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ORIGINAL ARTICLE

Pectinolytic activity secreted by yeasts isolated from fermented citrus molasses

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ABSTRACT

Aims: The aim of this study was to obtain improved strains of pectinolytic yeasts adapted to the conditions of an industrial fermentation process, which was continuously operated to convert citrus molasses into ethanol.

Methods and Results: The starter yeast of the industrial fermentation process was a commercial baker's yeast, which was capable of growing without forming any secretion halo of pectinase activity on solid medium. Nevertheless, isolates showing secretion of pectinolytic activity on plates were obtained from the fermentation process. The secretion of pectin-degrading activity by isolates on plates was repressed by galactose and improved as the result of colony aging on polygalacturonic acid plates at 30°C. Liquefaction of polygalacturonate gels as well as the splitting of the pectin-degrading activity into a wall-linked and a supernatant fraction were also observed when the starter yeast was propagated under agitation in liquid medium containing pectin.

Conclusions: Isolates capable of secreting pectinolytic activity on plates were predominant at the end of the citrus molasses fermentation. Nevertheless, the sizes of the secretion haloes on plates were not necessarily an indication of the levels of pectinolytic activity secreted in the liquid medium.

Significance and Impact of the Study: Improved pectinolytic strains of *Saccharomyces* can be used as a source of pectinases for a variety of applications. This organism also participates in plant deterioration processes.

Introduction

Pectin, the major constituent of plant cell walls, is a complex heteropolysaccharide mainly composed of D-polygalacturonic acid residues. Pectinolytic complexes have been isolated from plants, filamentous fungi and bacteria (Fogarty and Kelly 1983; Sakai *et al.* 1993). The major industrial application of pectic enzymes includes extraction and clarification of fruit juices, grape must, wine technology, maceration of vegetables and fruits, and extraction of vegetable oils (Kashyap *et al.* 2001; Gummadi and Panda 2003).

Yeasts and molds have been identified in spoiled citrus products (Parish and Higgins 1989). However, only a few strains of *Saccharomyces sensu stricto* showed the ability to catalyse the depolymerization of pectins (Gainvors *et al.* 1994a; Blanco *et al.* 1999) as well as to clarify fruit juices (Gainvors *et al.* 1994b). In a recent report, cells of *S. cerevisiae* invaded grapevine tissues causing cell death (Gognies *et al.* 2001). In addition, *S. cerevisiae* and *Penicillium membranifaciens* were the predominant yeasts responsible for the tray fermentation of West African cocoa beans rich in pectin (Jespersen *et al.* 2005).

Typical yeasts found in citrus juice are *Candida parapsilosis*, *C. stellata*, *S. cerevisiae*, *Torulaspora delbrueckii* and *Zygosaccharomyces rouxii* (Hatcher *et al.* 2000). However, there are only a few reports investigating the yeast species associated with citrus juice (Recca and Mrak 1952; Parish and Higgins 1989; Deak and Beuchat 1993). Yeast and bacteria appear to depend on symbiotic interactions during natural fermentation of reconstituted orange juice (Cancalon and Parish 1995). In the states of Florida

(USA) and São Paulo (Brazil), all citrus juice extraction plants are equipped with feed mills that produce citrus molasses.

Pectinase production by *S. cerevisiae* is not induced by pectin, polygalacturonic acid nor galacturonic acid but is regulated by monosaccharides and oxygen dissolved into the medium (Blanco *et al.* 1999). The secretion polygalacturonase of *S. cerevisiae* is induced by galactose and repressed by glucose (Blanco *et al.* 1994). In the present work, pectinolytic yeasts showing secretion of activity during growth on both polygalacturonic acid and pectin plates were isolated from fermented citrus molasses. In addition, recombinant cells, showing increased pectinolytic activity, arose during maturation of isolate colonies on polygalacturonic acid plates. Capacity to liquefy polygalacturonic acid gels and the effects of monosaccharides on the secretion of the pectinolytic activity produced by *Saccharomyces* were also determined for a part of the isolates. Lastly, the partitioning of the pectin-degrading activity at the cell surfaces was also studied in liquid cultures.

Materials and methods

Source of organisms, isolation and identification procedures

Yeasts were isolated from 15 samples of fermented-citrus molasses collected from six reactors linked in series in an alcohol plant, which was under continuous operation with cell recycling for 6 month before sampling. Citrus molasses, herein referred to as being the liquid concentrate resulting from the pressing of the orange peel (Rebeck 1990), was kindly supplied by Citrosuco S. A., (Matão, São Paulo, Brazil) as having the following composition: glucose (20·7 g l^{-1}), fructose (28·8 g l^{-1}), sucrose (22·3 g l^{-1}), total sugars (71·8 g l^{-1}), pH 4·77, hesperidine (8·75 g l^{-1}), total pectin (0·80 g l^{-1}), calcium (0·14 g l^{-1}), phosphate (0·43 g l^{-1}), ash (7·70 g l^{-1}), potassium (1·8 g l^{-1}) iron (17·0 mg l^{-1}), sodium (57·7 mg l^{-1}), oil (0·04%) and bottom pulp (22·2%).

Samples taken from the industrial reactors were diluted and plated on the isolation medium (YPD medium containing propionic acid and Rose of Bengal). A total of 201 colonies (Malavolta 2000) showing different sizes and morphologies were selected and maintained at 4°C on YP-pectin medium for further studies. The classical taxonomy assays recommended by Middelhoven (2001) as well as the API ID 32C kit (BioMérieux S.A., Marcy l'Etoile, France) were used to give a rapid identification of the isolates.

A commercial baker's yeast (supplied by Fleischmann & Royal, Jundiaí, Brazil) was used as starter yeast to produce ethanol in the industrial alcohol plant from which the isolates were obtained for the present study. Strain LTU-4, used as reference for the experiments reported in the present work, resulted from plating of commercial baker's yeast on solid YNB-PG medium.

Media composition

Six different solid media were used and prepared as follows: YPD medium (1% yeast extract, 2% peptone, 2% dextrose and 2% agar) containing Bengal Rose (0·035 g l^{-1}) and propionic acid (0·2%, v/v) used to obtain isolates; YNB-pectin medium containing 0·67% yeast nitrogen base (YNB), 1% citrus pectin (64–72% methoxylated pectin provided by Citrus Colloids, Limeira, São Paulo, Brazil, lot no. 8105) and 0·5% agarose (A-9539) from Sigma (St Louis, MO, USA) in 50 mmol l^{-1} potassium phosphate buffer at pH 5·5 plus monosaccharides (glucose or galactose) at concentrations varying from 0·2% to 1% (w/v) depending on the experiment; YNB-PG medium prepared to contain 1% polygalacturonic acid (PG from Sigma, P-3850) as described by McKay (1988) plus monosaccharides (glucose and galactose at concentrations varying from 0·2% to 1%, depending on the experiment) and 0·5% agarose from Sigma, A-9539; YP-pectin medium used for culture maintenance and prepared to contain 1% yeast extract, 2% peptone, 1% citrus pectin (64–72% methoxylated pectin) plus 2% agar; sporulation medium prepared and containing 1% potassium acetate plus 2% agar, as previously described by Jacket and Jauniaux (1999). YNB-PG and YNB-pectin media deprived of agarose and having the same composition as described above for the corresponding solid media were also used in the present work. The liquid YNB-PG medium jellified when its temperature was lowered to 27–28°C after sterilization.

Pectinolytic activity on plates

Isolates were inoculated by spreading the cells along a line marked (5-mm long) on the rear surface of the plates containing YNB-pectin (64–72% methoxylated pectin) or YNB-PG medium with added amounts of glucose or galactose as required by the experiment. After 5-days growth at 30°C, the plates were flooded with a 0·1% ruthenium red (Sigma, R-2757) solution and incubated at room temperature for one hour (Oliveira 2004). Then, the ruthenium red solution was removed from the plates and the surface of the medium was carefully rinsed with distilled water. The pectin degradation was indicated by a colourless halo contrasting with the red colour shown on the stained medium surface. The polygalacturonase activity was indicated by the purple halo formed around the colonies on polygalacturonic acid plates as described by

McKay (1988). Smaller haloes were visualized after 2–3 days growth, indicating that secretion of pectinases occurred during growth.

Colony aging on polygalacturonic acid plates

This procedure was based on the one described by Purnapatre and Honigberg (2002). The arising of recombinant cells was indicated by irregular edges visualized around the periphery of the isolate colonies after 15-days maturation on YNB-PG medium containing 0·2% glucose. In order to minimize the water evaporation, the plates were incubated at 30°C in a box containing pieces of paper soaked in water. Samples were removed with a toothpick from the periphery of the colonies, which had irregular edges, and plated on the same medium to obtain the single colonies used in polygalacturonase assays carried out on plates.

Sporulation procedure

Cells were transferred from stock cultures to the sporulation medium and incubated for 4–5 days at 30°C, as described by Jacket and Jauniaux (1999). Spore formation was observed under a microscope.

Assays for pectinolytic activity in supernatants and linked to intact cells

Assays were carried out in a reaction mixture containing the following (1·0 ml final volume): 0·5% commercial pectin (64–72% methoxylated pectin) in 25 mmol l^{-1} acetate buffer at pH 5·0 plus cell suspension (10^7 cells ml^{-1} in the reaction mixture) previously washed three times in the same buffer solution. The reaction mixture was incubated in horizontal water (agitated at 250 strokes min^{-1}) for 10 min at 50°C. Then, the reaction was stopped by adding 2 ml of 3,5-dinitrosalycilic acid solution as described by Miller (1959). After heating for 5 min in boiling water, the reaction mixture was diluted by adding 5·0 ml of water and was centrifuged (5000 ***g*** for 5 min) to separate out the insoluble pectinolytic materials formed during reaction. Then, the absorbance at 546 nm was determined in the supernatants. A control mixture deprived of pectin (to assay the reducing groups present in the enzymatic samples) and another mixture deprived of cells (to assay the reducing groups present in the substrate) were assayed in parallel. One enzymatic unit was expressed as μmol of reducing groups liberated per min per 10^7 cells in 1 ml of reaction mixture (U 10^7 $cells^{-1}$). Galacturonic acid was used as standard.

The same procedure was used to assay the pectinolytic activity in supernatants resulting from growth on YNB-pectin medium. In this case, samples of washed cells suspend in buffer solution were exchanged for samples of culture supernatant in order to start the enzymatic reaction.

Qualitative assay for gel liquefaction and cell counts

After sterilizing (sterilization for 20 min in an autoclave), 3 ml of YNB-PG medium containing added 0·2% monosaccharides (galactose or glucose), gels were formed when room temperature (27–28°C) was reached. Then, a loopful of cells was transferred from the stock cultures and deeply inserted into the gel. After 2 days at 30°C without shaking, the tubes, incubated in the vertical position, were inclined in order to show the gel liquefaction, which was indicated by the spreading of a viscous slurry over the side-wall of the test tubes. Cell counts were carried out in a Neubauer chamber and expressed as cell ml^{-1} suspensions.

Results

The enrichment of the yeast population with pectinase-producing cells was observed in industrial reactors linked in series and continuously operated with cell recycling, as described below. Assays for pectinase secretion, effects of simple sugars (glucose and galactose) on pectinase secretion on plates, liquefaction of polygalacturonic acid gels and the appearance of recombinants resulting from colony aging on polygalacturonic acid plates were described for some isolates. Partitioning of the pectin-degrading activity at the cell surfaces in liquid medium was also described.

Screening of isolates for pectinase secretion on plates

Polygalacturonase activity was assayed on plates containing polygalacturonic acid (McKay 1988) while pectin-degrading activity was assayed using pectin plates (present work). Figure 1 illustrates both pectin-degrading activity [indicated by transparent haloes of pectinase activity against a purple–red background on pectin plate (A)] and polygalacturonic acid-degrading activity [purple–red haloes of polygalacturonase activity against a lighter-coloured background on polygalacturonic acid plate (B)] by three yeast strains. Reference strain LTU-4 exhibited good growth, as shown in Fig. 1, without secreting pectinolytic activity on either plate containing pectin or polygalacturonic acid. The widest haloes of enzyme secretion were obtained on both plates and this was also confirmed by data shown in Table 2 for recombinant strain 15MB/2.

Two hundred and one isolates, resulting from plating of samples withdrawn from the industrial fermentation

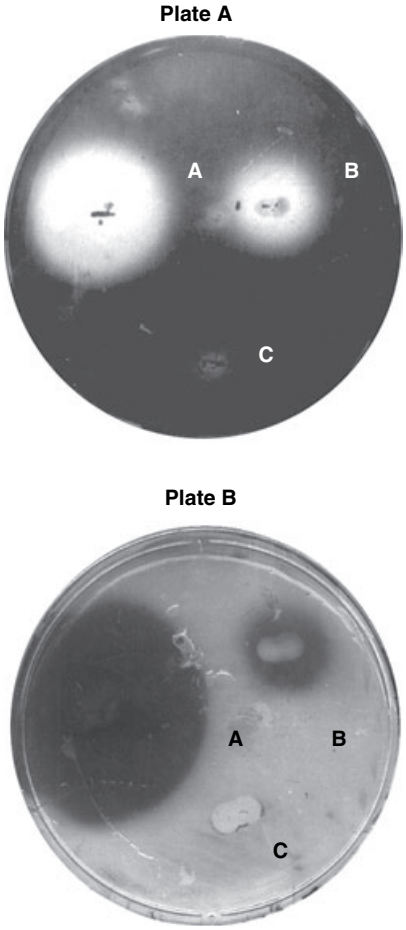

Figure 1 Secretion haloes formed on a pectin plate (plate A, containing 1% pectin and 0·2% glucose) and polygalacturonic acid plate (plate B, containing 1% polygalacturonate and 0·2% glucose) by the following isolates: (A) 15MB/2 (a recombinant strain derived from isolate 15MB), (B) isolate 5MV, (C) LTU-4 (reference strain).

system, were assayed on plates containing pectinolytic substrates and 1% glucose in order to indicate the secretion of both pectin-degrading and polygalacturonase activities. Based on the size of the haloes of pectinolytic activity, the isolates were arranged into 10 groups as shown in Table 1. A significant part of the isolates from these groups did not secrete any of the two-pectinolytic activities (31·5% of the total isolates, as described in Table 1 for group X) being the same observed for the reference strain LTU-4. A very small number of isolates (two isolates in group I representing 1·0% of the total isolates as shown in Table 1) secreted high levels of both activities on pectin and polygalacturonic acid plates. In addition, a greater number of isolates (15%, comprising isolates from groups IV and IX, Table 1) showed secretion of high pectin-degrading activity on plates containing pectin while polygalacturonase activity was not secreted

Table 1 Isolate grouping based on the halo sizes resulting from growth on solid screening media containing pectin (64–72% methoxylation) and polygalacturonic acid as pectic substrates

Groups (no. of isolates in each group)	Halo sizes on plates*		
	Pectin (YNB-pectin medium)†	Polygalacturonate (YNB-PG medium)†	Relative frequency (%)
Group I (2)	Large	Large	1·0
Group II (7)	Medium	Large	3·5
Group III (6)	ND	Large	3·0
Group IV (5)	Large	ND	2·5
Group V (8)	Large	Medium	4·0
Group VI (33)	Medium	Medium	16·5
Group VII (17)	Small	Medium	8·5
Group VIII (34)	ND	Medium	17·0
Group IX (25)	Medium	ND	12·5
Group X (64)	ND	ND	31·5
Reference strain (strain LTU-4)	ND	ND	–

ND, not detected.

*Large halos = 20–35 mm, medium halos = 10–20 mm and small halos = 3–10 mm.

†Medium containing 1% glucose, 0·67% yeast nitrogen base and 1% pectic substrate as pectin (Malavolta 2000) or polygalacturonic acid (McKay 1988).

on plates containing polygalacturonic acid. On the contrary, other isolates (20%, comprising isolates from Groups III and VIII as shown in Table 1) showed high secretion of polygalacturonase activity on plates without exhibiting any secretion of pectin degradation on the plates. However, a significant portion of the isolates (16·5%, comprising isolates from group VI as shown Table 1) showed haloes of medium sizes on both pectin and polygalacturonate plates.

Recombinants derived from aging of isolate colonies on plates

After colony maturation for 15 days on polygalacturonic acid plates (YNB-PG medium), single colony of isolates in groups I, II and III (groups are described in Table 2) showed irregular edges (data not shown), as previously described by Purnapatre and Honigberg (2002) for colonies of *S. cerevisiae*. Cell samples taken from the irregular edges were plated on YNB-PG medium and single colonies showing improved secretion of pectinolytic activity were mainly obtained for isolates 15MB and 16B, both described in group I as shown in Table 2. Four recombinants derived from strain 15MB (recombinant strains 15MB/1 to 15MB/4) showed the highest secretion of both pectin-degrading and polygalacturonase activities (see Table 2). Isolates 16B and 15MB showed high sporulation

Table 2 Effect of monosaccharides associated to the pectinolytic substrates on the sizes of ruthenium red halos formed on the plates

Isolates (Malavolta 2000)			Transparent halos [(mm) YNB-pectin medium]		Purple halos [(mm) YNB-PG medium]	
Groups	Strains	Recombinants derived from isolates	0·2% glucose*	0·5% galactose†	0·2% glucose*	0·5% galactose†
I	15MB	–	15	ND	5	18
	–	15MB/1	38	ND	37	42
	–	15MB/2	33	ND	38	41
	–	15MB/3	30	ND	35	40
	–	15MB/4	28	ND	32	38
	16B	–	18	ND	10	25
II	1MV	–	2	ND	7	25
	5MV	–	12	ND	7	35
	14BB	–	12	ND	7	23
	1BBM	–	ND	ND	ND	23
III	7G2	–	ND	ND	ND	25
	9BB2	–	8	ND	6	17
	14MB1	–	8	ND	7	23
IV	40C	–	13	ND	ND	23
V	1MB	–	3	ND	7	25
VII	2PV1	–	8	ND	ND	25
X	7PV2	–	2	ND	ND	15

ND, not detected.
*Medium containing 1% pectic substrate (pectin or polygalacturonate) plus 0·2% glucose.
†Medium containing 0·5% pectic substrate (pectin or polygalacturonate) plus 0·5% galactose.

frequencies (33% of the total cells in sporulated cultures of strain 15MB and 22% in the case of strain 16B), as an indication of genetic instability. Single colonies, derived from isolates of other groups described in Table 1, were also not able to show increases in polygalacturonase secretion.

Effects of monosaccharides on the pectinase activity secreted on plates

Table 2 shows that the secretion of pectinolytic activity on plates was dependent on the monosaccharide added to the medium. In the presence of 0·5% galactose, the secretion of polygalacturonase was induced while the secretion of pectin-degradation activity was suppressed. In the presence of 0·2% glucose, decreases in the polygalacturonase secreted on plates by isolates were observed. On the other hand, the secretion of pectinolytic activity by recombinant strains derived from isolate 15MB was not significantly affected by the presence of 0·2% glucose when degrading both pectin or polygalacturonic acid.

Isolates described in Table 2 as well as reference strain LTU-4 were identified as *S. cerevisiae* when taxonomically assayed using both the API ID 32C kit and an identification key proposed by Middelhoven (2001).

Gel liquefying activity

Liquefaction of polygalacturonate gels by the reference strain LTU-4 as well as by one of the isolates (strain 14BB) were qualitatively assayed (data not shown) using a fast procedure as described in the present work. Intact cells of these two strains promoted the gel liquefaction, when deeply inoculated into jellified YNB-PG medium containing polygalacturonic acid at pH 5·5. Gels were converted into a viscous solution except when 0·2% glucose was added to the medium. The endopolygalacturonase activity secreted by cells of the two yeast strains diffused through the gel causing its liquefaction. Gel liquefaction was observed when 0·2% galactose was added to the medium but it did not occur in the presence of 0·2% glucose (data not shown).

Partitioning of the pectinolytic activity in liquid cultures

Table 3 shows that the pectinolytic activity was secreted into liquid YNB-pectin medium by the reference strain LTU-4 as well as two isolates (strains 5MV and 15MB). The pectin-degrading activity was split into two parts, one part being accumulated onto the surface of intact cells while the other was freed into the culture supernatants of each organism. The partitioning of the activity

Table 3 Partitioning of the pectin-degrading activity between the surface of intact cells and the supernatant during yeast growth on YNB-pectin medium

Strains	Pectin degrading activity	
	Wall-bound activity (U 10^7 cells^{-1})	Supernatant activity (U ml^{-1})
LTU-4 (reference strain)	0·442 ± 0·048	0·592 ± 0·025
5MV	0·147 ± 0·046	0·107 ± 0·007
15MB	0·118 ± 0·018	0·596 ± 0·062
15MB/2 (recombinant derived from strain 15MB)	0·279 ± 0·022	0·679 ± 0·011

varied among strains as follows: being greater in the supernatant of strain LTU-4 than in the wall-linked fraction; greater in the wall-linked fraction of strain 5MV; and greater in the supernatant of recombinant strain 15MB/2. Despite the lack of halo formed on plates, strain LTU-4, showed the highest activity linked to the surface of intact cells while strain 15MB/2 showed the highest activity in its culture supernatant, as described in Fig. 1 and Table 1.

Discussion

As *S. cerevisiae* is a GRAS (generally recognised as safe) organism, its use as a source of pectinases is of great interest to the food industry. After 6-months operation of an alcohol plant fed with citrus molasses, a significant part of the isolates showed haloes of different sizes on plates, indicating the secretion of pectinolytic activity able to degrade either pectin or polygalacturonic acid. Such enrichment of the cell population of the industrial process with pectinolytic-producing yeasts can be because of yeast adaptation and/or wild yeast invasion. Thus, fermentation conditions of the citrus molasses created an appropriate environment for the proliferation of pectinolytic yeasts. The secretion of a pectinase system by *S. cerevisiae* was described in a previous work, in which the pectin-degrading activity was greatly dependent on the degree of substrate methoxylation (Gainvors *et al.* 1994b).

In the present work, secretion of polygalacturonase by the isolates (*Saccharomyces*) was indicated by the purple–red haloes obtained on plates (McKay 1988), while the pectin-degradation was indicated by a colourless halo possibly due to the simultaneous secretion of polygalacturonase and pectin methylesterase. Colourless bands on pectin gels were described as an indication of the polygalacturonase activity produced in cultures of filamentous fungi (Cruickshank and Wade 1980). Nevertheless, Gainvors *et al.* (1994a) have already described the secretion of polygalacturonase, pectin lyase and pectin methylesterase by *S. cerevisiae*. On the contrary, the presence of 20–50 g l^{-1} methanol in citrus ethanol (as information supplied by Citrosuco Paulista S. A., Matão, São Paulo, Brazil) is another evidence of the secretion of methylesterase during citrus molasses fermentation by *S. cerevisiae*.

Purnapatre and Honigberg (2002) observed that yeast cells transited from the cell division cycle into meiosis at a high frequency when growth ceased and spores were formed at the colony edges during maturation of *S. cerevisiae* colonies on plates containing a poor medium. Inspired by this work, colony aging of isolates on polygalacturonate plates was carried out giving rise to improved recombinants able to secrete increased amounts of pectinase. In the present work, isolates that gave rise to improved recombinants also showed greater frequency of asci formation on sporulation medium. The higher the sporulation frequency, the lower the genetic stability. If this should occur during the citrus molasses fermentation, such a type of instability would result in an increasing number of recombinant cells within the molasses medium.

Secretion of polygalacturonase by cells of *S. cerevisiae* was repressed by glucose and induced by galactose (Blanco *et al.* 1994). The same was observed in cultures of other yeasts such as *C. albidus* and *Kluyveromyces marxianus* (Blanco *et al.* 1999). In the present work, the secretion of polygalacturonase on plates by isolates obtained from the industrial reactor was induced by galactose. However, galactose repressed the secretion of pectin-degradation activity on pectin plates. In the presence of glucose added to the media, low levels were obtained for both the pectin-degrading and polygalacturonase activities by the isolates, indicating occurrence of glucose repression in both the media used in the present work. However, monosaccharides (glucose and galactose added to polygalacturonic acid plates) apparently did not have any effect on the secretion of polygalacturonase by the recombinants strains, resulting from the maturation of the colonies of isolate 15MB, as indicated by the wide haloes obtained on plates after staining with the ruthenium red reagent. This also indicated that recombination occurred during maturation of colonies on polygalacturonate plates, minimizing the repression effect of glucose during enzyme secretion on pectin and polygalacturonic acid plates. On the contrary, the galactose repression was not altered during growth of the recombinant strains on pectin plates. This suggests that the effects of both glucose and galactose on the secretion of pectinolytic enzymes are governed by different regulatory mechanisms.

Depending on strain, yeasts such as *Kluyveromyces*, *Saccharomycopsis* and *Saccharomyces* were reported as producing pectinolytic activity, notably polygalacturonase, when they were grown without shaking (Blanco *et al.* 1999). This suggests that levels of the oxygen dissolved into the

medium regulate the secretion of polygalacturonase activity. In the present work, the liquefaction of polygalacturonic acid gels was observed when the cells of the reference strain LTU-4 were deeply inoculated into the gels where the oxygen levels are low. In addition, liquefaction of polygalacturonic gels by strain LTU-4 was also repressed by glucose but not by galactose. The same was observed for the secretion of polygalacturonase activity on plates by the isolates.

Despite good growth obtained for the reference strain LTU-4, haloes of pectinase secretion were not observed in the present work for this strain on plates containing pectinolytic substrates. The presence of polygalacturonase activity on the cell wall of a strain of *S. chevalieri* was previously reported (Sanchez *et al.* 1984). The lack of a secretion halo on plates for strain LTU-4 was because of the accumulation of pectinolytic activity onto the surface of intact cells as indicated by pectinolytic assays carried out in suspensions of washed cells to determine the pectin-degrading activity immobilized onto the cell surfaces. However, partitioning of the pectinolytic activity at the cell surfaces was obtained with strain LTU-4 during growth in the liquid medium and this gave rise to two-pectinolytic fractions, a wall-linked and a soluble fraction. So, it can be assumed that the growth of strain LTU-4 on solid medium was due to the active fraction linked to the cell wall. The nutrient concentration available for use is greater around the cells (including oxygen) in a liquid culture mainly under agitation and this seems critical for the secretion of pectin-degrading activity. In addition, different media and conditions have been used for growth (propagation medium) and pectinase secretion (induction medium) by *P. frequentans* (Kawano *et al.* 1999). The present work shows that the partitioning of the pectin-degrading activity between the surface of intact cells and the culture supernatants is also dependent on the strain itself and culture medium. Actually, isolates and recombinants showing diminished capacity to differentiate between solid and liquid media for pectinase secretion was a contribution of the present work. This seems to be the case of starter strain LTU-4, which was capable of sensing some sort of extracellular signal that repressed the secretion of activity on solid medium. In addition, pectin-degrading activity was obtained with strain LTU-4 in agitated cultures and this suggests that the effect of oxygen on secretion can be low or absent in the case of this particular strain.

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