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Exploitation of bacterial pectinolytic strains for improvement of hemp water retting

Pectinolytic bacteria in water retting

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Summary

Retting is the major limitation to an efficient production of textile hemp fibres. Traditional retting has been carried out by autochthonous bacterial community. Aerobic and anaerobic pectinolytic strains were isolated from hemp or flax sources and characterised. Anaerobic pectinolytic strains had a wide range of acid polygalacturonase (PG) activity, whereas aerobic isolates did not produce any acid PG activity, but only an alkalophylic one, suggesting they could play a minor role in the retting process, except in the early stages. Analysis of 16S rDNA sequences assigned anaerobic strains to the *Clostridium* genus and aerobic isolates to the *Bacillus* and *Paenibacillus* genus. *C. felsineum* and *C. acetobutylicum* were confirmed as the main anaerobic agents. Nevertheless, a high proportion of anaerobic and aerobic pectinolytic strains was assigned to *C. saccharobutylicum* and *B. pumilus*, respectively, both species never being described as involved in water retting. Anaerobic and aerobic strains with high PG activity were selected and characterized. PG activity is well correlated with the strain retting efficiency and improvement of the process was obtained by inoculating the retting water with spores of selected aerobic and anaerobic bacteria. An advisable feature of retting strains is the absence of cellulosolytic activity. An aerobic strain with no cellulosolytic activity was identified. In contrast, all the anaerobic isolates showed cellulosolytic activity. Mutagenesis was ineffective for selection of Cel-Pec$^+$ mutants. Localization of the *C. felsineum* L1/6 PG activity was investigated.

Abbreviations: 16S rDNA: 16S rRNA gene, ARDRA: amplified ribosomal DNA restriction analysis, CBD: cellulose-binding domain, CMC: carboxymethylcellulose, CMCase: CMC-degrading, DNS: dinitrosalicylic acid, PG: polygalacturonase

Introduction

For textile productions, bast fibres of hemp (*Cannabis sativa*) stems must be released from the amorphous pectic matrix in a process known as retting. After retting, fibres are mechanically separated from other material by scutching.

During the past years, retting process has been carried out by microbiological or chemical treatments. Chemical retting included treatments with acids and bases (Dujardin, 1948), surfactants, and chelators (Henriksson et al., 1998). However, this process is considered expensive, due to the high consumption of chemicals and energy, and environmental unfriendly,

furthermore, it produces low-quality fibres (Van Sumere, 1992). Traditionally, two different types of microbiological retting were mainly adopted, that are dew and water retting. Our knowledge about microbiological retting mainly derives from studies on flax, while hemp was less investigated. During microbiological retting, depolymerisation of pectin is operated by pectinases, a mixture of primarily four enzymatic activities: polygalacturonase (PG), pectin and pectate lyase and pectin esterase; however, the primary retting agent is PG activity (Chesson, 1978; Akin et al., 2001; Zhang et al., 2000).

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 (Henriksson et al., 1997). Important properties of colonising fungi are the high level of pectinase activity, ability to attack non-cellulosic cell types without attacking cellulosic fibres, capacity to penetrate the cuticular surface of the stem, and efficient fibre release from the core (Henriksson et al., 1997).

In water retting, hemp straw is soaked in large tanks filled with water. Stalk soluble compounds (e.g., sugars) are solubilized, allowing the development of a bacterial community. Water penetration also detaches the bark and retting bacteria penetrate to degrade pectic substances. A bacterial succession was observed: the first to grow are aerobic bacteria, which, consuming all the oxygen present, are followed by anaerobic bacteria. The main pectinolytic agents are *Bacillus* spp., in the aerobic phase, and *Clostridium* spp., in the anaerobic one. During the course of retting, the pH value decreases to acid values (about 4.0) (Donaghy et al., 1990).

A modification of water retting is enzymatic treatment, where degrading enzymes are directly added to tank water (Sharma and Van Sumere, 1992). Despite the complexity of plant stem, the flax retting process was successfully carried out using the purified endo-PG of *Aspergillus niger*, demonstrating the importance of this enzymatic activity as primary retting agent (Zhang et al., 2000). However, at the present time, enzymatic retting is not yet feasible, due to the high cost of the process.

In Mediterranean regions, a return to hemp fibre production for textile industry should be based on water retting. Dew retting is usually less uniform than the water process, being affected by the fungal colonization along the plant stems (Akin et al., 1998). Moreover, risk of over-retting occurs due to the proliferation of cellulosolytic fungi, such as *Epicoccum nigrum* (Henriksson et al., 1997).

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

Since bacteria are the main retting agents, their properties affect the course of the process and the quality of the product. The aim of our work was the selection and characterisation of bacteria with high-retting activity and their use as *inoculum* of hemp water retting tanks for improvement of the process.

Materials and methods

Isolation of bacterial strains

Bacterial strains were isolated from the liquor of hemp or flax water retting tanks and from unretted or water retted fragments of flax and hemp. Straw was washed with sterile distillated water. Ret liquor and straw washing water were heated at 80 °C for 5 min to select spore-forming bacteria.

Aerobic strains were grown on solid rich medium (0.5% yeast extract, 0.5% peptone, 1% tryptone) at 30°C. Anaerobic strains were isolated on the same medium, supplemented with 0.05% cysteine and 2% glucose, and grown at 37 °C, under anaerobic conditions (Oxoid AnaeroGen, U.S.A.).

Enzymatic assays

Pectinolytic strains were identified by growth on solid rich medium containing 0.5% (w/v) pectin from citrus peel (Sigma-Aldrich, U.S.A.). After growth, plates were flooded with a 1% cetyltrimethyl ammonium bromide solution (Donaghy et al., 1990). A clearing around a colony indicated pectinolytic activity. Cellulosolytic strains were identified by growth on solid rich medium containing 0.1% (w/v) medium viscosity carboxymethylcellulose (CMC, Sigma-Aldrich, U.S.A.). After growth, plates were flooded with a 0.15% Congo red and washed twice with 1M

NaCl. A decolourised halo around a colony indicated CMC-degrading (CMCase) activity.

PG activity was measured in the supernatants of cell cultures by the dinitrosalicylic acid (DNS) method (Miller, 1959). Aerobic strains were grown for 2 days at 30 °C with shaking (250 rpm), while anaerobic strains were grown for 3 days at 37 °C in CO_2 atmosphere, in rich medium supplemented with 0.5% pectin. For anaerobic strains, PG activity was tested in a reaction mixture (1 ml volume) containing 50 mM citrate buffer pH 4.8 and 0.9% (w/v) polygalacturonic acid (Sigma-Aldrich, U.S.A.). After incubation for 20 min at 45 °C, the reaction was terminated by adding 3 ml of DNS and 1 ml of water, followed by heating at 100 °C for 10 min. The suspension was centrifuged and the supernatant's O.D. was measured at 640 nm. PG activity of aerobic strains was tested by DNS method as described by Kobayashi et al. (2001). CMCase activity was assayed by DNS method (Miller et al., 1960). PG and CMCase activities were determined by reference to standard galacturonic acid or glucose solutions, respectively. One unit (I.U.) corresponds to the release of 1 μmole of reducing groups (final products of the reaction) in 1 min at the assay temperature. Activities were determined in two separate experiments with two independent measurements each.

Molecular analysis of bacterial strains

Bacterial DNA was extracted from cells grown on solid rich medium using the FastDNA kit (Q BIO gene, Canada). The 16S rRNA gene (16S rDNA) was amplified with two universal primers for bacteria, as previously described (Tamburini et al., 2001).

For amplified ribosomal DNA restriction analysis (ARDRA), approximately 1.5 μg of amplified DNA was completely digested according to the manufacturer's specifications for 3 h with 3 units of the restriction endonucleases *Alu*I, *Rsa*I and *Hinf*I in single reactions (Invitrogen, U.S.A.). The restriction products were electrophoreted on a 2.5% (w/v) agarose gel in TAE buffer containing 0.5 μg ml^{-1} ethidium bromide. The nucleotide sequence of the amplified fragments was determined with an ABI 310 sequence analyser (Applied Biosystems, U.S.A.).

The 16S rDNA sequences were compared with the prokaryotic small subunit rRNA sequence database of the Ribosomal Database Project II (Maidak et al., 2001) and with GenBank by BLASTn program (Altschul et al., 1990).

Plant material and retting experiments

Plastic tanks filled with well water (about 10 l) were used. Each tank was filled with four bundles of hemp stalks, each formed of four stems. One tank was used as control; the remaining ones were inoculated with about 10^5 spores ml^{-1} of different strains of pectinolytic bacteria. When needed, aeration was obtained by bubbling air with a pump. Retting temperature was 24 °C.

Mutagenesis

Spores of the L1/6 strain were irradiated with UV radiation (260 nm) for 2 min. Spores were grown on solid medium at 37 °C under anaerobic conditions. Single colonies were tested for cellulosolytic and pectinolytic activities on solid medium, as previously described.

Absorption of PG and CMCase activities to Avicel

An aliquot (300 μl) of supernatant from a L1/6 culture, grown in rich medium with 0.5% pectin, was mixed with 1 mg bovine serum albumin and 10 mg of Avicel microcrystalline cellulose (FMC, U.S.A.). The suspension was incubated at 37 °C for 60 min and mixed every few minutes. After incubation, the sample was centrifuged and CMCase and PG activities were measured in the supernatant.

Results

Isolation and genotypic characterisation of pectinolytic aerobic and anaerobic strains

A collection of 104 anaerobic and 23 aerobic spore-forming pectinolytic bacteria were isolated from flax and hemp sources (Tamburini et al., 2003).

All the isolates were genotypically characterized using a well-established method of analysis of bacterial communities (Picard et al., 2000; Pukall et al., 2001), that is grouping of strains by ARDRA (Vaneechoutte et al., 1992) and sequencing the 16S rDNA of at least a member of each ARDRA group. ARDRA haplotypes are often species-specific (Grifoni et al., 1995; Heyndrickx et al., 1996).

The 104 pectinolytic anaerobic isolates were grouped in five different ARDRA haplotypes (Figure 1). Analysis of almost complete 16S rDNA sequences, of at least a strain from each ARDRA haplotype, confirmed all the anaerobic strains are members of the genus *Clostridium*.

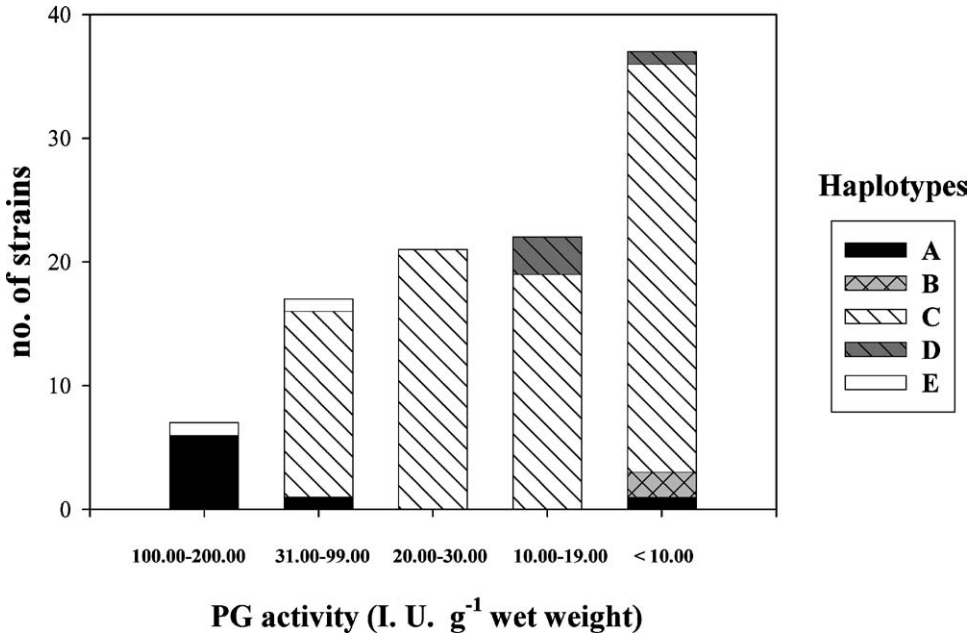

Figure 1. PG activity and ARDRA haplotypes of anaerobic strains. *C. felsineum* NCIMB 10690^T reference strain (haplotype A) has an activity of 30.00 I.U. g^{-1} cells wet weight.

The analysis showed that 84.6% of the pectinolytic isolates (haplotype C) are phylogenetically closely related to *C. saccharobutylicum* and 9.6% (haplotype A and E) to the *C. felsineum – C. acetobutylicum* cluster (Tamburini et al., 2001).

The 23 pectinolytic aerobic strains were grouped in three ARDRA haplotypes (Figure 2). All aerobic isolates were assigned to the *Bacillus* genus, except for a single strain (ROO32A), which clusters with *Paenibacillus amylolyticus* (haplotype H). Thirteen percent of the aerobic isolates were phylogenetically related to *B. subtilis* (haplotype G) and 82.6% to *B. pumilus* (haplotype F).

Characterisation of PG activity

The PG activity of all the pectinolytic strains was measured in the supernatant of liquid cultures grown in rich medium with 0.5% pectin and compared with the activity of the reference strains. This enzymatic activity was chosen since it is considered the most important one in the retting process (Chesson, 1978; Zhang et al., 2000; Akin et al., 2001).

The activity of anaerobic strains covered a wide range; 24 anaerobic isolates showed a PG activity higher than *C. felsineum* reference strain (>31.00 I.U. g^{-1} cells) and, among them, seven showed an activity >100.00 I.U. g^{-1} cells.

Sequence and molecular analysis assigned the most active strains to the *C. felsineum – C. acetobultyicum* cluster or to *C. saccharobutylicum*. Isolates related to *C. saccharobutylicum* showed a wide range of PG activity, whereas all the strains, except one, related to the *C. felsineum – C. acetobultyicum* cluster have a high PG activity. Strains with low PG activity were assigned to two minor haplotypes B and D.

None of the aerobic pectinolytic isolates showed a detectable PG activity under the conditions used for anaerobic bacteria (citrate buffer pH 4.8, 45 °C). The same result was obtained at different incubation temperatures (32, 37 and 40 °C). On the other hand, most of the isolates showed a detectable PG activity under the experimental condition previously described by Kobayashi et al. (2001), Tris-HCl buffer pH 8.0 and 30 °C. Nine aerobic isolates showed a PG activity higher than the reference strain *B. subtilis* 168 (>39.00 I.U. g^{-1} cells). All the strains with high PG activity are related to *B. subtilis* or *B. pumilus*.

Retting tests with selected strains

Eleven anaerobic strains selected for their high level of PG activity were previously used in laboratory tests

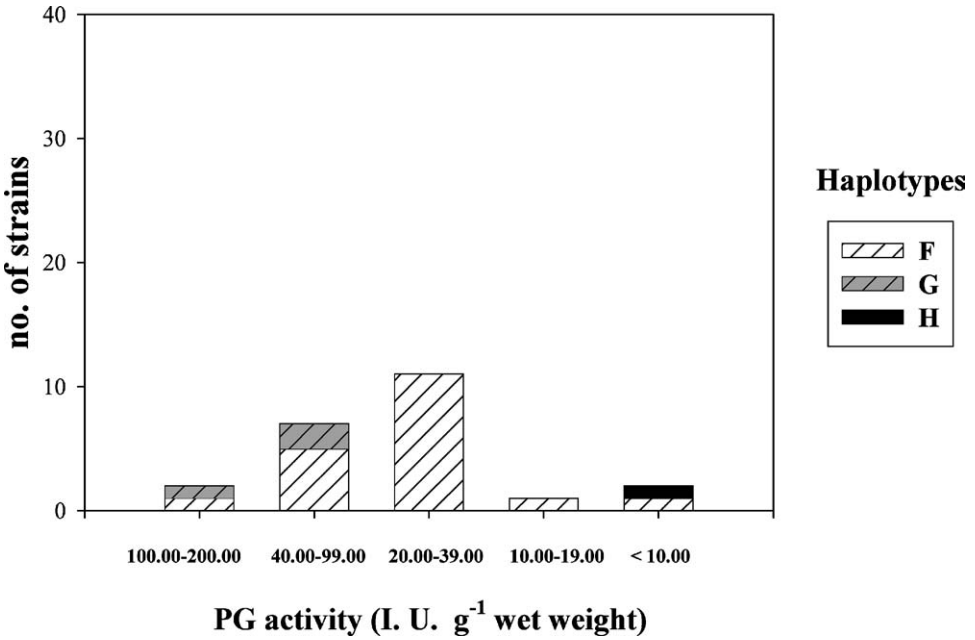

Figure 2. PG activity and ARDRA haplotypes of aerobic strains. *B. subtilis* 168 reference strain (haplotype G) has an activity of 39.00 I.U. g^{-1} cells wet weight.

of water retting (Di Candilo et al., 2000). Two of these strains, belonging to the *C. felsineum – C. acetobutylicum* cluster (L1/6 and C1/6), significantly reduced retting time, giving the optimal degree of retting within the sixth day. The control (no *inoculum*) required 12 days (Di Candilo et al., 2000).

L1/6, which has good retting ability, was also tested together with an *inoculum* of an aerobic strain with high PG activity (ROO2A). Retting tests were also performed with ROO2A alone and aeration during the whole process. Observation of the retted stalks (Figure 3) showed that the best results were obtained with the double *inoculum* (aerobic and anaerobic bacteria together). Under these conditions, retting took place in four days. In the tank inoculated with the anaerobic strain only the process required 6 and 12 days in the control. Retting under aerobic conditions (aerobic strain plus aeration) took as long as the control, but the fibre was of poor quality and showed a dark staining already after eight days of incubation (Figure 3B).

Characterisation of CMCase activity and distribution of PG activity

An advisable feature of pectinolytic strains to be used in retting is the absence of cellulosolytic activity. All the pectinolytic aerobic and anaerobic isolates were characterised for CMCase activity on solid medium. All the clostridial strains and the strains related to *B. subtilis* and *P. amylolyticus* are cellulosolytic (Cel^+), while 57.9% of the strains belonging to the major haplotype F, phylogenetically related to *B. pumilus*, showed no CMCase activity (Table 1).

The absence of CMCase activity in the aerobic strains was also confirmed by measuring the enzymatic activity in the supernatant of liquid cultures. Interestingly, strain ROO40B, which displays the highest PG activity, has no cellulosolytic activity. This strain showed high retting efficiency in laboratory tests when inoculated with the anaerobic strain L1/6, and a complete retting was obtained in four days (data not shown).

Since all the Pec^+ anaerobic strains screened were Cel^+, spores of L1/6 were subjected to UV mutagenesis. A total of 7000 colonies were screened for pectinolytic and cellulosolytic activity on solid medium. A total of 16 Cel^- mutants were identified, however, all of them were Pec^-. The difficulty to isolate Pec^+ Cel^- mutants could be due to the association of PG and CMCase activities in the same multi-enzymatic complex. Several clostridial species produce a multicomponent enzymatic complex, the cellulosome (Doi et al., 2003). In order to determine the distribution of PG activity, the supernatant of L1/6 cultures, grown on pectin, was

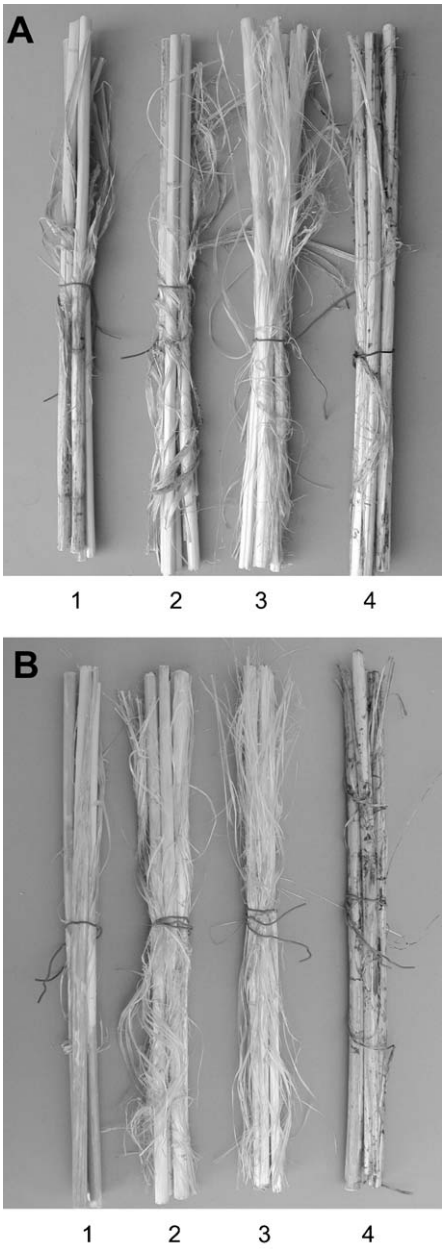

Figure 3. Hemp stems retted in water tanks for: (A) 4 days; (B) 8 days. 1. Control, tank not inoculated; 2. tank inoculated with spores of anaerobic strain L1/6; 3. tank simultaneously inoculated with spores of aerobic (ROO2A) and anaerobic (L1/6) strains; 4. tank inoculated with spores of aerobic strain ROO2A and aeration maintained all the time.

fractionated by binding to Avicel. The fraction that did not bind to cellulose was the noncellulosomal one. Under this condition, 79.5% of PG activity was found in the noncellulosomal fraction, compared to 46.8% of

Table 1. Pectinolytic and cellulosolytic activity of aerobic strains belonging to haplotype F

Strain	CMC degrading activity	PG activity[a]
ROO40B	−	169.0
ROO71AI	−	80.2
ROO9A	+	52.6
ROO71AII	−	50.8
ROO72B	−	46.8
ROO37A	+	42.0
ROO43A	−	36.5
ROO35BII	−	33.5
ROO31A	−	33.4
UNO51AI	++	30.7
ROO62A	++	30.1
ROO14A	−	29.2
UNO51AII	+	27.5
ROO78BI	−	26.0
ROO66B	+	22.5
ROO66A	++	21.5
ROO9B	−	20.5
ROO62B	+	18.5
ROO37B	−	7.8

[a]Expressed as I.U. g^{-1} of cells wet weight.

Table 2. Adsorption to Avicel of PG and CMC-degrading (CMCase) activities of L1/6

PG activity (I.U. ml^{-1})		CMCase activity (I.U. ml^{-1})	
Total	Unbound	Total	Unbound
2.24	1.78 (79.5%)	0.47	0.22 (46.8%)

CMCase activity (Table 2). The cell associated PG activity was also measured. Cell-associated PG activity was 170.00 I.U. g^{-1} of cells wet weight, a value that is comparable with the supernatant fraction.

Discussion

Traditionally hemp retting has been carried out by autochthonous bacterial communities present both in soil and on plants. However, an industrial application needs improvement and standardisation of the process. This work goes in this direction.

Our strategy involved the isolation of spore-forming pectinolytic bacteria from hemp or flax sources

and the selection of strains with high PG activity, which appears to be the main retting agent. Indeed PG activity, with an optimum pH close to that of the ret liquor, predominates throughout water retting (Chesson, 1978). Furthermore, retting of flax was achieved by using the purified endo-PG of *Aspergillus niger* (Zhang et al., 2000). A total of 23 aerobic and 104 anaerobic pectinolytic strains were isolated. Phylogenetic characterisation by 16S rDNA sequence analysis assigned the anaerobic strains to the *Clostridium* genus and the aerobic isolates to the *Bacillus* or to the *Paenibacillus* genus. Determination of acid PG activity showed that the anaerobic pectinolytic strains were highly heterogeneous. However, none of the pectinolytic aerobic isolates showed PG activity at acid pH, that is close to that of the ret liquor (Chesson, 1978; Donaghy et al., 1990). This suggests that PG enzymes produced by aerobic bacteria play a minor role in the retting process, except in the early stages before the ret liquor becomes acid. On the other hand, the aerobic strains showed PG activity at pH 8.0.

Anaerobic (24) and aerobic (9) isolates with an enzymatic activity higher than the reference strains *C. felsineum* and *B. subtilis* were selected. Anaerobic isolates with high PG activity were related to *C. acetobutylicum–C. felsineum* and *C. saccharobutylicum* species, whereas aerobic isolates were related to *B. subtilis*^{T} and *B. pumilus*^{T}.

This characterisation confirms *C. felsineum* and *C. acetobutylicum* as the main anaerobic agents and *B. subtilis* as an important pectinolytic species in water retting (Donaghy et al., 1990). Nevertheless, the highest proportion of anaerobic and aerobic pectinolytic strains was assigned to *C. saccharobutylicum* and *B. pumilus*, respectively. Both species has never been described as involved in water retting. However, the importance of *C. saccharobutylicum* was probably underestimated due to taxonomic errors; indeed several strains, previously classified as *C. acetobutylicum* on the basis of phenotypic traits, have been recently included in this species by genotypic characterisation (Keis et al., 2001).

PG activity is well correlated with retting efficiency. Inoculation of water tanks with spores of the most active strains reduced by half the time required for the process, from 12 to 6 days (Di Candilo et al., 2000).

A further improvement of the process was obtained when the tanks were simultaneously inoculated with spores of aerobic and anaerobic bacteria. Under these conditions retting was completed in four days. The double *inoculum* probably improves the process, because aerobic bacteria rapidly generate the anoxic conditions needed by anaerobic bacteria to grow and degrade pectin. Hemp retting with the aerobic strain only, and with aeration, took as long as the control and the fibre had a dark colour.

To further improve retting control, it is desirable to select strains free of cellulosolytic activity. Prolonged treatment of hemp stems with cellulosolytic bacteria could otherwise result in the loss of fibre strength, due to a direct attack to cellulosic fibres. Thus, one of the scopes of our work was the selection of PG producing strains deficient in cellulase activity. From the screening of aerobic pectinolytic strains, we identified ROO40B, which has no CMCase activity both in liquid and solid medium. On the contrary, all the pectinolytic anaerobic isolates showed cellulosolytic activity.

UV mutagenesis of L1/6 spores was an ineffective strategy for selection of CMCase negative strains, since all the Cel^{-} mutants were also Pec^{-}. A possible reason for the difficulty to isolate Pec^{+} Cel^{-} mutants could be the association of PG and CMCase activities on the cellulosomal complex. At present, our knowledge about the structure and genetics of clostridial pectinases is limited to the pectate lyase, PelA, of *C. cellulovorans*. PelA is associated to the cellulosome by dockerin domains and contains a putative CBD (Tamaru and Doi, 2001). Two putative PG-coding genes (CAC0355 and CAC3684) have been identified in the completely sequenced genome of *C. acetobutylicum* (Noelling et al., 2001). Analysis of their protein structures revealed the putative PG AAK81605.1 presents an N-terminal signal peptide, whereas none of the putative PG contains dockerin domains or CBD. *C. felsineum* L1/6 PG activity has probably similar structural properties; indeed the majority of PG does not bind to crystalline cellulose under our experimental conditions. Thus, the PG secreted by L1/6 does not seem to be associated to the cellulosome or to contain any CBD. A cell-associated activity was also found. The high frequency of double Cel^{-} and Pec^{-} mutants could be due to a common regulatory mechanism of cellulose and pectin degradation. Elucidation of these processes is essential to obtain strains free of cellulosolytic activity.

Acknowledgments

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