Bacillus amyloliquefaciens ANT1 activity against *Pennicillum* spp. as bread spoliage mould.

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Moulds are main responsible for bread spoilage even because of its low water activity (wa). In the course of six months, we analized different kind of breads, with the aim to evaluate their content in moulds. *Penicillum decumbens*, *Penicillum crysogenum* and *Pennicillum* spp. were isolated from all breads sample. A modified susceptibility test was carried out to ascertain mould isolates sensitivity to *Bacillus amyloliquefaciens* ANT1 antimicrobials. ANT1 antimicrobial activity against potentially pathogenic bacteria and moulds was proved and the results reported elsewhere [3][4] Bread samples were artificially contaminated with the moulds isolates and ANT1. The moulds content in samples was measured after 24 h. *Aspergillus niger* ATCC9642 was used as positive control microorganism in test susceptibility because of its sensitivity to ANT1 antimicrobials [1][2]. ANT1 production of NRPS, PKS was also ascertained [5]. The results of our survey proved the antimicrobial activity of ANT1 against moulds responsible for bread spoilage. A possible utilization of ANT1 as bread preservative is hypothesized.

Keywords Food spoliage; *Pennicillum* spp.; *Aspergillus niger*; *Bacillus amyloliquefaciens* ANT1; NRPS; PKS.

1. Introduction

Bread is one of the most important staple foods in the world can be spoiled by many moulds, of which *Penicillium* spp. are by far the most common[6]. *Aspergillus* spp. are quite often isolated from stored cereal products as well [1]. Besides of the spoilage activity itself, the main hazard correlated to the moulds proliferation in foods consists in thermo-resistant mycotoxins production. This hazard is significant for children and newbornes [6]. In the course of the time, different techniques were proposed to preserve wheat and bread from moulds proliferation: from the addiction of spices [7] and chemical compounds like benzoate, propionate, sorbate [8] to the conservation through the use of a modified atmosphere [9]. Antifungal compounds like Phenyllactic acid (PLA) produced by Lactic Acid Bacteria (LAB) caused a delay in bread moulds isolates proliferation, thus suggesting a possible utilization of natural antifungal compounds for the increase of bread shelf life [9]. The possible application of *B.amyloliquefaciens* ANT1 (a strain isolated in hospital environment in Southern Italy) to the conservation of bread is explored in this survey.

2. Materials and Methods

2.1. Samples collection and analyses.

Samples of fresh baked whole, white and sourdough bread were collected weekly in a backery. On the whole, eighteen samples were collected in the course of 6 weeks and analyzed after 24 and 48 hours for their content in moulds, *Bacillus* spp. and mesophilic bacteria growing at 30°C. The analyses were carried out according to standardized techniques [10][11][12]. Briefly, 10g of sample were homogenated by a Stomacher 400 Circulator (Seward) in 90mL of Buffered Peptone Water (pH 7) and opportunely diluted. The media used in this survey were by Oxoid©, if not otherwise specified. Yeast Extract Dextrose Chloramphenicol Agar (YEDCA; Difco©) added with chloramphenicol, Mannitol Egg Yolk Polimixin Agar (MEYPA), Plate Count Agar (PCA) Tryptic Soy Broth (TSB) were used for the analyses. Landy Medium (LM) was preparared according to Landy M.et al. [11]. 100 µl or 1 mL of sample (or its dilutions) were plated according to the specific analysis. After plated, the media were incubated at suitable time and temperature, according to the ISO procedures.

Moulds isolates were identified by rDNA16S and ITS region amplification by PCR. Sequencing similarity search was performed using BLAST algorithm against the GenBank database.

2.2. Susceptibility test

ANT1 antimicrobial activity against isolated moulds was estimated by an agar diffusion assay (Guida et al 2011). A positive control was prepared with *A.niger* ATCC9642, whose susceptibility to ANT1 antimicrobials was previously proved [1]. Control plates seeded just with moulds (without the addiction of ANT1) were incubated at 30°C. TSA plates were first homogeneously surface inoculated with 100 µl (0,5 McFarland unit) of the moulds cell suspensions.

Once the plates were dried, *B. amyloliquefaciens* ANT1 was spotted just at the center of plates in double replica. As previous studies proved the dependence of ANT1 antimicrobial activity from temperature [2], the tests were carried out at 30°C for 48 hours and at 37°C for 24 hours. The antimicrobial activity was estimated by measuring the diameter (in mm) of the growth inhibition zone.

2.3. Bread samples contamination

Bread slices of 100g were artificially contaminated with a suspension of each mould and ANT1 previously grown for 72 hours in LM at 30°C and 37°C respectively. In all cases, a final concentration of 10^4 CFU per gram of bread was achieved. Negative controls without ANT1 addiction were also prepared. The samples were saved in sterile bags at room temperature (25±2°C) and analyzed soon after the contamination and again after 24 hours of incubation. We compared the moulds concentration after 24 hours with the initial concentration, at t°.

2.4. Identification of active compounds

Identification of active compounds was carried out as described in [4]. ANT1 cultures in Landy medium (72 hours at 30°C and 37°C) were prepared. The antimicrobial compounds isolation procedure included a solid phase extraction, a reverse-phase high-pressure liquid chromatography coupled with single quad mass spectrometer as described elsewhere [5]. Lipopeptides were eluted as described by Toure and collaborators [15] whereas polyketides were eluted as described elsewhere [16]. All the analyses where carried out at the Centre of Protein Engineering, University of Liege and at the Bio-Industry Unit, Gembloux Agro-Bio Tech, University of Liege under the supervision of Professor Patrick Fickers, Université libre de Bruxelles.

3. Results

The microbiological analyses revealed the absence of *Bacillus* spp. in all samples. All values was in accordance with the EC Regulation 1441/2007 concerning the microbiological criteria to assess foodstuffs quality.

Table 1 Bread samples microbiological analyses results.

Samples	CBT 30°C	*Bacillus cereus*	Moulds
White bread	3,7^04	0	5,5 ^02
Whole bread	3,59^04	0	1,41^02
Sourdough bread	3,18^04	0	9,00^02

The moulds were isolated and identified as *Pennicillum decumbens* (predominant isolate), *Pennicillum crysogenum, Pennicillum* spp. While *P.crysogenum* and *Pennicillum* spp. didn't show significant susceptibility to ANT1 antimicrobials, while *P.decumbens* was positive to the tests. In table 2 the results of *P.decumbens* susceptibility test are reported.

Table 2 Results of the susceptibility tests using different incubation temperature.

Coculture at 30°C	Inhibition halo (mm)	Coculture at 37°C	Inhibition halo (mm)
P.decumbens	26±2.48	*P.decumbens*	22±2.03
A.niger ATCC9642	34±2.48	*A.niger* ATCC9642	28±2.03

The results of moulds count in not antificially contaminated sourdogh bread samples as well as slices containing *P.decumbens* and *A.niger* ATCC9642 are reported in Fig.1,2,3.

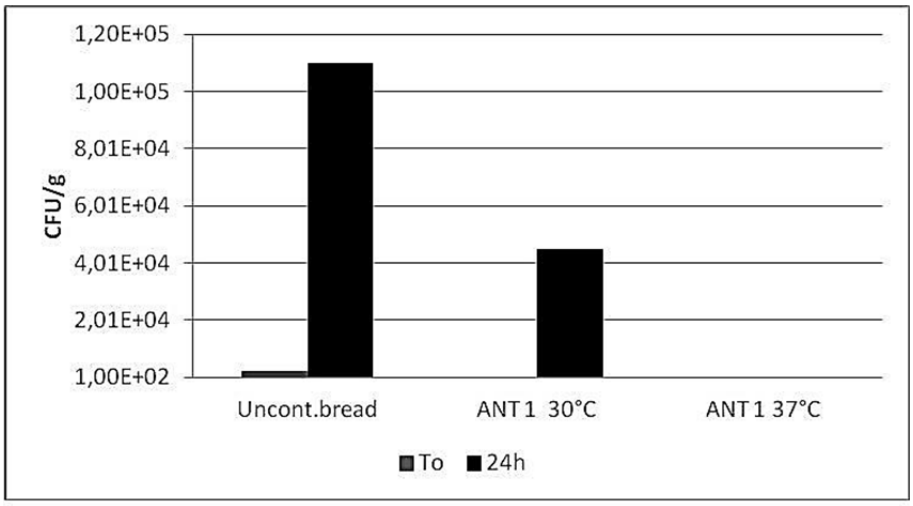

Fig. 1 Moulds concentration in control sourdough bread samples.

The inhibition of moulds after 24 hours ranged from the 77,7% in samples contaminated with *P.decumbens* in presence of ANT1 strain grown at 30°C till the 99,9% of the control bread (not artificially contaminated) inoculated with ANT1 grown at 37°C. In all cases, the addiction of ANT1 to the bread contaminated with moulds caused a decrease of one logarithm at least. It is interesting to notice the effect of ANT1 on bread samples not added with moulds: in this case, the decrease was of more than 3 logarithms for the samples added with ANT1 cultured at 37°C. The analyses of ANT1 supernatants in Landy medium evidenced the production of difficidin, surfactin, iturin A, macrolatin and fengycin classified as NRPS and PKS.

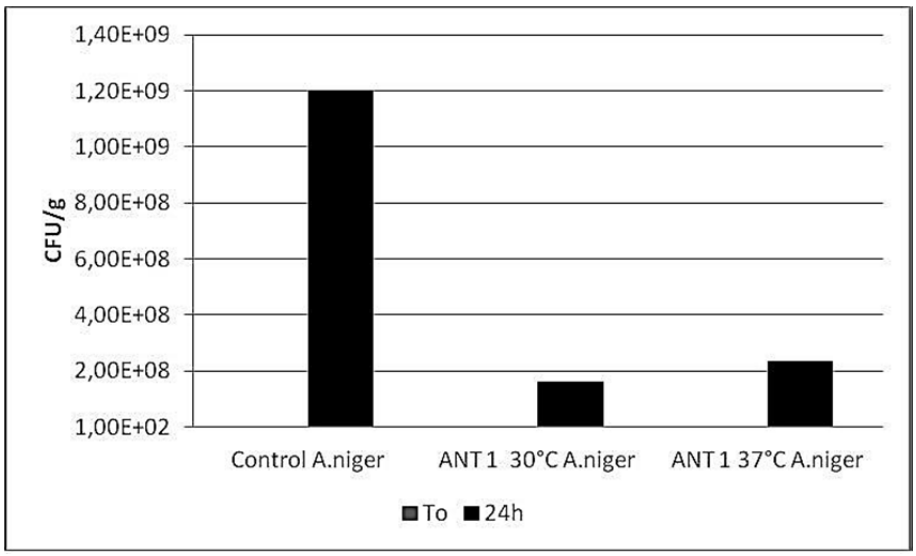

Fig. 2 *A.niger* concentration trend in samples contaminated with ANT1 pre-incubated at 30°C and 37°C.

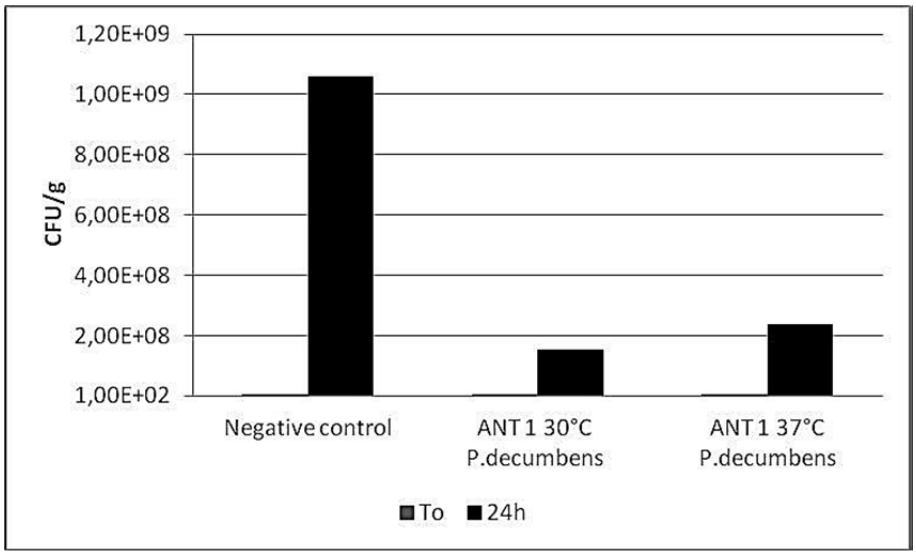

Fig. 3 *P. decumbens* concentration in bread samples.

4. Conclusions

Food spoliage is still an actual concern. Moulds belonging to *Pennicillum* genus act an important role in the biodeterioration of different food-stuffs, ceral based foods included. In the present survey, we reported the findings of an experiment aimed to test the ability of *Bacillus amyloliquefaciens* ANT1 to inhibit the growth of *Pennicillum* species in sourdogh bread. From previous studies, fengycin and surfactin as a secure antifungal effect. Even if there are evidences of a significant effect on *P.decumbens* and *A.niger* strains even in bread samples, further studies are needed in order to state the real effect of ANT1 activity on bread shelf life elongation.

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