OH-propiofenone	(Seca <i>et al.</i> , 2001b)	Stem	grossamide-K	(Seca <i>et al.</i> , 2001a)
Seed	3-OH-alpha-calacorene	(Stipanovic <i>et al.</i> , 2006)	Stem	guaiacyl-beta-aryl	(Kuroda <i>et al.</i> , 2002)
Leaf	3-octanon	(Kobaisy <i>et al.</i> , 2001)	Stem	guaiacyl-lignine	(Nishimura <i>et al.</i> , 2002)
Leaf	3-p-menthene	(Kobaisy <i>et al.</i> , 2001)	Stem	hibiscanal	(Bell <i>et al.</i> , 1998)
Leaf	4-keto-isophorone	(Kobaisy <i>et al.</i> , 2001)	Leaf	indole	(Kobaisy <i>et al.</i> , 2001)
Leaf	5-methyl-furfural	(Kobaisy <i>et al.</i> , 2001)	Leaf	isobutanol	(Osman <i>et al.</i> , 1976)
Leaf	6-methyl-5-epiten-2-one	(Kobaisy <i>et al.</i> , 2001)	Stem	kaempferol-3,7-rhamnoxyde	(Seca <i>et al.</i> , 2001a)
Leaf	acetophenone	(Kobaisy <i>et al.</i> , 2001)	Stem	keto-pinoresinol	(Seca <i>et al.</i> , 2001b)
Stem	3,4-dimethoxy-cinnamic acid	(Kuroda <i>et al.</i> , 2002)	Leaf	kusimone	(Kobaisy <i>et al.</i> , 2001)
Stem	4-methoxy-cinnamic acid	(Kuroda <i>et al.</i> , 2002)	Stem	lariciresinol	(Seca <i>et al.</i> , 2001a)
Seed	cis-12-13-epoxy-oleic acid	(Mohamed <i>et al.</i> , 1995)	Leaf	limonene	(Osman <i>et al.</i> , 1976)
Stem	docosanoic acid	(Seca <i>et al.</i> , 2000)	Seed	lyso-p-coline	(Mohamed <i>et al.</i> , 1995)
Stem	eicosanoic acid	(Seca <i>et al.</i> , 2000)	Seed	lyso-p-inositol	(Mohamed <i>et al.</i> , 1995)
Seed	phosphatidic acid	(Mohamed <i>et al.</i> , 1995)	Stem	lupeol	(Seca <i>et al.</i> , 2000)
Stem	lauric acid	(Seca <i>et al.</i> , 2000)	Leaf	m-mentha-1-7,8-diene	(Kobaisy <i>et al.</i> , 2001)
Seed	linoleic acid	(Mohamed <i>et al.</i> , 1995)	Leaf	m-tolualdehyde	(Kobaisy <i>et al.</i> , 2001)
Stem	linoleic acid	(Seca <i>et al.</i> , 2000)	Stem	medioresinol	(Seca <i>et al.</i> , 2001b)
Stem	myristic acid	(Seca <i>et al.</i> , 2000)	Leaf	methyl-hexadecaonate	(Kobaisy <i>et al.</i> , 2001)
Seed	oleic acid	(Mohamed <i>et al.</i> , 1995)	Stem	motiol	(Seca <i>et al.</i> , 2000)
Stem	oleic acid	(Seca <i>et al.</i> , 2000)	Stem	motiol	(Seca <i>et al.</i> , 2000)
Seed	palmitic acid	(Mohamed <i>et al.</i> , 1995)	Flower	myricetin	(Pakudina <i>et al.</i> Maksudova, 1977)
Stem	palmitic acid	(Seca <i>et al.</i> , 2000)	Leaf	myrtenol	(Kobaisy <i>et al.</i> , 2001)
Stem	palmitoleic acid	(Seca <i>et al.</i> , 2000)	Flower	myrtillin	(Rakhimkhanov <i>et al.</i> , 1974)
Stem	pentacosanoic acid	(Seca <i>et al.</i> , 2000)	Seed	n-acyl-iso-p-ethanolamine	(Mohamed <i>et al.</i> , 1995)
Stem	pentadecanoic acid	(Seca <i>et al.</i> , 2000)	Seed	n-acyl-p-ethanolamine	(Mohamed <i>et al.</i> , 1995)
Stem	stearic acid	(Seca <i>et al.</i> , 2000)	Leaf	n-decanal	(Kobaisy <i>et al.</i> , 2001)
Stem	tetracosanoic acid	(Seca <i>et al.</i> , 2000)	Leaf	n-nonadecane	(Kobaisy <i>et al.</i> , 2001)



Position	Molecule	Reference	Position	Molecule	Reference
Stem	tricosanoic acid	(Seca <i>et al.</i> , 2000)	Leaf	<i>n</i> -nonanal	(Kobaisy <i>et al.</i> , 2001)
Stem	uncosanoic acid	(Seca <i>et al.</i> , 2000)	Leaf	<i>n</i> -octan	(Kobaisy <i>et al.</i> , 2001)
Stem	uronic acid	(Neto <i>et al.</i> , 1996); (Nacos <i>et al.</i> , 2006)	Leaf	<i>n</i> -octanol	(Kobaisy <i>et al.</i> , 2001)
Leaf	<i>alpha</i> -bisababol oxide-B	(Kobaisy <i>et al.</i> , 2001)	Leaf	<i>n</i> -pentacosan	(Kobaisy <i>et al.</i> , 2001)
Leaf	<i>alpha</i> -calacoren	(Kobaisy <i>et al.</i> , 2001)	Leaf	<i>n</i> -tetracosan	(Kobaisy <i>et al.</i> , 2001)
Leaf	<i>alpha</i> -terpenyl-acetate	(Osman <i>et al.</i> , 1976)	Stem	<i>n</i> -trans-feruloil-tyramine	(Seca <i>et al.</i> , 2001a), (Seca <i>et al.</i> , 2001b)
Leaf	<i>alpha</i> -terpinene-7-al	(Kobaisy <i>et al.</i> , 2001)	Leaf	<i>n</i> -tricosan	(Kobaisy <i>et al.</i> , 2001)
Leaf	<i>alpha</i> -terpineol	(Kobaisy <i>et al.</i> , 2001)	Stem	nonacosan	(Seca <i>et al.</i> , 2000)
Leaf	allo-aromadendrene	(Kobaisy <i>et al.</i> , 2001)	Seed	o-hibiscanone	(Stipanovic <i>et al.</i> , 2006)
Leaf	benzene acetaldehyde	(Kobaisy <i>et al.</i> , 2001)	Stem	o-hibiscanone	(Bell <i>et al.</i> , 1998)
Leaf	benzyl-benzoate	(Kobaisy <i>et al.</i> , 2001)	Stem	octacosanyl-eicsonoate	(Seca <i>et al.</i> , 2000)
Leaf	<i>beta</i> -cadinene	(Kobaisy <i>et al.</i> , 2001)	Stem	octacosanol	(Seca <i>et al.</i> , 2000)
Leaf	<i>beta</i> -camigrene	(Kobaisy <i>et al.</i> , 2001)	Leaf	<i>p</i> -cimenene	(Kobaisy <i>et al.</i> , 2001)
Leaf	<i>beta</i> -E-ionone	(Kobaisy <i>et al.</i> , 2001)	Seed	<i>p</i> -coline	(Mohamed <i>et al.</i> , 1995)
Leaf	<i>beta</i> -pinene oxide	(Kobaisy <i>et al.</i> , 2001)	Seed	<i>p</i> -ethanolamine	(Mohamed <i>et al.</i> , 1995)
Seed	<i>beta</i> -sitosterol	(Mohamed <i>et al.</i> , 1995)	Seed	<i>p</i> -glycerol	(Mohamed <i>et al.</i> , 1995)
Stem	<i>beta</i> -sitosterol	(Seca <i>et al.</i> , 2000)	Seed	<i>p</i> -inositol	(Mohamed <i>et al.</i> , 1995)
Stem	boehmenan	(Seca <i>et al.</i> , 2001b)	Leaf	<i>p</i> -mentha-1,5-dien-8-ol	(Kobaisy <i>et al.</i> , 2001)
Stem	boehmenan-D	(Seca <i>et al.</i> , 2001b)	Stem	<i>p</i> -OH-benzaldehyde	(Seca <i>et al.</i> , 2001b)
Stem	boehmenan-H	(Seca <i>et al.</i> , 2001b)	Stem	<i>p</i> -OH-phenil-lignine	(Nishimura <i>et al.</i> , 2002)
Stem	boehmenan-K	(Seca <i>et al.</i> , 2001b)	Seed	<i>p</i> -serine	(Mohamed <i>et al.</i> , 1995)
Leaf	cadalene	(Kobaisy <i>et al.</i> , 2001)	Leaf	perilla-aldehyde	(Kobaisy <i>et al.</i> , 2001)
Seed	campesterol	(Mohamed <i>et al.</i> , 1995)	Stem	pinoresinol	(Seca <i>et al.</i> , 2001a), (Seca <i>et al.</i> , 2001b)
Stem	campesterol	(Seca <i>et al.</i> , 2000)	Stem	protoantocianidine-B5	(Thkin <i>et al.</i> , 1982)
Flower	cannabinine	(Rakhimkhanov <i>et al.</i> , 1974)	Stem	protoantocianidine-B6	(Thkin <i>et al.</i> , 1982)
Seed	cardiolipin	(Mohamed <i>et al.</i> , 1995)	Seed	sphingomyelin	(Mohamed <i>et al.</i> , 1995)
Leaf	<i>cis</i> - <i>beta</i> -elemone	(Kobaisy <i>et al.</i> , 2001)	Stem	sitosterol	(Seca <i>et al.</i> , 2001a)
Leaf	<i>cis</i> -linalolo-oxide	(Kobaisy <i>et al.</i> , 2001)	Stem	stigmast-4-en-3,6-dione	(Seca <i>et al.</i> , 2000)
Leaf	<i>cis</i> -pinocampnone	(Kobaisy <i>et al.</i> , 2001)	Stem	stigmast-4-en-3,6-one	(Seca <i>et al.</i> , 2000)
Leaf	citral	(Osman <i>et al.</i> , 1976)	Stem	stigmast-4-en-3-one	(Seca <i>et al.</i> , 2001b)
Stem	Docosanol	(Seca <i>et al.</i> , 2000)	Stem	stigmast-4-en-3-one	(Seca <i>et al.</i> , 2000)
Leaf	E,E-2-4-decadienal	(Kobaisy <i>et al.</i> , 2001)	Seed	stigmasterol	(Mohamed <i>et al.</i> , 1995)
Leaf	E,Z-2-4-decadienal	(Kobaisy <i>et al.</i> , 2001)	Stem	stigmasterol	(Seca <i>et al.</i> , 2000)

Position	Molecule	Reference	Position	Molecule	Reference
Leaf	E-2-decenal	(Kobaisy <i>et al.</i> , 2001)	Stem	syringaldehyde	(Seca <i>et al.</i> , 2001b)
Leaf	E-2-hexenal	(Kobaisy <i>et al.</i> , 2001)	Stem	syringaresinol	(Seca <i>et al.</i> , 2001a) (Seca <i>et al.</i> , 2001b)
Leaf	E-beta-damascenone	(Kobaisy <i>et al.</i> , 2001)	Stem	syringyl-beta-aryl	(Kuroda <i>et al.</i> , 2002)
Leaf	E-phytol	(Kobaisy <i>et al.</i> , 2001)	Stem	syringyl-lignine	(Nishimura <i>et al.</i> , 2002)
Stem	eicsanol	(Seca <i>et al.</i> , 2000)	Stem	threo-carolignan-E	(Seca <i>et al.</i> , 2001b)
Stem	threo-carolignan-E	(Seca <i>et al.</i> , 2001b)	Stem	threo-carolignan-H	(Seca <i>et al.</i> , 2001b)
Stem	threo-carolignan-K	(Seca <i>et al.</i> , 2001b)	Stem	threo-carolignan-K	(Seca <i>et al.</i> , 2001b)
Stem	erythrocanabisin-H	(Seca <i>et al.</i> , 2001a)	Leaf	<i>trans</i> -carveol	(Kobaisy <i>et al.</i> , 2001)
Stem	hexacosanol	(Seca <i>et al.</i> , 2000)	Stem	<i>trans</i> -coniferyl-aldehyde	(Seca <i>et al.</i> , 2001b)
Leaf	ethanol	(Osman <i>et al.</i> , 1976)	Leaf	<i>trans</i> -p-mentane	(Kobaisy <i>et al.</i> , 2001)
Leaf	ethyl-E-cinnamate	(Kobaisy <i>et al.</i> , 2001)	Leaf	<i>trans</i> -pinocamphone	(Kobaisy <i>et al.</i> , 2001)
Leaf	ethyl-esadecaonate	(Kobaisy <i>et al.</i> , 2001)	Stem	<i>trans</i> -sinapaldehyde	(Seca <i>et al.</i> , 2001b)
Leaf	phellandrene	(Osman <i>et al.</i> , 1976)	Stem	trictanol	(Seca <i>et al.</i> , 2000)
Leaf	furfural	(Kobaisy <i>et al.</i> , 2001)	Stem	hentriacontane	(Seca <i>et al.</i> , 2000)
Leaf	gamma-eudesmol	(Kobaisy <i>et al.</i> , 2001)	Leaf	Z-fitol	(Kobaisy <i>et al.</i> , 2001)

## 6.2. Appendix of tables of isolates from different maceration test in bioreactor

### 6.2.1. Tables of spore-forming isolates obtained from liquor retting of maceration K2 and K3

Isolate	Homology	Taxon	Accession number	Reference
<b>K2H1</b>	100%	<i>Bacillus</i> sp.	EU864323	Comparison of chitinase and 16S rRNA gene diversity in suppressive soil isolates, DNA extracts and a metagenomic library
		<i>Bacillus altitudinis</i>	FJ973524	Cultivable Diversity of <i>Bacillus</i> and <i>Bacillus</i> Derived Genera in Paper and Pulp Mill Effluent Treated Soils
		<i>Bacillus pumilus</i>	GQ169785	Screening of antagonistic bacterium against poplar tree ulcer and its colonization
		<i>Bacillus pumilus</i>	GQ220330	Isolation of a new strain of <i>Bacillus pumilus</i> from mangrove soil
<b>K2H2</b>	100%	<i>Bacillus subtilis</i>	GQ150487	Phylogenetic Analysis of Denitrogenated Bacteria in Sewage
		<i>Bacillus subtilis</i>	FJ445392	Drought stress tolerant EPS-producing plant growth promoting <i>Bacillus subtilis</i> POSB1
<b>K2H3</b>	100%	<i>Bacillus pumilus</i>	EU869263	Spore forming bacteria in cattle manure, slaughterhouse waste and substrates from biogas plants
		<i>Bacillus</i> sp.	AM988968	High diversity of cultivable heterotrophic bacteria in association with cyanobacterial water blooms
		<i>Bacillus pumilus</i>	GQ131871	Production of PHB under nutrient limiting conditions from an unknown distillery waste isolate
<b>K2H4</b>	99%	<i>Bacillus circulans</i>	EF100968	<i>Bacillus circulans</i> WZ-12 - a newly discovered aerobic dichloromethane-degrading methylotrophic bacterium
		<i>Bacillus benzoovorans</i>	DQ333291	Cultivable bacterial diversity of alkaline Ionar lake, India
		<i>Bacillus benzoovorans</i>	DQ346732	Papaya shoot tip associated endophytic bacteria isolated from in vitro cultures and host-endophyte interaction in vitro and in vivo
<b>K2H5</b>	99%	<i>Bacillus</i> sp.	FM877591	Phylogenetic analysis of anaerobic bacteria from environmental samples of North India
		<i>Bacillus</i> sp.	DQ988155	Isolation and molecular characterization of an epiphytic population from transgenic poplar leaves
		<i>Bacillus licheniformis</i>	GQ131872	Production of PHB under nutrient limiting conditions from an organism, <i>Bacillus licheniformis</i> isolated from soil near to distillery waste
<b>K2H6</b>	98%	<i>Paenibacillus lactis</i>	FN429978	Bacterial strain of <i>Paenibacillus lactis</i> IB-188D producing <i>beta</i> -CGTase ( <i>beta</i> -cyclodextrin glucanotransferase) and dextranase
		<i>Paenibacillus lactis</i>	AY257869	<i>Paenibacillus lactis</i> sp. nov., isolated from raw and heat-treated milk
		<i>Paenibacillus lactis</i>	FJ445392	Isolation and characterization of a cellulose-decomposing bacteria from soil
<b>K2H7</b>	99%	<i>Bacillus</i> sp.	FM958162	The Study of Prokaryotic diversity of a landfill environment using a polyphasic taxonomic approach
		<i>Bacillus licheniformis</i>	AY601721	Characterization of extracellular alpha-galactosidase produced by <i>Bacillus licheniformis</i> YB-42
		<i>Bacillus licheniformis</i>	EU256501	Molecular taxonomic study on <i>Bacillus licheniformis</i>

## 6.2.2. Tables of spore-forming isolates obtained from liquor retting of maceration K4 and K5

Isolate	Homology	Taxon	Accession number	Reference
<b>K401</b>	98%	<i>Pantoea</i> sp.	EU816766	Copper-resistant endophytic bacteria isolation from the plant grown in copper mine
		<i>Enterobacteriaceae</i> bacterium	AB461718	Diversity of endophytic bacteria isolated from stems of field-grown soybeans with different nodulation phenotypes
		<i>Pantoea ananatis</i>	DQ777968	Plant pathogenic bacterium, causing center rot of onion in Georgia, USA
<b>K402</b>	99%	<i>Paenibacillus</i> sp.	AM910222	Comparative analysis of the diversity of aerobic spore-forming bacteria in raw milk from organic and conventional dairy farms
		<i>Paenibacillus lactis</i>	FJ445392	Isolation and characterization of a cellulose-decomposing bacteria from soil
		<i>Paenibacillus</i> sp.	AF500311	Isolation and characterization of a diverse group of phenylacetic acid degrading microorganisms from pristine soil
<b>K403</b>	97%	<i>Paenibacillus</i> sp.	EU497637	Gram-negative bacteria forming endospores found in the soil and hot springs of the Valley of Geysers, Kamchatka, Russia
		<i>Paenibacillus</i> sp.	AB190129	Microbial community in compost processing under cold climate
		<i>Bacterium</i>	AJ250319	Aerobic endospore-forming bacteria from geothermal environments in northern Victoria Land, Antarctica, and Candlemas Island, South Sandwich archipelago, with the proposal of <i>Bacillus fumarioli</i> sp. nov
<b>K404</b>	100%	<i>Bacillus subtilis</i>	FJ485826	Isolation of mannanase-producing microorganisms for the treatment of palm kernel cake (PKC)
		<i>Bacillus</i> sp.	FJ755946	Study on prokaryotic diversity in cold spring of the Shawan Xin Jiang
		<i>Bacillus subtilis</i>	FJ460480	Plant growth promoting rhizobacteria
<b>K405 K409</b>	99%	<i>Bacillus megaterium</i>	FJ839691	Distribution and diversity of endophytic bacteria in rice plants from Guangxi
		<i>Bacillus megaterium</i>	EF690396	Plant growth promoting rhizobacteria
		<i>Bacillus</i> sp.	EF017043	Characterization of an Interesting Consortium Developed in an Alkaline Environment and Its Application to Black Liquor Treatment
<b>K406</b>	100%	<i>Bacillus licheniformis</i>	FJ914624	Abiotic stress tolerant exopolysaccharide producing plant growth promoting <i>Bacillus licheniformis</i> strain HYTAB18 growth under drought stress
		<i>Bacillus subtilis</i>	FJ864727	Drought stress tolerant EPS-producing plant growth promoting <i>Bacillus subtilis</i> POSB1
		<i>Bacillus subtilis</i>	FJ772087	Detection of Microbial Populations in a Forest Soil and Culture Collections of Effective Microorganisms
<b>K407</b>	99%	<i>Bacillus</i> sp.	FJ899759	Endophytic bacterial diversity in <i>Rhizoma Dioscoreae</i>
		<i>Bacillus</i> sp.	FM877591	Phylogenetic analysis of anaerobic bacteria from environmental samples of North India
		<i>Bacillus</i> sp.	FJ413051	16S rRNA gene sequences of <i>Bacillus</i> strains isolated from chloride tank of a sewage treatment plant
<b>K408</b>	99%	<i>Bacillus</i> sp.	EF584540	Isolation and identification of phenol-degrading strains and their degradation character
		<i>Lysinibacillus sphaericus</i>	EU869266	Spore forming bacteria in cattle manure, slaughterhouse waste and substrates from biogas plants
		<i>Bacillus</i> sp.	DQ279753	Microbial Azorespiration with Azo Compounds as the Sole Electron Acceptor

## 6.2.3. Tables of no spore-forming isolates obtained from liquor retting of maceration K6

Isolat2	Homology	Taxon	Accession number	Reference
K601	100%	<i>Pantoea</i> sp.	FJ646663	Environmental isolates from New Zealand pulp and paper wastewater
		<i>Enterobacter</i> sp.	AB461767	Diversity of endophytic bacteria isolated from stems of field-grown soybeans with different nodulation phenotypes
		<i>Enterobacter</i> sp.	EU438971	Characterisation of aerobically grown non-spore-forming bacteria from paper mill pulps containing recycled fibres
K602	100%	<i>Klebsiella pneumoniae</i>	FJ040189	Isolation and characterization of some EPS-producing biofilm bacteria on the materials coated by different antifouling dyes in the marinas
		<i>Klebsiella pneumoniae</i>	EU661376	Characterization and purification of indole acetic acid from <i>Klebsiella</i> species isolated from rhizosphere of wheat and its effect on plant growth
		<i>Klebsiella</i> sp.	FJ646653	Environmental isolates from New Zealand pulp and paper wastewater
K603	99%	<i>Enterobacter</i> sp.	EU438990	Characterisation of aerobically grown non-spore-forming bacteria from paper mill pulps containing recycled fibres
		<i>Enterobacter</i> sp.	FJ025770	Identification of marine bacteria isolated from marine environment
		<i>Enterobacter hormaechei</i>	AM943033	Microbial diversity of an electroactive biofilm generated in the anoxic zone of waste water treatment plant
K604	100%	<i>Acinetobacter</i> sp.	FJ816054	Isolation, identification and characterization of <i>Acinetobacter</i> species from the rhizosphere of <i>Pennisetum glaucum</i> (pearl millet)
		<i>Acinetobacter</i> sp.	FJ268994	Characterization of unculturable bacteria from rhizospheric soil of <i>Phragmites communis</i> growing in wetland ecosystem
		<i>Acinetobacter</i> sp.	EU073091	Genes involved in arsenic transformation and resistance associated with different levels of arsenic-contaminated soils
K605	100%	<i>Klebsiella</i> sp.	FM164637	Studies on Culture dependant and culture independent bacterial diversity of a natural spring water from mid-western Ghats region in India
		<i>Klebsiella pneumoniae</i>	EU360791	Dark fermentative hydrogen production by a new isolated <i>Klebsiella pneumoniae</i> ECU-21
		<i>Klebsiella</i> sp.	AY435403	<i>Klebsiella</i> sp. bacteria associated to stem rot disease in henequen ( <i>Agave fourcroydes</i> Lem)
K606	99%	<i>Bacterium</i>	FN179283	Expression of sulfur-oxidizing genes in Bioreactors
		<i>Pseudomonas</i> sp.	FJ897723	Identification, Oil Degrading Potential and Bio-film Forming Ability of Novel <i>Pseudomonas</i> Species Isolated from Water Sources near Kolkata Port in India
		<i>Pseudomonas aeruginosa</i>	FJ786006	Effect of rhamnolipids on degradation of anthracene by two newly isolated strains, <i>Sphingomonas</i> sp. 12A and <i>Pseudomonas</i> sp. 12B
K607	99%	<i>Enterobacter</i> sp.	EU855207	Microbial diversity of traditional soy paste and soy sauce during fermentation
		<i>Enterobacter</i> sp.	DQ855282	Conversion of sucrose into isomaltulose by <i>Enterobacter</i> sp. FMB1, an isomaltulose-producing microorganism isolated from traditional Korean food
		<i>Escherichia coli</i>	FJ422470	Characterization of unculturable bacterial isolates growing in tannery effluent common treatment plant in initial aeration tank
K608	99%	<i>Enterobacter</i> sp.	FJ587226	Hydrogen production from autofermentation of vegetable refuses
		<i>Enterobacter amnigenus</i>	AB362305	Phylogenetic and enzymatic diversity of deep subseafloor aerobic microorganisms in organics- and methane-rich sediments off Shimokita Peninsula
		Uncultured gamma proteobacteria	AJ318112	High bacterial diversity of a waste gas-degrading community in an industrial biofilter as shown by a 16S rDNA clone library

K609	100%	<i>Enterobacter</i> sp.	EU438972	Characterisation of aerobically grown non-spore-forming bacteria from paper mill pulps containing recycled fibres
		<i>Enterobacter sakazakii</i>	EF614997	The rhizosphere as a reservoir for the opportunistic pathogen <i>Cronobacter</i> spp.
		<i>Cronobacter sakazakii</i>	FJ947061	Phylogenetic Analysis of Denitrrogenated Bacteria in Sewage

### 6.3. Appendix of tables of main DGGE profile's bands from different maceration test in bioreactor

#### 6.3.1. Tables of main bands of total Eubacteria of maceration K2

Band	Homology	Taxon	Accession number	Reference
K2.01	100%	<i>Uncultured bacterium</i>	AB461301	Community analysis of stem-associated bacteria in soybeans by a bacterial cell enrichment method
		<i>Acinetobacter</i> sp.	EU260123	Molecular identification and analysis of antimicrobial resistance of bacteria isolated from oligotrophic lakes in a tropical region
K2.02	100%	<i>Klebsiella oxytoca</i>	GQ150485	Phylogenetic Analysis of Denitrrogenated Bacteria in Sewage
		<i>Uncultured bacterium</i>	AB485412	Community analysis of stem-associated bacteria in soybeans with different nodulation phenotypes
K2.03	100%	<i>Exiguobacterium</i> sp.	EU438949	Characterisation of aerobically grown non-spore-forming bacteria from paper mill pulps containing recycled fibres
		<i>Uncultured Exiguobacterium</i> sp.	EF593053	Identification of cultivable and non-cultivable bacteria in the predigester slurry of Nisargruna biogas plant
K2.04	99%	<i>Achromobacter</i> sp.	EF617310	Algae-lysing bacterium <i>Achromobacter</i> sp. ALB1
		<i>Uncultured bacterium</i>	AB485538	Community analysis of stem-associated bacteria in soybeans with different nodulation phenotypes
K2.05	100%	<i>Clostridium beijerinckii</i>	EF446166	Isolation of a <i>Clostridium beijerinckii</i> sLM01 cellulosome and the effect of pH, temperature, metal ions and thiols on cellulosomal xylanase activities
		<i>Clostridium</i> sp.	DQ196630	Bacterial diversity of an acidic Louisiana groundwater contaminated by dense nonaqueous-phase liquid containing chloroethanes and other solvents
K2.06	100%	<i>Uncultured Bacteroidetes bacterium</i>	CU926767	Towards the definition of a core of microorganisms involved in anaerobic digestion of sludge
		<i>Uncultured bacterium</i>	FJ950730	Molecular diversity of planctomycetes in biological wastewater treatment system
K2.07	99%	<i>Uncultured bacterium</i>	FJ535007	Effect of pH on volatile fatty acids accumulation during co-fermentation of waste activated sludge and carbohydrate
	98%	<i>Uncultured Clostridium</i> sp.	DQ232857	Fermentative hydrogen production by anaerobic sludge bacteria using foodwaste as a substrate
K2.08	100%	<i>Uncultured sulfate-reducing bacterium</i>	EF592737	Characterization of the Methanogen and Sulfate-Reducing Bacteria Populations in Anaerobic Biofilm Reactors Treating a Low-Temperature, High-Sulfate Wastewater
		<i>Uncultured Bacteroidales bacterium</i>	EU073787	Microbial diversity of subsurface coal beds and methanogenic coal enrichment cultures
K2.09	100%	<i>Bacillus licheniformis</i>	EU935597	Molecular characterization of thermophilic bacteria isolated from Erzurum City Pasinler Town hot spring
	99%	<i>Bacillus licheniformis</i>	GQ131872	Production of PHB under nutrient limiting conditions from an organism, <i>Bacillus licheniformis</i> isolated from soil near to distillery waste

6.3.2. Tables of main bands of *Bacillus* sp. of maceration K2

Band	Homology	Taxon	Accession number	Reference
K2.B01	100%	<i>Bacillus</i> sp.	FJ755946	Characterization of culturable bacterial community in aerated lagoons of common effluent treatment plant (CETP) for tannery wastewater
		<i>Bacillus subtilis</i>	FJ800367	Simultaneous cloning and expression of two cellulase genes from <i>Bacillus subtilis</i> newly isolated from Golden Takin ( <i>Budorcas taxicolor Bedfordi</i> )
K2.B02	99%	<i>Bacillus koreensis</i>	AY667496	<i>Bacillus koreensis</i> sp. nov., a spore-forming bacterium, isolated from the rhizosphere of willow roots in Korea
	98%	<i>Bacillus</i> sp.	AB181664	Community structure of culturable bacteria on tomato leaves based on 16S ribosomal DNA sequencing
K2.B03	100%	<i>Bacillus oleronius</i>	FJ973531	Cultivable Diversity of <i>Bacillus</i> and <i>Bacillus</i> Derived Genera in Paper and Pulp Mill Effluent Treated Soils
		<i>Bacillus oleronius</i>	AB441626	Microbial community in composting process
K2.B04	100%	<i>Bacillus licheniformis</i>	EU935597	Molecular characterization of thermophilic bacteria isolated from Erzurum City Pasinler Town hot spring
	99%	<i>Bacillus licheniformis</i>	GQ131872	Production of PHB under nutrient limiting conditions from an organism, <i>Bacillus licheniformis</i> isolated from soil near to distillery waste
K2.B05	99%	<i>Bacillus licheniformis</i>	AM932277	DGGE and T-RFLP Analysis of Bacterial Succession during Mushroom Compost Production and Sequence-aided T-RFLP Profile of Mature Compost
		Uncultured bacterium	DQ083102	Functional and molecular responses of soil microbial communities of century-old field
K2.B06	99%	Uncultured Clostridiales bacterium	FJ808967	Enumeration and identification of dominant types of sulfate-reducing bacteria in pulp from a paper recycling plant – a multiphasic approach
		<i>Clostridium aciditolerans</i>	DQ114945	<i>Clostridium aciditolerans</i> sp. nov., an acid-tolerant spore-forming anaerobic bacterium from constructed wetland sediment
K2.B07	99%	<i>Bacillus licheniformis</i>	FJ914624	Abiotic stress tolerant exopolysaccharide producing plant growth promoting <i>Bacillus licheniformis</i> strain HYTAB18 growth under drought stress
		<i>Bacillus licheniformis</i>	GQ169783	Screening of antagonistic bacterium against poplar tree ulcer and its colonization
K2.B08	99%	<i>Bacillus nealsonii</i>	FJ544393	16S rDNA of the microorganism resources for herbaceous fibers extracting
	98%	<i>Bacillus</i> sp.	AB291889	Culturable Endophytic Bacterial Flora of the Maturing Leaves and Roots of Rice Plants ( <i>Oryza sativa</i> ) Cultivated in a Paddy Field

## 6.3.1. Tables of main bands of total Eubacteria of maceration K3

Band	Homology	Taxon	Accession number	Reference
K3.01	100%	Uncultured <i>Acinetobacter</i> sp.	AB485058	Community analysis of stem-associated bacteria in soybeans with different nodulation phenotypes
		Uncultured <i>Acinetobacter</i> sp.	GQ129961	Genetic inventory of 'JPL-SAF' spacecraft assembly clean room during MSL mission
K3.02	99%	<i>Achromobacter</i> sp.	EF617310	Algae-lysing bacterium <i>Achromobacter</i> sp. ALB1
		Uncultured bacterium	AB485538	Community analysis of stem-associated bacteria in soybeans with different nodulation phenotypes



K3.03	100%	<i>Uncultured bacterium</i>	AB461301	Community analysis of stem-associated bacteria in soybeans by a bacterial cell enrichment method
		<i>Acinetobacter</i> sp.	EU260123	Molecular identification and analysis of antimicrobial resistance of bacteria isolated from oligotrophic lakes in a tropical region
K3.04	100%	<i>Clostridium beijerinckii</i>	EF446166	Isolation of a <i>Clostridium beijerinckii</i> sLM01 cellulosome and the effect of pH, temperature, metal ions and thiols on cellulosomal xylanase activities
		<i>Clostridium</i> sp.	DQ196630	Bacterial diversity of an acidic Louisiana groundwater contaminated by dense nonaqueous-phase liquid containing chloroethanes and other solvents
K3.05	100%	<i>Bacterium enrichment culture</i>	FJ804464	Partial 16S rDNA sequence of microbe obtained from enriched consortium of sulfate-reducing bacteria from mining soil
		<i>Escherichia coli</i>	FJ950549	Multiple antibiotic resistance of environmental bacteria isolated from an oxytetracycline production wastewater treatment plant and the receiving river
K3.06	100%	<i>Pseudomonas</i> sp.	FJ755950	Characterization of culturable bacterial community in aerated lagoons of common effluent treatment plant (CETP) for tannery wastewater
		<i>Uncultured Pseudomonas</i> sp.	EU073818	Microbial diversity of subsurface coal beds and methanogenic coal enrichment cultures
K3.07	100%	<i>Bacillus licheniformis</i>	EU935597	Molecular characterization of thermophilic bacteria isolated from Erzurum City Pasinler Town hot spring
	99%	<i>Bacillus licheniformis</i>	GQ131872	Production of PHB under nutrient limiting conditions from an organism, <i>Bacillus licheniformis</i> isolated from soil near to distillery waste
K3.08	99%	<i>Uncultured bacterium</i>	FJ535007	Effect of pH on volatile fatty acids accumulation during co-fermentation of waste activated sludge and carbohydrate
	98%	<i>Uncultured Clostridium</i> sp.	DQ232857	Fermentative hydrogen production by anaerobic sludge bacteria using foodwaste as a substrate

### 6.3.2. Tables of main bands of *Bacillus* sp. of maceration K2

Band	Homology	Taxon	Accession number	Reference
K3.B01	100%	<i>Bacillus silvestris</i>	FJ482027	Phylogenetic and physiological analyses of <i>inoculums</i> for use in anaerobic reactor
		<i>Bacterium enrichment culture</i>	FJ948279	Isolation and identification of endophytic bacteria in three medicinal plants
K3.B02	100%	<i>Bacillus</i> sp.	FJ755946	Characterization of culturable bacterial community in aerated lagoons of common effluent treatment plant (CETP) for tannery wastewater
		<i>Bacillus subtilis</i>	FJ800367	Simultaneous cloning and expression of two cellulase genes from <i>Bacillus subtilis</i> newly isolated from Golden Takin ( <i>Budorcas taxicolor Bedfordi</i> )
K3.B03	100%	<i>Bacillus pumilus</i>	GQ131871	Production of PHB under nutrient limiting conditions from an unknown distillery waste isolate
		<i>Bacillus</i> sp.	FJ755949	Characterization of culturable bacterial community in aerated lagoons of common effluent treatment plant (CETP) for tannery wastewater
K3.B04	99%	<i>Uncultured Clostridiales bacterium</i>	FJ808967	Enumeration and identification of dominant types of sulfate-reducing bacteria in pulp from a paper recycling plant – a multiphasic approach
		<i>Clostridium aciditolerans</i>	DQ114945	<i>Clostridium aciditolerans</i> sp. nov., an acid-tolerant spore-forming anaerobic bacterium from constructed wetland sediment

K3.B05	99%	<i>Bacillus koreensis</i>	AY667496	<i>Bacillus koreensis</i> sp. nov., a spore-forming bacterium, isolated from the rhizosphere of willow roots in Korea
	98%	<i>Bacillus</i> sp.	AB181664	Community structure of culturable bacteria on tomato leaves based on 16S ribosomal DNA sequencing
K3.B06	100%	<i>Bacillus oleronius</i>	FJ973531	Cultivable Diversity of <i>Bacillus</i> and <i>Bacillus</i> Derived Genera in Paper and Pulp Mill Effluent Treated Soils
		<i>Bacillus oleronius</i>	AB441626	Microbial community in composting process
K3.B07	100%	<i>Bacillus licheniformis</i>	EU935597	Molecular characterization of thermophilic bacteria isolated from Erzurum City Pasinler Town hot spring
	99%	<i>Bacillus licheniformis</i>	GQ131872	Production of PHB under nutrient limiting conditions from an organism, <i>Bacillus licheniformis</i> isolated from soil near to distillery waste
K3.B08	100%	<i>Bacillus</i> sp.	FJ876432	A novel microbial habitat of the alkaline black liquor with very high pollution load: Microbial diversity and key members in potential applications
		<i>Bacillus licheniformis</i>	EU718490	Cloning of <i>Bacillus licheniformis</i> xylanase gene and characterization of recombinant enzyme
K3.B09	98%	<i>Bacillus nealsonii</i>	FJ544393	16S rDNA of the microorganism resources for herbaceous fibers extracting
		<i>Bacillus</i> sp.	AB291889	Culturable Endophytic Bacterial Flora of the Maturing Leaves and Roots of Rice Plants ( <i>Oryza sativa</i> ) Cultivated in a Paddy Field
K3.B10	100%	<i>Bacillus pumilus</i>	GQ131871	Production of PHB under nutrient limiting conditions from an unknown distillery waste isolate
		<i>Bacillus</i> sp.	FJ755949	Characterization of culturable bacterial community in aerated lagoons of common effluent treatment plant (CETP) for tannery wastewater

