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Effects of selected pectinolytic bacterial strains on water-retting of hemp and fibre properties

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Bacillus, bacteria, *Clostridium*, fibre quality, hemp, pectin, retting, SEM analysis.

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Abstract

Aims: To study the effect of selected bacterial strains on hemp water-retting and properties of retted fibre.
Methods and Results: The trials were performed in laboratory tanks. The traditional water-retting process, without *inoculum* addition, was compared to a process modified by inoculating water tanks with two selected pectinolytic bacteria: the anaerobic strain *Clostridium* sp. L1/6 and the aerobic strain *Bacillus* sp. ROO40B. Six different incubation times were compared. Half the fibre obtained from each tank was combed. Micromorphological analyses were performed by scanning electron microscopy on uncombed and combed fibres. Moreover, organoleptic and chemical analyses of uncombed fibres were performed.
Conclusions: The *inoculum*, besides speeding up the process, significantly improved the fibre quality. The fibre was not damaged by mechanical hackling, thanks to the good retting level obtained by the addition of selected strains, differently to what happened with the traditionally retted fibre. The best fibre quality was obtained after 3–4 days of retting with the addition of the bacterial *inoculum*.
Significance and Impact of the Study: Retting is the major limitation to an efficient production of high-quality hemp fibres. The water-retting process and fibre quality were substantially improved by simultaneously inoculating water tanks with two selected pectinolytic strains.

Introduction

Bast fibre of hemp (*Cannabis sativa*) and flax (*Linum usitatissimum*) have a long history of usage in textile industry (Ranalli and Venturi 2004). They have also high-value markets in paper/pulp and composites, such as automobile components (Foulk *et al.* 2002). The relaunching of hemp fibre production may be successful if it is possible to set up a competitive production chain, reducing the production costs and increasing the quality of the fibre. To achieve this goal, it is essential, amongst other things, to set up a suitable method for extracting the fibre from the stems. At present, the most promising process would appear to be microbiological retting. Among the other methods, the chemical retting and the mechanical separation of fibres are not suitable because of the poor quality of the obtained fibres (Van Sumere 1992). The enzymatic method has provided encouraging results even though it is not yet industrially applicable because of high costs (Akin *et al.* 2007).

Microbiological retting can be performed using two different procedures: water-retting and dew-retting. In dew-retting, straw is spread on the ground, and pectins are broken down by pectinolytic micro-organisms, mainly

filamentous fungi (Henriksson *et al.* 1997). In Mediterranean climate, unlike central-northern European countries, dew-retting is not possible because of the high temperatures and the low humidity in the summer (Cavallero *et al.* 1997), which heavily curtails the development of pectinolytic microflora (Reyneri and Cavallero 1997). On the other hand, the use of irrigation to compensate for the insufficient dew would not be economically viable because of the large number of operations required (Reyneri and Cavallero 1997). Furthermore, it should also be emphasized that even where the climatic conditions are suitable, the fluctuations in the seasonal trends and the fibre aggression by fungi, such as *Alternaria alternata*, *Fusarium culmorum* and *Phoma* spp., lead to uneven retting (Courtney and Robinson 1982; Fraser *et al.* 1982; Brown and Sharma 1984; Mercer and Fraser 1986).

In water-retting, straw is submerged in large water tanks, where a pectinolytic bacterial community develops (Donaghy *et al.* 1990). During the first stage of the process, the soluble compounds in the stems (sugars, nitrogenous substances, etc.) dissolve, allowing the development of a bacterial community. The penetration of water into the stems causes the detachment of the bast fibres, thus allowing the entry of retting bacteria, which demolish the fibre-binding pectins (Donaghy *et al.* 1990). The water-retting process is initiated by aerobic bacteria; as the air in the rettery diminishes, the anaerobic bacteria predominate. The main aerobic retting agents belong to the genus *Bacillus*, whereas the main anaerobic retting agents belong to the genus *Clostridium* (Donaghy *et al.* 1990; Tamburini *et al.* 2003).

The retting methods are a crucial element for the quality of the fibre. The information available as regards linen production indicates that the retting has a strong influence on several fibre properties: (i) the removal of unwanted elements, such as residues of other tissues, cuticle and encrustations (Akin *et al.* 1996), (ii) the easy separation of the fibres from other tissues, (iii) the fibre quantity (Akin *et al.* 1997) and (iv) the residual pectin, hemicellulose and lignin content of the fibre walls (Focher 1992).

In Italy, hemp-retting was traditionally performed by placing the bundles of stems in the rettery full of stagnant water. The quality of Italian fibre was famous throughout the world, thanks to the excellent autochthonous germoplasm and, above all, to the skill of the farmers in performing rustic retting. However, the results obtained were heavily influenced by the quality of the water used on the various farms and, even more so, by seasonal temperature fluctuations. Thus, the fibre characteristics could vary markedly from farm to farm and from 1 year to the next. To obtain a good quality fibre, retting must be performed in industrial centres following criteria designed to considerably reduce the duration of the process; increase its control; and guarantee, as much as possible, reproducibility, standardization of the fibre characteristics and reduction in process costs.

In a previous study, we have carried out the isolation of a wide range of aerobic and anaerobic bacterial strains, their molecular characterization and the assessment of their pectinolytic activity (Tamburini *et al.* 2003). Furthermore, anaerobic bacterial strains, selected for their high pectinolytic activity, have been tested in hemp-retting trials (Di Candilo *et al.* 2000). Two *Clostridium* strains, L1/6 and C1/6, displayed the best retting ability. Their addition to the water tanks significantly reduced the process time, giving an optimal degree of retting in half the time when compared with the traditional retting without *inoculum* addition (6 *vs* 12 days) (Di Candilo *et al.* 2000).

Bacteria are the main retting agents; thus, their metabolic properties affect the course of the process and the quality of the product. The aim of this work was to evaluate the applicability of selected aerobic and anaerobic pectinolytic strains for the improvement of hemp water-retting process. Scanning electron microscopy (SEM) analysis was carried out to evaluate the effects induced by microbiological degradation and mechanical treatment on the fibre micromorphology. We also compared the organoleptic and chemical properties of the fibres retted with and without the bacterial *inoculum*.

Materials and methods

Plant material

The stems used for the retting trials were obtained from a field set up at Anzola Emilia (Bologna, Italy), using the dioecious Fibranova variety. The cultivation techniques adopted were the same envisaged for textile hemp in this environment. On soil specially prepared and fertilized, a machine was used to sow the seed in rows 18 cm apart, with a seed density of 50 kg ha^{-1}, for an envisaged investment of 130–140 plants per m^2. According to Bócsa and Karus (1998), the harvest was performed at full flowering (August 10), as fibre lignification occurs rapidly after flowering, causing a reduction in quality (Struik *et al.* 2000). Four areas of 4 m^2 were harvested manually, cutting the stems 5 cm above the soil. Stems were air-dried, leaves were removed, and then they were gathered in bundles and placed under cover until retting trials were performed.

During the growing season, plants were inspected for uniformity of growth, phytosanitary state, resistance to prebloom and lodging. During these observations, the 'nonstandard' plants, those with stunted development and

those damaged by *Pyrausta nubilalis* were all removed from the field. The purpose of this operation was to obtain a uniform set of plants for the retting tests.

Water-retting tests

The retting trials were performed with 10 l plastic tanks with lids, to reduce air exchange. Tanks were incubated at 35°C. The water used to fill the tanks was taken from an artesian well, to prevent any interference with the microbial activity by the chlorine present in drinking water.

The trials compared two types of water-retting: (i) with bacterial *inoculum* and (ii) without bacterial *inoculum* (control). Six different incubation times were compared (1, 2, 3, 4, 5 and 6 days). Bacterial strains used as *inoculum* were the anaerobic strain *Clostridium* sp. L1/6 and the aerobic strain *Bacillus* sp. ROO40B. Isolation and characterization of these strains have been already reported (Tamburini *et al.* 2003). Spores were added to water tanks at the beginning of the process.

Each tank was used to ret 15 stems of approximately the same height (220–230 cm) and diameter (7·5–8·0 mm), and cut into 50 cm segments. After retting, stems were washed in running water and placed to dry in a kiln at 50°C. Subsequently, they were manually scutched. Finally, half the fibre obtained from each tank was combed using three small combs with two rows of teeth, with a distance between the teeth of 2·0, 1·6 and 1·2 mm. During this final operation, each comb, starting with the one with the widest teeth, was passed five times through the fibres of each sample.

A total of 24 water-retted fibre samples were obtained, of which 12 retted with bacterial *inoculum* (six combed and six not combed) and 12 without bacterial *inoculum* (six combed and six not combed). Micromorphological fibre analyses were performed on these samples using SEM. Moreover, the 12 uncombed fibre samples underwent organoleptic and chemical analyses.

Cultural condition and *inoculum* preparation

Strain L1/6 was grown on reinforced clostridial agar (RCA; Oxoid, Cambridge, UK) in CO_2 atmosphere (Oxoid AnaeroGen) at 37°C. For spore production, a cell suspension was plated on solid medium and incubated for 5 days. Spores were collected with sterile physiological solution, centrifuged and washed once with the same solution and then stored at −20°C in 40% (v/v) glycerol. The *inoculum* for retting tanks was prepared by injecting the spore suspension into potatoes, washed and autoclaved for 10 min at 100°C. Injected potatoes were incubated for 5 days in sterile water at 37°C, and the resulting cell culture was used to inoculate water tanks at about 10^5 cells ml^{-1} (final concentration).

Strain ROO40B was grown on solid rich medium MA (Medium A) (0·5% yeast extract, 0·5% peptone, 1% tryptone) at 37°C. Spores were prepared on the same medium, following the procedure described for strain L1/6. Water tanks were inoculated with the spore suspension at about 10^4 cells ml^{-1} (final concentration). In all the procedures, anaerobiosis was maintained only during cell growth.

Determination of bacterial titre and reducing sugars

Liquor samples from retting tanks were stored at −20°C, for biochemical analysis, or mixed with an equal volume of 40% (v/v) glycerol and then stored at −20°C, for microbiological analysis.

The total aerobic and anaerobic heterotrophic cultivable bacteria were enumerated as colony-forming units (CFUs) onto MA and RCA plates, respectively. Colonies were counted after incubation for 5 days at 37°C. For the determination of anaerobic titre, plates were incubated in CO_2 atmosphere. CFUs were determined in triplicate, and the average values were determined.

The amount of reducing sugars present in the ret liquor was determined by the dinitrosalicylic acid (DNS) method (Miller 1959). One millilitre of sample was mixed with 3 ml of DNS solution and 1 ml of distilled water. After boiling for 10 min, the optical density was measured spectrophotometrically at 640 nm. A solution of galacturonic acid was used as a standard.

SEM analysis

Morphological analyses were performed on samples of retted fibres fixed in phosphate-buffered 2·5% glutaraldehyde, acetone dehydrated and critical point dried. Samples mounted on aluminium stubs were coated with a 10-nm gold film and observed under a Philips XL-30 SEM operating between 10 and 20 kV. At least ten specimens for each experimental condition were examined.

Organoleptic analysis

An overall evaluation, based on an organoleptic analysis, was made on swingled fibres using the CIPALIN classification system (I.T.L. 1992; Cavallero *et al.* 1997). This system attributes a quality value (index) that ranges from 0 to 700, indicating a merchandize value that increases as the value increases. In our case, this classification system was modified using a quality value that ranges from 1 (undesirable) to 5 (optimum). The traits examined were scutching, colour, degree of retting, homogeneity, presence of residual core and fineness. The lowest and the highest scores were attributed as following: (i) one corresponding to fibres characterized by hard scutching, dark

colour, insufficient retting, presence of residual core, lack of homogeneity and roughness; (ii) five corresponding to fibres characterized by easy scutching, light colour, correct retting, absence of residual core, homogeneity and fineness.

Chemical analysis

All chemical analyses were carried out on three fibre samples with two independent measurements, and the results are the average of six determinations. Technical Association of the Pulp and Paper Industry (TAPPI) standard methods were used to evaluate the content of the ash (TAPPI T 204 om-88 Solvent extractives of wood and pulp), the extractives (TAPPI T 211 om-93 Ash in wood and pulp), the pentosans (TAPPI T 233 om-84) and the soluble and Klason lignin (TAPPI T 222 om-88 acid insoluble lignin in wood and pulp). The acid soluble lignin was determined spectrophotometrically at 205 nm with an extinction coefficient 110 l g^{-1} m^{-1} (Schoening and Johanson 1965).

Results

Raw materials

The field cultivation gave good results, revealed by sufficiently uniform development of the plants, good final plant density (132 ± 10·7 plants per m^2), high production of fresh biomass (51·4 ± 2·58 t ha^{-1}) and stems (14·5 ± 0·81 t ha^{-1}), as well as by a good average yield in fibre (2·55 ± 0·22 t ha^{-1}).

Selection of bacterial strains

Bacterial strains used as *inoculum* were the anaerobic strain *Clostridium* sp. L1/6 and the aerobic strain *Bacillus* sp. ROO40B. The strain L1/6, isolated from unretted flax, has been assigned to the *Clostridium felsineum–Clostridium acetobutylicum* cluster by 16S rRNA gene analysis, whereas the strain ROO40B, isolated from hemp ret liquor, belongs the *Bacillus pumilus* cluster (Tamburini *et al.* 2003). Both strains were selected based on their polysaccharide degrading activities. Particularly, polygalacturonase (PG) activity appears to be the primary retting agent. This activity predominates throughout water-retting and shows an optimum pH close to that of the ret liquor (pH 4·5) (Chesson 1978).

Among 225 anaerobic pectinolytic strains, L1/6 has been demonstrated to be one of the seven strains displaying the highest PG activity (pH 4·8, 100·00 UI g^{-1} cells) (Tamburini *et al.* 2003). Among 104 aerobic pectinolytic isolates, none have showed PG activity under the assay conditions used for the anaerobic strains (Tamburini *et al.* 2003). Nevertheless, the aerobic strains displayed a pectinolytic activity at pH 8·0 under the condition previously described by Kobayashi *et al.* (2001), and the strain ROO40B showed the highest value (169 UI g^{-1} cells). Moreover, ROO40B did not display a cellulosolytic activity. This feature is important for strain selection because a prolonged treatment of hemp stems with cellulosolytic bacteria could otherwise result in loss of fibre strength, because of a direct attack to cellulosic fibres. On the contrary, all the pectinolytic anaerobic strains characterized so far showed a cellulosolytic activity (Tamburini *et al.* 2004).

Bacterial concentration and reducing sugars

Aerobic and anaerobic bacteria were monitored during the retting process by determining their CFUs. Furthermore, the production of reducing sugars was evaluated as a measure of the whole microbial depolymerizing activities in the ret liquor. Figure 1 shows data obtained for the 6-day retting process. Similar results were also obtained by repeating the retting trials and with shorter process times (data not shown).

The number of aerobic and anaerobic bacterial cells present in the retting liquor showed the same pattern both in the control and in the inoculated tank (Fig. 1). This is probably because of the fact that the *inoculum* did not significantly change the total amount of bacteria present in the water tanks. On the other hand, the anaerobic strain L1/6 has a typical colony morphology, and colonies with this morphology were only observed with cells from the inoculated tank. Furthermore, anaerobic bacteria were most probably underestimated because vegetative cells are oxygen sensitive and might be killed during sampling. These data also showed that aerobic bacteria were predominant in the first phases of the process, while anaerobic strains became predominant at later times. No significant differences between the two trials were observed in the production of reducing sugars.

Micromorphological fibre analyses

In water-retting without bacterial *inoculum,* the material obtained by manual scutching of stems retted for 24 h appeared as large compact ribbons, where both external and internal bast tissues largely maintained their structural integrity (Fig. 2a,b). Scattered bacterial cells were observed on the cuticular layer. Remnants of epidermis still persisted on days 3 and 4 (Fig. 2c); however, discrete fibre bundles were perceptible on the internal bast strip surface, because of the differential tissue destruction (Fig. 2d, arrows). The fibre bundles were generally well separated and almost free from extraneous tissues after

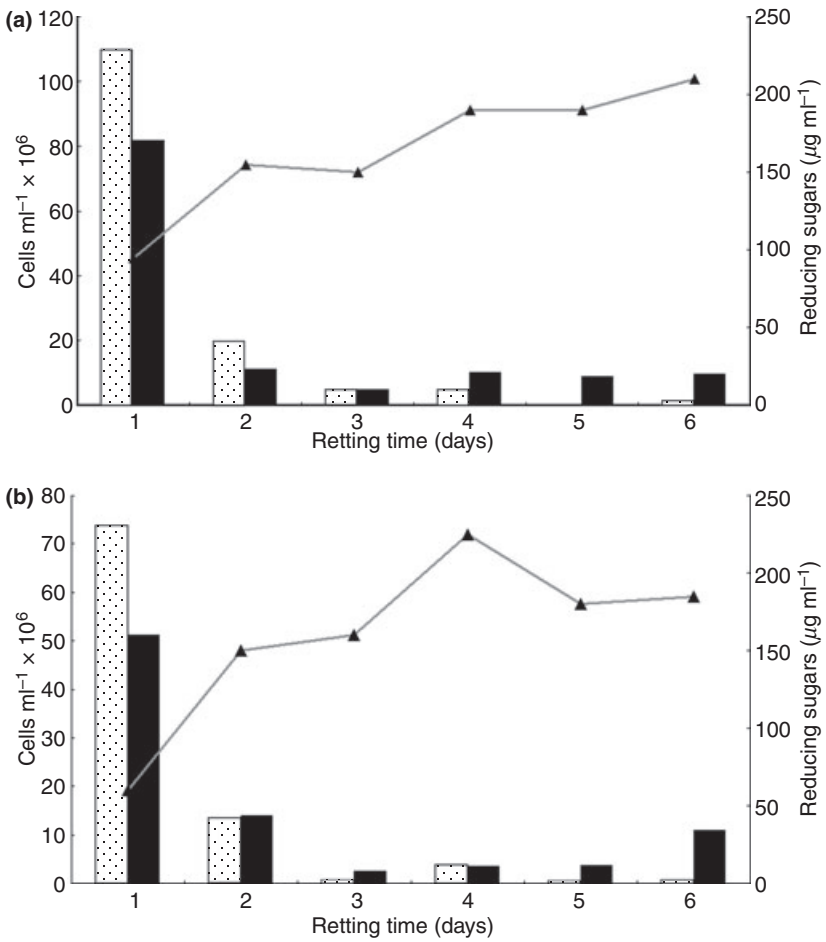

Figure 1 Anaerobic and aerobic bacterial cell number and reducing sugar concentration for the 6-day retting process: (a) tank not inoculated (control) and (b) tank inoculated with the selected bacterial strains. (□) Aerobic bacteria; (■) anaerobic bacteria and (—▲—) reducing sugar.

the longest water-retting times (5–6 days) (Fig. 2e). At this time, the fibre wall showed longitudinal wrinkles; encrusting substances were observed where conspicuous bacterial colonization occurred (Fig. 2f, arrowheads).

In water-retting with the *inoculum* of selected bacterial strains, no substantial differences were shown in the samples retted for 24 h if compared with the respective control; several micro-breaks, however, hosted dense bacterial population on the epidermal layer (Fig. 3a,b). Extraneous tissues were notably reduced on day 2. Within 72 h, most fibre bundles appeared to be sharply defined and tended to further split into individual fibres (Fig. 3c). Retting with bacterial *inoculum* for 5–6 days led to major changes in the ultimate fibres: the fibrous wall texture became more prominent, suggesting a loss of matrix components (Fig. 3d), and intense degradation was evident in the location of bacterial clusters (Fig. 3e,f). Moreover, crackling and localized burst frequently occurred under the electron beam during SEM analysis.

The hackling efficacy was related to the retting extent, and it obviously resulted in a higher degree of fibre separation. Some extraneous tissues were, however, still present in specimens hackled after water-retting for 6 days without bacterial *inoculum*; moreover, fibres showed wall fibrillation and delamination (Fig. 2g,h). Conversely, the specimens hackled after retting with bacterial *inoculum* showed mechanical damage not later than day 2. Ultimate fibres with intact smooth surface were recovered by hackling bast strips after 3–4 days of retting (Fig. 3g,h).

Fibre organoleptic and chemical characteristics

Starting from the second day of retting, the fibre samples from the tanks inoculated with the two selected bacterial strains clearly showed better properties than the corresponding samples retted without *inoculum* (Table 1). The fibres retted with the bacterial *inoculum* obtained an average maximum score of 4·8 after just 3 days of retting, while the ones from the control tanks obtained a maximum score of 4·0 after 6 days of retting. As shown in Table 1, besides halving the process time, the bacterial

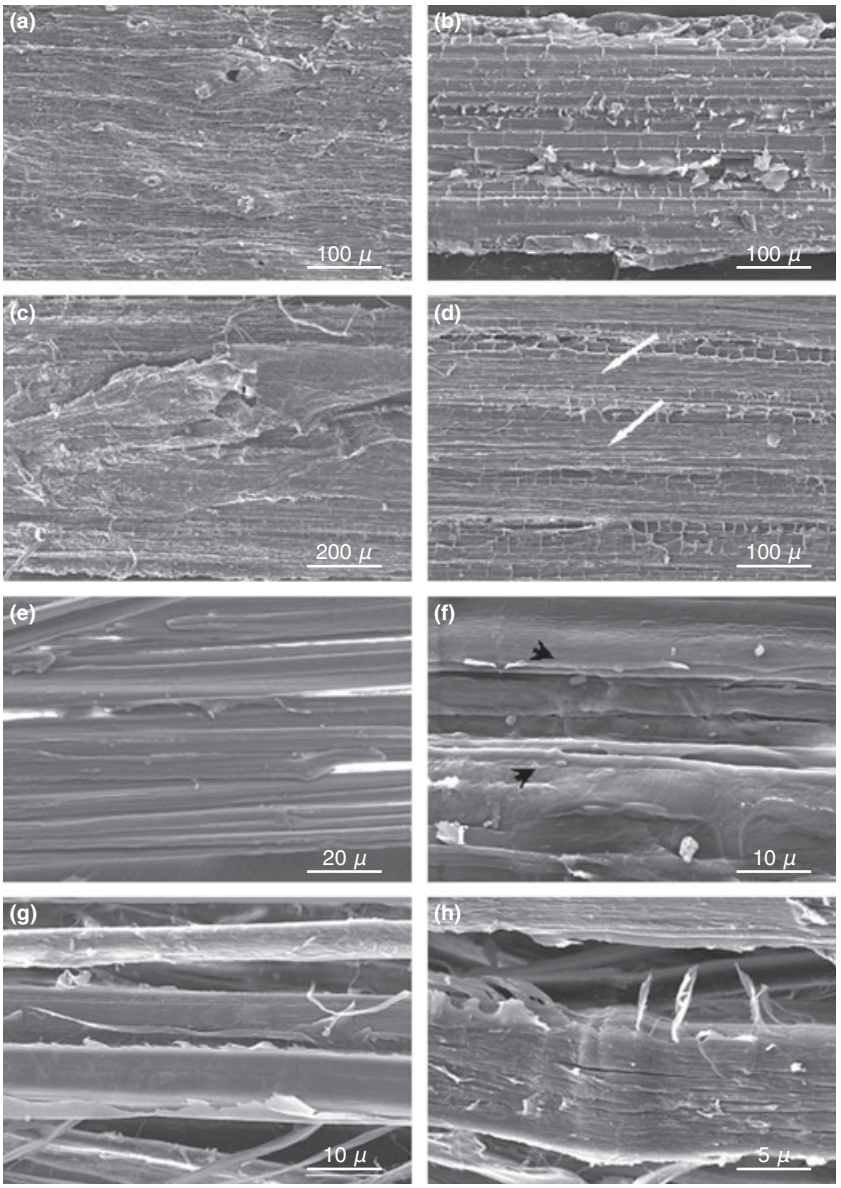

Figure 2 SEM micrographs of hemp fibre water-retted in tanks without bacterial *inoculum*. (a–f) Not combed samples; (g–h) combed samples. (a) Intact epidermal layer on the outer bast strip surface after 24 h retting; (b) remnants of nonfibrous tissues on the internal bast strip surface after 24 h retting; (c) outer surface of a bast strip after 3 days of retting; (d) fibre bundles are perceptible on the internal strip surface after 3 days of retting; (e) ultimate fibres obtained after 6 days of retting; (f) bacteria adhering to the fibre surface after 6 days of retting; (g) fibre cell wall delamination induced by hackling after 6 days of retting; (h) severe mechanical damage induced by hackling to the fibre wall after 6 days of retting.

inoculum improved all the examined parameters. Similar results were obtained in repeated water-retting tests (data not shown).

The percentages of the single components of the water-retted fibres are reported in Table 2. Fibres obtained by retting with and without *inoculum* showed a decrease in pentosan content during the retting time. However, the *inoculum* specifically facilitated the degradation of structurally amorphous pentosans.

The extractive content was determined at the beginning of each retting level, while the pentosan, lignin and ash contents were determined on the extracted samples. Decreasing the pentosans content during the retting increased the lignin percentage in the fibre obviously, while the ash loss is likely because of their complexation with pentosans.

Discussion

In this study, we investigated the effect on hemp water-retting and on retted fibre quality of two selected pectinolytic bacteria: the anaerobic strain *Clostridium* sp. L1/6 and the aerobic strain *Bacillus* sp. ROO40B. The results demonstrate that the bacterial *inoculum* substantially improved the hemp-retting process. This occurs because of the high pectinolytic activity of the selected strains and

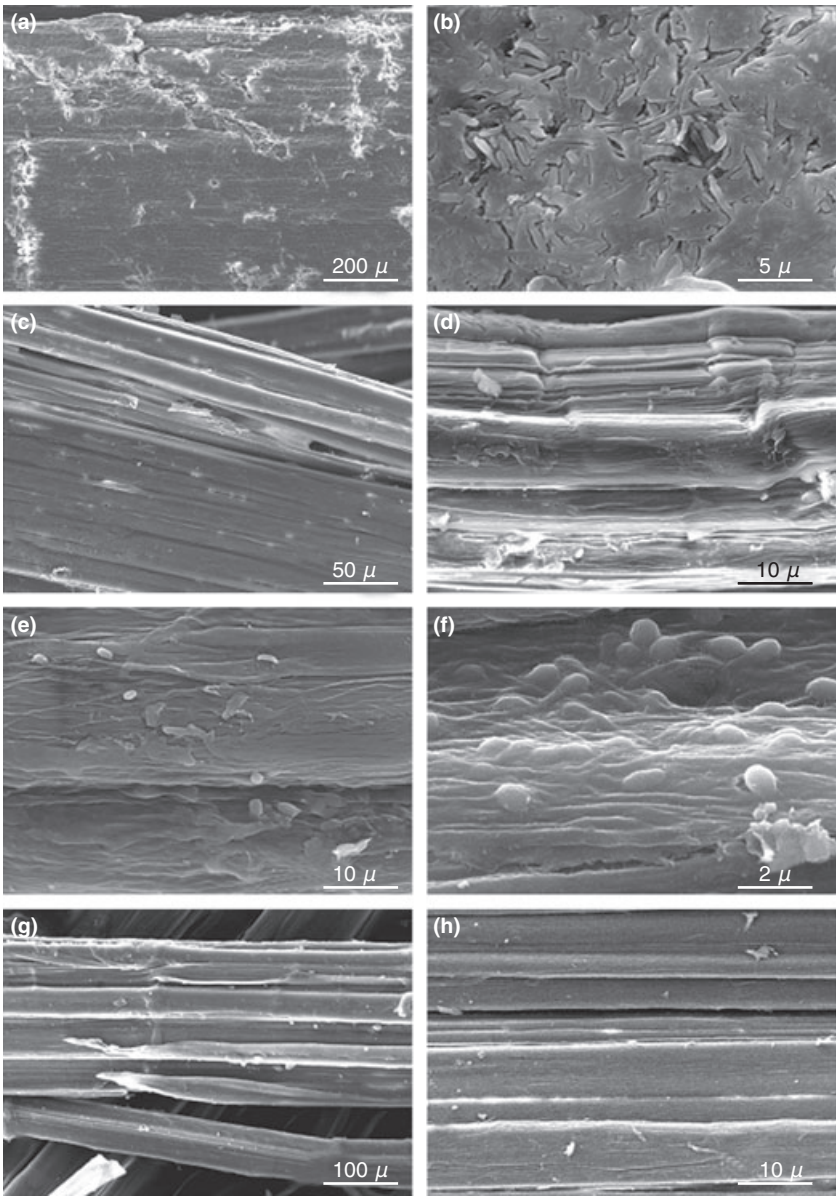

Figure 3 SEM micrographs of hemp fibre water-retted in tanks with the bacterial *inoculum*. (a–f) Not combed samples; (g–h) combed samples. (a) Epidermal layer on the outer bast strip surface after 24 h retting; (b) bacterial clusters in epidermal microbreaks after 24 h retting; (c) clean fibres obtained after 3 days of retting; (d) fibre wall showing prominent fibrous texture after 5 days of retting; (e,f) heavy fibre degradation and bacterial colonization after 6 days of retting; (g) undamaged fibres obtained by hackling after 3 days of retting; (h) fibres with smooth surface obtained by hackling after 4 days of retting.

because anaerobic bacterial spore germination requires the setting up of anaerobic conditions, generated by growth of aerobic bacteria.

In hemp stem retting, the enzymatic bacterial activities act on heterogeneous material, as for cell type, age, maturation degree and plant sex (Horkay and Bócsa 1996; Keller *et al.* 2001; Mediavilla *et al.* 2001; Medeghini Bonatti *et al.* 2002, 2004). Moreover, the air-dried stems host a natural population of micro-organisms, whose potential enzymatic activity towards various cell wall components could determine uneven retting. We summarized the observed morphological modifications and related them to three sequential retting steps, whose duration was clearly influenced by the applied experimental procedures (Fig. 4).

Step 1: separation of the fibrous components from the other bast tissues. This is mainly because of the pectinolytic dissolution of the middle lamellas joining cells with nonlignified wall, facing intercellular spaces. In both our experimental conditions, parenchyma, collenchyma and phloem cells were easily detached from the fibre bundles. The epidermis was also early detached from the underlying tissues, while keeping its integrity because of its constitutive histological compactness and cuticular covering. This preliminary step lasted more than 3 days when hemp was retted in tanks without bacterial addition but it was shortened up to 2 days by the bacterial *inoculum*.

Step 2: fibre elementarization. After the detachment of the surrounding tissues, fibre bundles can be directly

Table 1 Fibre organoleptic classification. Each parameter was assessed awarding a score of between 1 (undesirable) and 5 (optimum), as envisaged by the CIPALIN classification

	Water-retting without bacterial *inoculum*						Water-retting with bacterial *inoculum*					
	Time of retting (days)						Time of retting (days)					
Parameter	1	2	3	4	5	6	1	2	3	4	5	6
Scutching	2·0	2·0	2·0	3·0	3·5	4·0	2·0	3·0	5·0	5·0	5·0	5·0
Colour	1·0	1·0	1·0	2·0	3·3	4·0	1·0	3·0	5·0	4·4	4·0	4·0
Degree of retting	2·0	2·0	2·0	3·0	3·0	4·0	2·0	3·0	4·5	5·0	4·0	4·0
Homogeneity	1·0	1·0	2·0	3·0	3·0	4·5	1·0	3·0	5·0	5·0	5·0	5·0
Residual core	1·0	1·0	1·0	2·0	3·2	3·5	1·0	2·0	5·0	5·0	5·0	5·0
Fineness	1·0	1·0	2·0	2·0	3·0	4·0	1·0	2·0	4·4	5·0	5·0	5·0
Mean	1·3	1·3	1·7	2·5	3·2	4·0	1·3	2·7	4·8	4·9	4·7	4·7

Table 2 Chemical fibre composition at different retting times

Sample	Time of retting (days)	Extractives (%)	Soluble lignin (%)	Insoluble lignin (%)	Ash (%)	Pentosans (%)	Cellulose* (%)
Unretted	–	4·4 ± 0·28	2·2 ± 0·22	0·7 ± 0·03	2·3 ± 0·21	8·4 ± 0·39	82·0 ± 1·13
Water-retted without bacterial *inoculum*	1	3·2 ± 0·27	2·2 ± 0·28	0·7 ± 0·02	2·2 ± 0·18	6·3 ± 0·24	85·4 ± 0·99
	2	3·2 ± 0·39	2·0 ± 0·23	0·7 ± 0·03	1·9 ± 0·25	5·8 ± 0·21	86·4 ± 1·11
	3	3·2 ± 0·21	3·0 ± 0·20	0·6 ± 0·02	1·6 ± 0·23	5·2 ± 0·21	86·4 ± 0·87
	4	3·2 ± 0·36	3·2 ± 0·27	0·6 ± 0·02	1·4 ± 0·24	5·0 ± 0·16	86·6 ± 1·05
	5	3·1 ± 0·23	3·2 ± 0·31	0·6 ± 0·04	1·0 ± 0·24	4·7 ± 0·20	87·4 ± 1·02
	6	3·1 ± 0·21	3·7 ± 0·34	0·4 ± 0·02	1·0 ± 0·23	4·5 ± 0·24	87·3 ± 1·04
Water-retted with bacterial *inoculum*	1	3·2 ± 0·19	1·5 ± 0·17	0·7 ± 0·03	2·6 ± 0·23	6·5 ± 0·28	85·5 ± 0·90
	2	3·2 ± 0·27	1·7 ± 0·18	0·6 ± 0·02	2·0 ± 0·26	5·5 ± 0·25	87·0 ± 0·98
	3	3·2 ± 0·24	2·1 ± 0·20	0·6 ± 0·03	0·7 ± 0·09	4·8 ± 0·18	88·6 ± 0·74
	4	3·1 ± 0·24	2·4 ± 0·36	0·6 ± 0·05	0·7 ± 0·06	4·6 ± 0·17	88·6 ± 0·88
	5	3·1 ± 0·29	2·9 ± 0·23	0·5 ± 0·04	0·7 ± 0·04	4·4 ± 0·21	88·4 ± 0·81
	6	2·8 ± 0·18	3·1 ± 0·19	0·5 ± 0·05	0·6 ± 0·03	4·1 ± 0·22	88·9 ± 0·67

*Determined as difference.

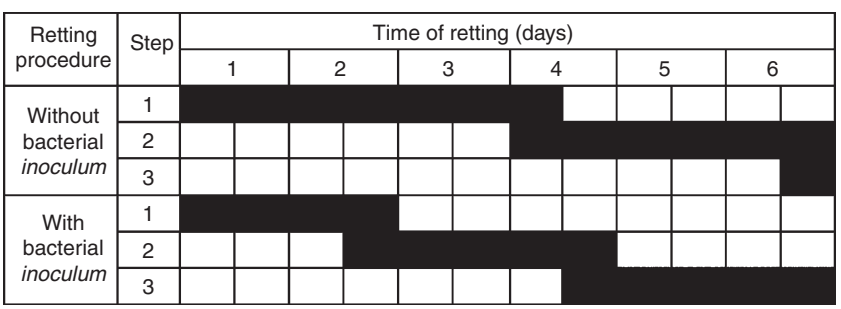

Figure 4 Timing of the modifications induced in hemp fibre morphology by the tested retting procedures.

subjected to enzymatic attack. However, because the middle lamellas joining the fibres are modified by lignin and generally composed of less methylated pectins (Carpita and Gibeaut 1993), they offer higher resistance to pectinolytic enzymes. Although this step lasted for a relatively longer time (more than 2 days) both in water and in bacterial supplemented liquor, clean ultimate fibres were obtained within 3 days, from the beginning of the bacterial retting procedure.

Step 3: fibre cell wall structural modifications. The third step was characterized by a significant increase in bacterial cells adhering to the fibre surface and by cell wall modifications suggesting over-retting. It is conceivable that increased pectinolytic activity affects the matrical pectins also, at least in the outermost fibre wall layers; new available carbon sources may then induce the activity of other enzymes (i.e. hemicellulases and cellulases) responsible for the inappropriate loss of cell wall structural

polysaccharides and unveiling of the cellulose network (Doi and Kosugi 2004; Han *et al.* 2004). Actually, the bacterial *inoculum* greatly accelerated the occurrence of morphological alterations related to the over-retting phase. Features such as bacterial anchoring, wall erosion and decreased resistance to the electron beam indicate a deep attack to the cell wall structure.

The bacterial *inoculum*, besides speeding up the retting process, significantly improved the fibre quality, as easy scutching, bright colour, retting degree, cleanliness, homogeneity and fineness. Moreover, the fibre was not damaged by mechanical hackling, thanks to the good retting level obtained with the *inoculum*, differently to what happened without *inoculum*. Chemical analysis confirmed such positive effects, showing a reduction of pentosans. The SEM analysis, even if limited to the fibre surface micromorphology and applied to nonhomogeneous materials, provided a reliable framework of the main material characteristics and was useful for the assessment of the efficacy of the applied retting protocols.

The best fibre quality was obtained after 3–4 days of retting with the addition of the combined bacterial *inoculum* to water tanks. However, the fibre wall structural architecture can be altered and weakened by excessive bacterial proliferation and/or too long-lasting retting. Integration with data drawn from biochemical and physical measurements is, therefore, needed for a more comprehensive fibre characterization and technological performance assessment.

References

Akin, D.E., Gambe, G.R., Morrison, H., Rigsby, L.L. and Dodd, R.B. (1996) Chemical and structural analysis of fibre and core tissues from flax. *J Sci Food Agric* **72**, 155–165.

Akin, D.E., Morrison, W.H., Gamble, G.R., Rigsby, L.L., Henriksson, G. and Eriksson, K.E.L. (1997) Effect of retting enzymes on the structure and composition of flax cell wall. *Text Res J* **67**, 279–287.

Akin, D.E., Condonb, B., Sohna, M., Foulk, J.A., Dodd, R.B. and Rigsby, L.L. (2007) Optimization for enzyme-retting of flax with pectate lyase. *Ind Crops Prod* **25**, 136–146.

Bócsa, I. and Karus, M. (1998) *The Cultivation of Hemp: Botany, Varieties, Cultivation and Harvesting*. Sebastopol, CA: Hemptech.

Brown, A.E. and Sharma, H.S.S. (1984) Production of polysaccharide-degrading enzymes by saprophytic fungi from glyphosate treated flax and their involvement in retting. *Ann Appl Biol* **105**, 65–74.

Carpita, N.C. and Gibeaut, D.M. (1993) Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth. *Plant J* **3**, 1–30.

Cavallero, A., Reyneri, A. and Rondi, G. (1997) Fibre flax production and quality in North Italy. II Comparison between dew retting and retting methods controlled by field irrigation. *Ital J Agron* **1–5**, 73–80.

Chesson, A. (1978) The maceration of linen flax under anaerobic conditions. *J Appl Bacteriol* **45**, 219–230.

Courtney, A.D. and Robinson, E. (1982) Glyphosate (N-(Phosphonomethyl) glycine) as a preharvest retting agent in flax (*Linum usitatissimum*). In *Proceedings of the 1982 British Crop Protection Conference: Weeds (Brighton)* pp. 231–236. Croydon, UK: British Crop Protection Council.

Di Candilo, M., Ranalli, P., Bozzi, C., Focher, B. and Mastromei, G. (2000) Preliminary results of tests facing with the controlled retting of hemp. *Ind Crops Prod* **11**, 197–203.

Doi, R.H. and Kosugi, A. (2004) Cellulosomes: plant-cell-wall-degrading enzyme complexes. *Nat Rev Microbiol* **2**, 541–551.

Donaghy, J.A., Levette, P.N. and Haylock, R.W. (1990) Changes in microbial populations during anaerobic flax retting. *J Appl Bacteriol* **69**, 634–641.

Focher, B. (1992) Physical characteristics of flax fibre. In *The Biology and Processing of Flax* ed. Sharma, H.S. and Van Sumere, S.F. pp. 329–342. Belfast: M. Publications.

Foulk, J.A., Akin, D.E., Dodd, R.B. and McAlister, D.D. (2002) Flax fiber: potential for a new crop in the Southeast. In *Flax Fiber: Potential for a New Crop in the Southeast Trends in New Crops and New Uses* ed. Janick, J. and Whipkey, A. pp. 361–370. Alexandria, VA: ASHS Press.

Fraser, T.W., Courtney, A.D. and Harvey, B.M.R. (1982) Pre-harvest retting of flax: a light microscope study of the effects of glyphosate treatment on the maturation of stem tissue. *Ann Appl Biol* **101**, 533–537.

Han, S.O., Cho, H.Y., Yukawa, H., Inui, M. and Doi, R.H. (2004) Regulation and expression of cellulosomes and noncellulosomal (hemi)cellulolytic enzymes in *Clostridium* cellulovorans during growth on different carbon sources. *J Bacteriol* **186**, 4218–4227.

Henriksson, G., Akin, D.E., Hanlin, R.T., Rodriguez, C., Archibald, D.D., Rigsby, L.L. and Eriksson, K.L. (1997) Identification and retting efficiencies of fungi isolated from dew-retted flax in the United States and Europe. *Appl Environ Microbiol* **63**, 3950–3956.

Horkay, E. and Bócsa, I. (1996) Objective basis for the evaluation of differences in fibre quality between male, female and monoecious hemp plants. *J Int Hemp Ass* **3**, 67–69.

I.T.L. (1992) *La culture du Lin Fiber*. Paris: Institute Technique Agricole du Lin.

Keller, A., Mediavilla, V., Leupin, M. and Wintermantel, E. (2001) Influence of the growth stage of industrial hemp on chemical and physical properties of the fibre. *Ind Crops Prod* **13**, 35–48.

Kobayashi, T., Higaki, N., Suzumatsu, A., Sawada, K., Hagihara, H., Kawai, S. and Ito, S. (2001) Purification and properties of a high-molecular-weight, alkaline exopolygalacturonase from a strain of *Bacillus*. *Enzyme Microb Technol* **5**, 70–75.

Medeghini Bonatti, P., Focher, B., Grippo, C., Ferrari, C. and Pellacani, G. (2002) Caratterizzazione strutturale e istologica di fibre di canapa (*Cannabis sativa* L.). *Agroindustria* **1**, 59–63.

Medeghini Bonatti, P., Ferrari, C., Focher, B., Grippo, C., Torri, G. and Cosentino, C. (2004) Histochemical and supramolecular studies in determining quality of hemp fibres for textile applications. *Euphytica* **140**, 55–64.

Mediavilla, V., Leupin, M. and Keller, A. (2001) Influence of the growth stage of industrial hemp on the yield formation in relation to certain fibre quality traits. *Ind Crops Prod* **13**, 49–56.

Mercer, P.C. and Fraser, T.W. (1986) Microorganisms associated with retting of flax treated with the herbicide glyphosate. *Ann Appl Biol* **109**, 509–521.

Miller, G.L. (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal Chem* **31**, 426–428.

Ranalli, P. and Venturi, G. (2004) Hemp as a raw material for industrial applications. *Euphytica* **140**, 1–6.

Reyneri, A. and Cavallero, C. (1997) Fiber flax production and quality in northern Italy: I. Effects of environment, seeding rate and nitrogen fertilization. *Ital J Agron* **1–2**, 81–88.

Schoening, A.G. and Johanson, G. (1965) Absorptionmetric determination of acid soluble lignin in semichemical bisulphite pulps and in some woods and plants. *Svensk Papperstid* **68**, 607–610.

Struik, P.C., Amaducci, S., Bullard, M.J., Stutterheim, N.C., Venturi, G. and Cromack, H.T.H. (2000) Agronomy of fibre hemp (*Cannabis sativa* L.) in Europe. *Ind Crops Prod* **11**, 107–118.

Tamburini, E., Gordillo Leon, A., Perito, B. and Mastromei, G. (2003) Characterization of bacterial pectinolytic strains involved in the water retting process. *Environ Microbiol* **5**, 730–736.

Tamburini, E., Gordillo León, A., Perito, B., Di Candilo, M. and Mastromei, G. (2004) Exploitation of bacterial pectinolytic strains for improvement of hemp water retting. *Euphytica* **140**, 47–54.

Van Sumere, C.F. (1992) Retting of flax with special reference to enzyme retting. In *Biology and Processing of Flax* ed. Sharma, H.S.S. and Van Sumere, C.F. pp. 157–198. Belfast, UK: M Publications.

