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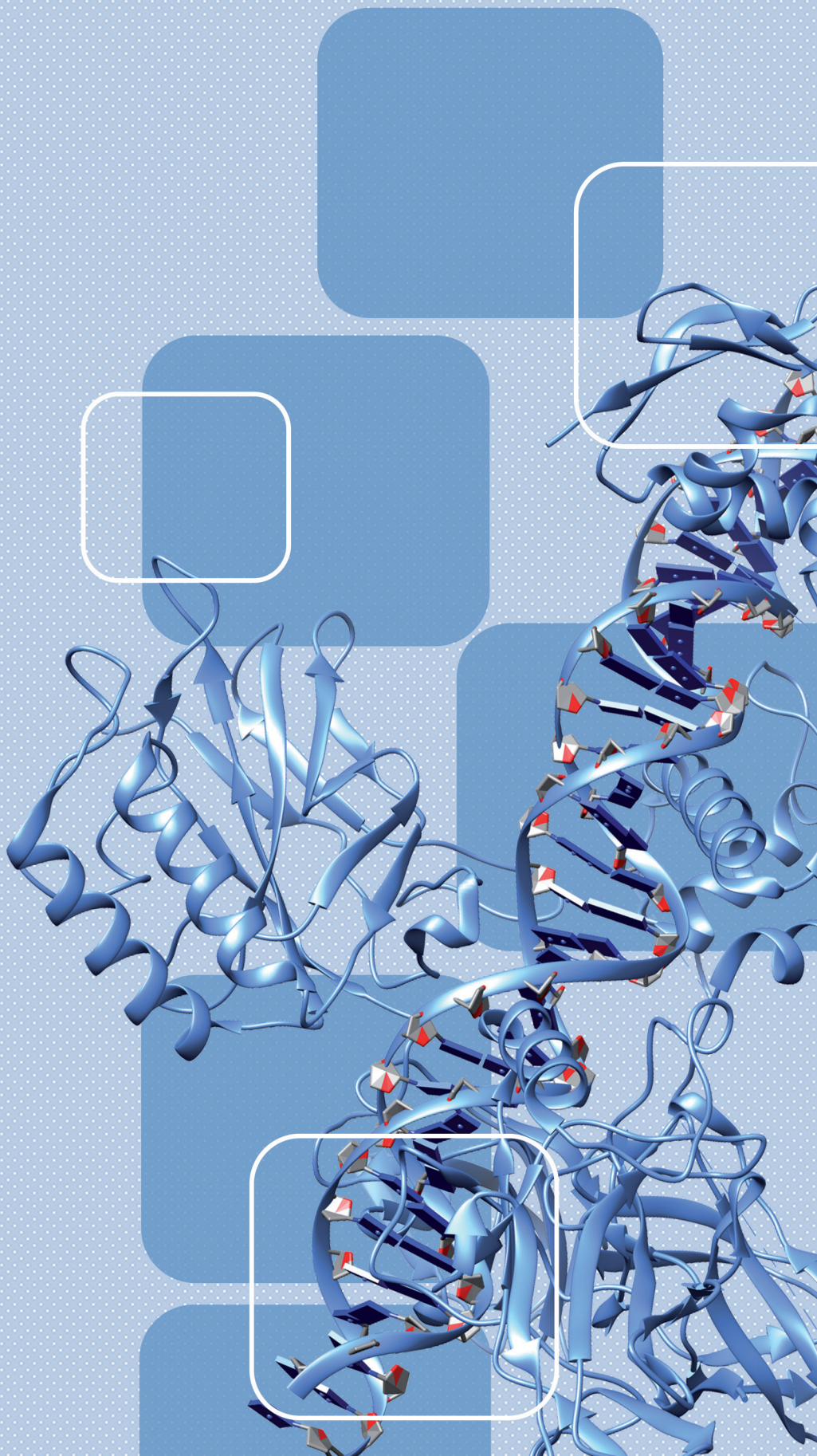

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GUEST EDITORIAL

The international conference Biotech 2014 and 6th Czech-Swiss Symposium with Exhibition was held from June 11 to 14, 2014 in Prague, Czech Republic. For more information about the meeting as well as the Book of Abstracts and photo gallery, please visit http://www.biotech2014.cz/. The history of the conference dates back to 1999, when Czech and Swiss biotechnologists decided to organize joint meetings to exchange knowledge and experiences in a dynamic field of biotechnology and foster academic and industrial collaborations. The focus of the 2014 meeting, which hosted 265 participants from 32 countries, was on microbial biotechnology as the key technology for bio-based technologies. A total 77 lectures delivered in 6 conference sections, including Large and Small Molecules for Pharma, Food, Feed and Nutrition, Biomaterials and Biochemicals, Environmental Biotechnology, Biorefinery, and Microalgae Biotechnology, were complemented with 120 poster presentations. As the guest editor, I am pleased to present in this Special Issue of *Bioprospect* a snapshot of the poster sessions – 2 review and 6 original papers contributed from the conference participants. At this point, I would like to thank all the authors and reviewers for their fine work and commitment to this issue.

Pavel Kotrba

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PURIFICATION AND CHARACTERIZATION OF PROTEASE, STABLE IN ORGANIC SOLVENT, FROM NEW THERMOPHILIC ACTINOMYCETE ISOLATE

Ivelina Hristova[1], Plamena Nedelcheva[2], Georgi Dobrev[2], Albert Krastanov[1]

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Introduction

Peptidases are enzymes that hydrolyze peptide bonds. They are necessary for the survival of all living organisms[1], participating in cell growth and differentiation processes[2]. These enzymes are the single class that occupies a primary position with respect to their applications in both physiological and commercial fields[3]. Nowadays, they represent 60 % of the total enzyme sales in the world market[4]. Proteases are extensively used in food[5], pharmaceutical[6], leather[7], silk[8], silver recovery[9] and production of protein hydrolysates[5]. Recently, proteases were also used in development of several bioremediation processes[10].

The application of commercially available proteases is strongly dependent on pH and temperature optimum for catalysis. The application of commercially available proteases is strongly dependent on pH and temperature optimum for catalysis. In this regard, one of the most common application of proteolytic enzymes with alkaline pH optimum is in detergent formulations[11]. These kind of proteases are mostly active in the pH range 8 –12 and at temperature range 50 – 70°C[11,3].

The protein nature of proteases is an obstacle for their successful application in harsh process conditions. In this regard, searching for active and stable enzymes in hostile environment is of primary importance. Klibanov reported that enzymes exhibit high activity and specificity not only in conventional aqueous solutions but also in non-aqueous reaction media[12]. These kind of enzymatic reactions provide multiple attractive advantages, such as increased solubility of non-polar substrates, reversal of the thermodynamic equilibrium of hydrolysis reactions, suppression of water-dependent side reactions, alternation of substrate specificity and enantioselectivity, and elimination of microbial contamination[13]. Proteases in organic solvents can catalyze water sensitive reactions, such as esterification, transesterification, and peptide synthesis, which are usually impossible in aqueous media[14]. However, this kind of reactions is limited because of the prompt enzyme inactivation or lower catalytic activity. Beside the existence of immobilization and chemical modification methods for maintaining the enzymes stable and active in organic solvents[15,13], researchers attempt to find natural sources of such enzymes. As microorganisms are proved sources of enzymes with diverse pH, temperature, chemicals and other stabilities, they are promising sources to explore[6].

Actinomycetes are Gram-positive, filamentous microorganisms commonly known as antibiotic producers[16]. Their capacity for enzyme production is well recognized but not extensively studied[17]. However, a number of actinomycete strains were already reported to produce alkaline proteases with a potential application in detergent industry[18,9,19].

The aim of this study was to purify and partially characterize protease from new thermophilic actinomycete isolate in order to discover new enzymatic activities and stabilities.

Materials and methods

Isolation, media and cultivation

The actinomycete strain, producer of the investigated protease, was isolated from Antarctic penguin excrements samples and was provided by the Institute of microbiology of Bulgarian Academic of Science. Previously it was characterized as thermophile. The isolate was cultivated on maize-peptone broth reported by Gushterova et al.[20]. Batch fermentation was lead at 45°C for 48h with constant shaking at 200 min^{-1}. The media was subsequently centrifuged and the supernatant used for further purification.

Determination of protease activity and protein content

The protease activity was measured according to the method reported by Jain et al.[4] with casein, buffered with phosphate buffer to pH 7.0, as substrate. The reaction mixture was incubated at 60°C for 10 min. The proteolytic activity was calculated in Unit per mL that is are defined as the amount in micromoles (μmol) of tyrosine released from casein per minute and per milliliter (mL) of partially purified enzyme.

The protein content was determined by the Bradford method [21] using bovine serum albumin as standard.

Enzyme purification

The culture supernatant was concentrated and partially purified by ultrafiltration using a 25 kDa MW cut-off polyacrylonitrile ultrafiltration membrane. The

concentrated enzyme was subsequently subjected to gel filtration on a Sephadex G-75 column (K26/70, 2.6 x 70 cm) equilibrated with 0.05 M sodium chloride aqueous solution. Fractions of 10 mL were collected at a flow rate of 20 mL/h. Active fractions were pooled together for further analysis.

Polyacrylamide gel electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method described by Laemmli[22] using 12 % crosslinked polyacrylamide gel on a Mini-PROTEAN 4 Cell electrophoresis system (Bio-Rad,USA). The gel was stained with Coomassie brilliant blue for 30 min and then destained overnight.

Effect of temperature on protease activity

The temperature optimum of the enzyme was evaluated by measuring the protease activity at different temperatures (40°C to 90°C) according to the method described above.

Effect of pH on protease activity

The pH optimum of the purified protease was studied over a range of pH 5.0 –12.0 at 60°C. The following 50 mM buffer systems were used: citrate buffer for pH 5.0; phosphate buffer for pH 6.0 – 8.0 and pH 12.0; carbonate – bicarbonate for buffer pH 9.0 – 10.0.

Effect of metal ions and organic solvents on protease activity

The purified enzyme was pre-incubated for 60 min at 30°C in various concentrations (5, 10, 15 mM) of different metal ions ($Hg^{2+}/HgCl_2$, $Zn^{2+}/ZnSO_4$, K^+/ /KCl, $Fe^{2+}/FeSO_4$, $Fe^{3+}/FeCl_3$, $Mn^{2+}/MnSO_4$, $Cu^{2+}/CuCl_2$, $Ag^+/AgNO_3$, $Co^{2+}/Co(NO_3)_2$, $Cd^{2+}/CdCl_2$, $Mg^{2+}/MgSO_4$, $Ca^{2+}/CaCl_2$, Na^+/NaCl, $Pb^{2+}/Pb(CH_3COO)_2$). The remaining activity was measured afterwards.

The impact of 20, 40 and 60 % (v/v) of ethanol, acetone, toluene, iso-propanol, DMF, DMSO for 60 min at 30°C on protease activity was examined. The influe ence of 5 mM EDTA and β–mercaproethanol was also studied. The residual activity measurement (%) was performed under optimal assay conditions.

Kinetic constants determination

K_m and V_{max} were determined by measuring the activity in various concentrations of casein as substrate (1.2 – 6.0 mg/mL) using Lineweaver-Burke plot.

All experiments were done in triplicate and the results were statistically analyzed using ANOVA (Microsoft Excel, USA).

Results and Discussion

Fig. 1 represents a single colony of the actinomycete isolate on maize peptone agar medium. It forms a large whitish colored colony with striated surface. Thermophilic microflora originating from Antarctica was already reported in the literature. Gushterova et al investigated the microflora of Antarctic penguin's excrements and reported 44 actinomycetes strains, 22 of which are thermophiles[23]. The same authors suggested that the thermophilic nature of the Antarctic strains originated from neighboring Deception Island possessing volcano activity. Therefore, it is likely that thermophilic microorganisms are transported by ash particles from Deception Island to Livingston Island[24]. The cultivation for protease production in the current study was conducted at temperature of 45°C for 48 hours. These conditions were chosen based on preliminary studies. The relatively short cultivation time of 48h is in agreement with the report of Burkholder[25] that thermophylic actinomycetes grow faster than mesophilic ones[26].

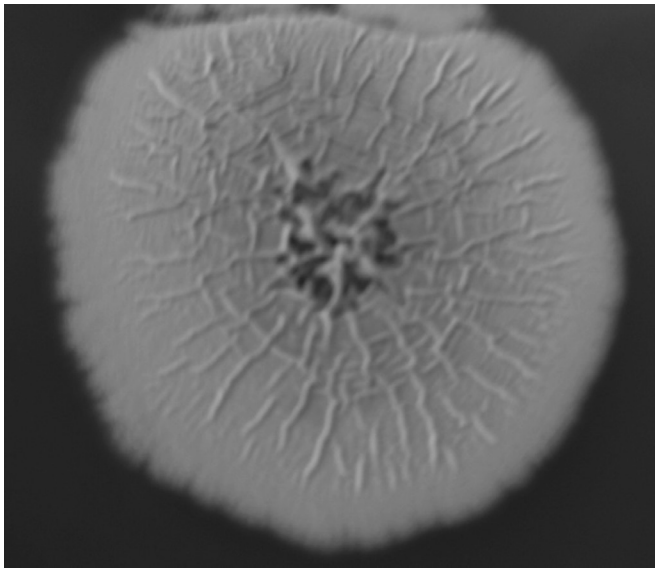

Fig. 1: Colony of actinomycete isolate

The advances in ultrafiltration membranes and systems technology have expanded the capability of this technique from concentration and buffer exchange applications to more complex biological separations[27]. That is why in the current study the first step in the purification process was ultrafiltration. The second step used was size exclusion chromatography. The protein elution profile is presented on Fig. 2. The active fractions corresponded exactly to one protein peak, eluted between the 20th and 25th fraction. The pooled active fractions were used for further characterization.

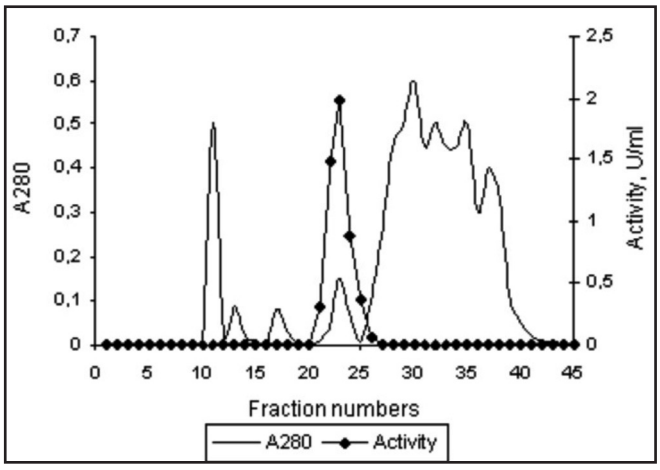

Fig. 2: Protein elution profile after SEC on Sephadex G75 and proteolytic activity of obtained fractions expressed in Units per mL.

The purification processes was tracked out with SDS-PAGE. After the second step of the process two protein bands were visualized (Fig. 3). It was estimated that one of them corresponds to 26 kDa, which is a confirmation of the approximate molecular weight determined by size exclusion chromatography, while the other band corresponded to 31 kDa. Most likely, one of the two bands is an accompanying protein. Although, it is not a complete purification process, the presence of the substrate often stabilizes the protease's structure.

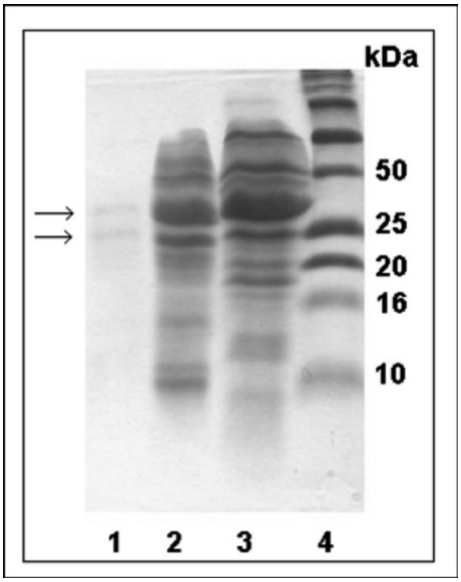

Fig. 3: SDS–PAGE of purified protease produced by actinomycete isolate. Cell free supernatant, retentate after ultrafiltration PAN 25 and active fraction after Sephadex G75 purification steps were gel loaded and, after electrophoresis, strained with Coomassie brilliant blue R-250. Line 1: active fractions after Sephadex G75 SEC; Line 2: retentate after ultrafiltration PAN 25; Line 3: crude enzyme; Line 4: protein molecular mass markers, expressed in kilodaltons.

The results of purification of extracellular alkaline protease are summarized in Table I. The enzyme was purified from the culture supernatant employing ultrafiltration on PAN 25 and size-exclusion chromatography on Sephadex G75. After the first step only 0.97 purification fold was obtained with high enzyme recovery (86 %). At the same time two-step purification method resulted in 13.67 fold of purification with a yield of 43 % and specific activity at 833.16 U/mg protein. Thumar et al. also purified alkaline protease from *Streptomyces clavuligerus* strain with two-step purification process including ammonium sulfate precipitation and ion-exchange chromatography[28]. They reported 11 % yield and 141.66 purification fold.

Kinetic constants were determined as well using Lineweaver-Burke plot (Fig. 4). The Michaelis constant was calculated to be 5.799 mg/mL casein and the maximal reaction velocity – 0.201 µmol/min. The affinity of purified enzyme from actinomycete isolate appeared to be lower compared to the affinity of other actinomycete proteases. Gohel and Singh purified highly thermostable alkaline protease from alkaliphilic actinomycete *Nocardiopsis alba* OK-5 having K_m – 0.50 mg/mL [29].

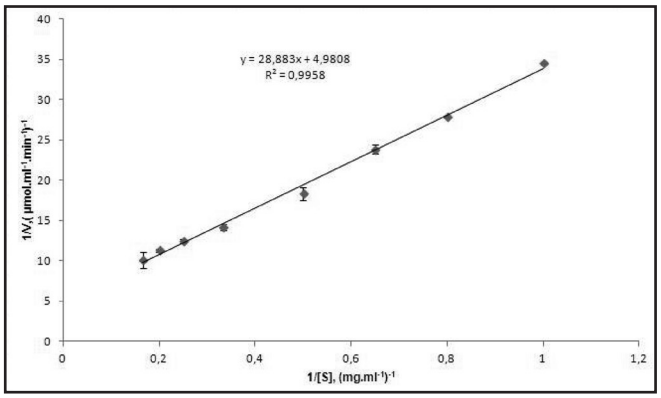

Fig. 4: Lineweaver-Burke plot. The presented data is the mean of three repetitions

The effect of temperature on protease activity is shown in Fig. 5. The temperature optimum for the maximum protease activity was established to be 70°C. At both 50 and 60°C, the protease was quite active, with nearly 60 % of its maximum activity. At 80°C the enzyme showed more than 80 % activity and dropped down to 20 % afterwards. Such a broad temperature optimum is desirable for biotechnological application[30].

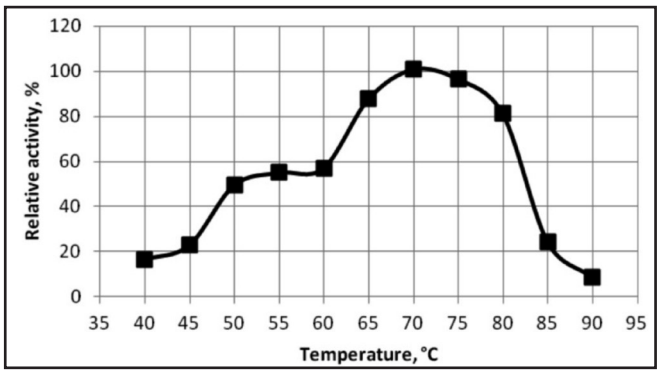

Fig. 5: Effect of temperature on protease activity. The activity of purified enzyme was measured at the temperature range 40 – 90°C at an interval of 5°C. The presented data is the mean of three repetitions.

Table I: Purification summary table

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Fold purification	Yield, %
Crude enzyme	9.60	585.15	60.95	1.00	100
Ultrafiltration PAN 25	8.58	505.85	58.95	0.97	86
Sephadex G-75	0.30	249.95	833.16	13.67	43

The potential application of newly isolated enzymes is strictly related to the temperature and pH optimum for their activity. Broader the pH range is, larger the application might be. The studied purified protease exhibited high activity over the pH range between 5 and 12 (Fig. 6). Habbeche et al. also reported broad pH active protease. The enzyme was more than 75 % active in the pH range 6 – 11[26].

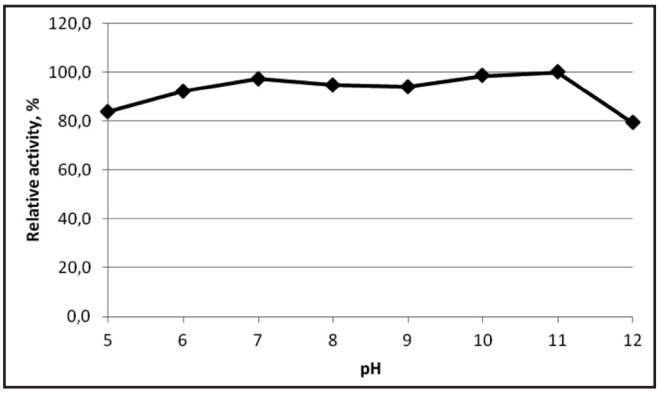

Fig. 6: The effect of pH 5-12 on caseinolytic activity of the purified enzyme. The presented data is the mean of three repetitions.

In order to study the organic solvent response of the purified protease a various solvents were studied. Unexpectedly the enzyme was significantly more active in the presence of ethanol, acetone, toluene, iso-propanol, DMF, DMSO compared to the enzyme in aqueous solution only (Fig. 7). A strong positive correlation between organic solvent concentration and proteolytic activity was observed. The presence of organic solvents changes the dielectric constant. This provokes a modification of enzyme conformation and favors the enzyme action. In the presence of 60 % (v/v) acetone and ethanol 2.37 and 2.59 higher activity was detected, respectively. These results suggested that the purified protease has a strong potential to be used in peptide synthesis.

Li et al. studied the effect of organic solvent at a concentration of 50 % on the bacterial protease YP1A and trypsin stability [32]. Toluene and acetone affected slightly the activity of the protease YP1A, and DMF and DMSO didn't affected it at all. At the same time trypsin's activity was inhibited in the presence of DMF and DMSO with 40 and 20 % respectively. Doukyu and Ogino summarized the organic solvent stable proteases know so far[13]. The presented data indicated that *Pseudomonas aeruginosa* and *Bacillus* strains are promising sources of organic solvent active proteases.

The impact of selected metal ions on proteolytic activity was also studied in three concentrations (5, 10, 15 mM). The temperature of 30°C was chosen in order to study only the effect of metal ion over the enzyme activity. The results shown in Table II indicated that the purified enzyme was reasonably stable and active in the presence of most of the metal ions studied. The proteolytic activity was significantly enhanced in a presence of Mn^{2+}. The enzyme remained more than 90 % active in the presence of 5, 10 and 15 mM of Pb (II), Zn (II), K (I), Fe (II), Co (II), Cd (II), Mg (II), Ca (II), Fe (II) ions for 1h at 30°C. Surprisingly the presence of Fe (III) ions enhanced the proteolytic activity by 26 %. A moderate inactivation (up to 34 %) was observed when Cu (II) ions were used. The presence of Hg (II) and Ag (II) inhibited the enzyme resulting to less than 10 % remaining activity.

Gohel and Singh reported full inhibition of thermostable protease from a salt-tolerant alkaliphilic actinomycete by the ions of Cu (II), Cd (II) and Hg (II) at a concentration of 5[33]. Gupta et al. also studied the impact of some metal ions over the alkaline protease purified from a haloalkaliphilic *Bacillus* sp. They demonstrated that Ca (II) in 1 mM enhanced significantly the proteolytic activity (1.4 times) but 5 mM inhibited the enzyme to nearly 60 % of the initial activity. 5 mM of Zn (II) and Mn (II) didn't affect the enzyme while Cu (II) increased the activity to 1.2 times.

At concentration of 5 mM of EDTA and β-mercaptoethanol the remaining proteolytic activity was 100 %, thereby suggesting that the purified enzyme is neither a metalloprotease nor a thiolprotease.

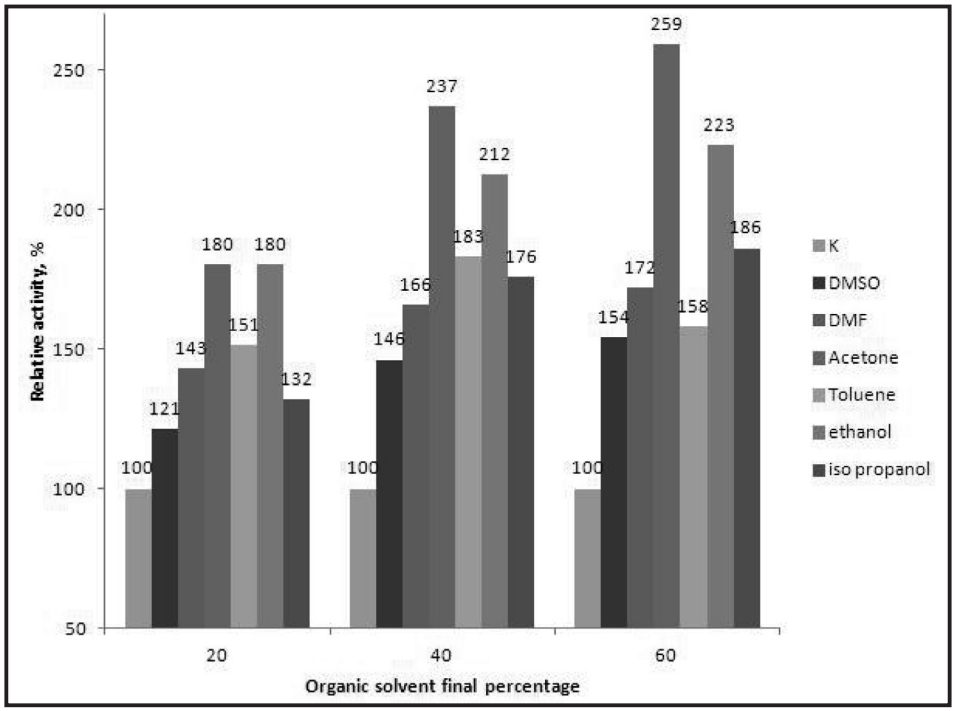

Fig. 7: Effect of DMSO, DMF, acetone, toluene, ethanol and iso propanol at concetration of 20,40 and 60 % (v/v) on the proteolytic activity of the purified protease, incubated for 1h at 30°C. The activity of enzyme in aquous solution is used as 100 %. The presented data is the mean of three repetitions.

Table II. The influence of 5, 10 and 15 mM of selected metal ions over proteolytic activity of purified protease.

Metal	5 mM	10 mM	15 mM
Enzyme without supplements	100	100	100
Pb^{2+}	91	86	83
Zn^{2+}	92	90	89
K^{+}	96	99	98
Fe^{2+}	112	114	115
Mn^{2+}	130	149	147
Cu^{2+}	70	72	66
Co^{2+}	102	107	105
Cd^{2+}	92	96	91
Mg^{2+}	98	99	99
Ca^{2+}	95	92	96
Fe^{3+}	74	117	126
Hg^{2+}	10	8	4
Ag^{2+}	8	4	4
EDTA	99	-	-
β-mercaptoethanol	100	-	-

Conclusions

In the present study a stable in organic solvents protease was purified from a new thermophilic actinomycete isolate. After SDS-PAGE of the purified fractions two protein bands were detected. The approximate molecular weights were established to be 26 and 31 kDa. The kinetic constants were determined as well.

Partial characterization was carried out in order to determine whether the protease will be a potential target for technological use. The study of the impact of the metal ions over the proteolytic activity showed a significant increase of the activity in the presence of Mn^{2+} and more than 90 % in the presence of 5, 10 and 15 mM Pb^{2+}, Zn^{2+}, K^{+}, Fe^{2+}, Co^{2+}, Cd^{2+}, Mg^{2+}, Ca^{2+}, Fe^{3+} ions for 1h at 30°C. At the same time the protease exhibited an increased activity and stability in the presence of 20, 40 and 60 % (v/v) organic solvents such as DMSO, DMF, acetone, ethanol, iso-propanol and toluene when incubated for 1h at 30°C. The purified protease showed maximum activity at 70°C and exhibited broad pH optimum (5.0 – 12.0). The displayed characteristics make the enzyme a potential detergent ingredient or biocatalyst in peptide synthesis.

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Summary

Hristova I., Nedelcheva P., Dobrev G., Krastanov A.: Purification and characterization of protease, stable in organic solvent, from new thermophilic actinomycete isolate

In the present research, stable in organic solvent protease from a new thermophilic actinomycete isolate, was purified and characterized. The approximate molecular mass of 26 kDa was determined by SDS-PAGE and SEC. The purified protease showed maximum activity at 70°C and exhibited broad pH optimum (5.0 – 12.0). After treatment with 5 mM EDTA and β-mercaptoethanol the enzyme remained fully active. The protease showed an increased activity and stability in the presence of 20, 40 and 60 % (v/v) organic solvents such as DMSO, DMF, acetone, ethanol, iso-propanol and toluene when incubated for 1h at 30°C. The proteolytic activity was significantly enhanced in presence of Mn^{2+} and remained more than 90 % active in the presence of 5, 10 and 15 mM Pb^{2+}, Zn^{2+}, K^{+}, Fe^{2+}, Co^{2+}, Cd^{2+}, Mg^{2+}, Ca^{2+}, Fe^{3+} ions for 1h at 30°C. The kinetic constants were also determined.

Keywords: actinomycetes, proteases, organic solvets

Souhrn

Hristova I., Nedelcheva P., Dobrev G., Krastanov A.: Purifikace a charakterizace proteasy, stabilní v organických rozpoštědlech, z nových termofilních aktinomycet

Byla purifikována a charakterizována protease stabilni v organických rozpoštědlech. Její molekulová hmotnost byla stanovena pomocí SDS-PAGE a SEC na 26 kDa. Purifikovaná proteasa měla nejvyšší aktivitu při 70°C a její pH optimum bylo velmi široké (5,0 – 12.0). Po ošetření 5 mM EDTA a β-mercaptoethanolem zůstával enzym aktivní. Proteasa měla po hodinové inkubaci při 30°C v přítomnosti 20, 40 a 60 % (v/v) organických rozpouštědel, jako jsou například DMSO, DMF, aceton, ethanol, iso-propanol a toluen vyšší aktivitu a stabilitu. Proteolytická aktivita byla v přítomnosti Mn^{2+} iontů prokazatelně vyšší a v přítomnosti 5, 10 a 15 mM Pb^{2+}, Zn^{2+}, K^{+}, Fe^{2+}, Co^{2+}, Cd^{2+}, Mg^{2+}, Ca^{2+}, Fe^{3+} iontů po dobu 1h při 30°C si zachovávala více než 90 % aktivity.

Klíčová slova: aktinomycety, proteasy, organická rozpouštědla

THE PROBIOTIC PROPERTIES OF AUTOCHTHONOUS *Streptococcus thermophilus* ISOLATED FROM TRADITIONAL ALGERIAN FOODS

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Introduction

Lactic acid bacteria (LAB) are widely used in fermentative food processes[1]. *Streptococcus thermophilus* (*St*) is frequently isolated from dairy environments, but strains have been isolated from plant samples in Bulgaria. Yogurt, a nutrient-dense food, is one of the most popular fermented milk products worldwide[2]. *St. thermophilus* has been traditionally used as starters with *Lactobacillus delbrueckii* subsp. *bulgaricus* (*Lb*) for milk fermentation in yogurt production[3].

In some cases, the beneficial health effects of yogurt are attributed to the starter cultures *Lb. delbrueckii* subsp. *bulgaricus* and *St. thermophilus*. However, disagreements exist whether yogurt starter cultures are considered as probiotics or not [4]. One of the main preconditions for a bacterial strain to be called probiotic is its ability to survive in the gastrointestinal environment, although the importance of viability for the beneficial effects of probiotics is not well defined since inactivated and dead cells also have immunological and health-promoting effects [5,6].

However, *St. thermophilus* and *Lb. delbrueckii* should also be considered as probiotics. Recent studies reported the ability of these organisms to release, among other compounds, enzymes improving the digestion of nutrients in the intestine[7], as well as the contribution to the normal development of the gut mucosal immune system[8], that also plays a positive role in human health.

Considering the traditional use of *St. thermophilus* and *Lb. delbrueckii* in fermented dairy products, it is interesting to asses, the probiotic potential of individual strains for these species, as the literature contains controversial observations with respect to their proposed benefits[9].

The present study was dedicated to investigate new *St. thermophilus* strains for the development of Algerian thermophilic starters, microbiological procedures are required to identify strains and assess their technological and probiotics properties.

Material and Methods

Bacterial strains and culture conditions

Two *St. thermophilus* strains (designated R_4 and R_{14}) isolated from traditional fermented milk cold "Raib" were used in this study (Collection Laboratory of Biotechnology, Environment and Health, university of Jijel). The strains were cultured on M17 broth[10].

A commercial starter freeze-dried culture routinely used in production of yogurt (ST-M6, CHR HENSEN, France) was used in this study. *Lactobacillus delbrueckii* subsp. *bulgaricus* (*Lb*) was isolated from this commercial starter.

Technological properties

pH and titratable acidity: Sterilized skim milk (120g skim milk powder. L^{-1}distilled water) was inoculated with active culture 1 % (v/v) of each strain and incubated at 42°C for 3h and 6 hours. The pH was measured with pH meter (Hanna Instrument pH211). The

electrode was calibrated with pH 7.00 and 4.00 buffer solutions prior to use. The total acidity (g. lactic acid/l) was performed by titration of 10 mL of fermented milk with N/9 NaOH in the presence of phenolphtalein[11]. Triplicates of each test were done.

Exopolysaccharide (EPS) production and proteolytic activity

The EPS production was recorded in M17 agar (20 g sucrose/l). After incubation for 16 to 24 h mucoid colony formation on agar medium is related to EPS production[12]. The total carbohydrate content of EPS was determined according to Dubois et al.[13] by phenol-sulfuric acid procedure.

The proteolytic activity was evaluated in Yeast Milk Agar. The diameter of proteolysis zone was determined after incubation at 42°C for 24 hours[12]. Triplicates of test were done.

Syneresis

Milk was prepared by dissolving 120 g of skim milk powder (Spray processed, Belgium) per liter of distilled water. The mix was pasteurized at 90°C for 10 min, cooled to 42°C and inoculated with 3 % (v/v) *St. thermophilus* culture. The inoculated milk was transferred to cups of 110 mL of capacity (4.5 cm/4.5 cm/6.5 cm) and fermentation was carried out at 42°C for 6 hours. Syneresis was measured as described by Minto et al.[14] with minor modifications.

For each strain, six cups of fermented milk were firstly weighed and maintained at an angle of 45° for 2 h at 4°C. The whey was removed from the surface with a syringe, and the cups with product were re-weighed. Syneresis was reported in terms of the percentage (%) of whey lost. Triplicates of test were done.

Water holding capacity

The method described by Parnell-Clunies et al.[15] was used with minor modification. First, the milk was prepared as described above (syneresis method) and inoculated with 3 % (w/w) *St. thermophilus*. The inoculated milk directly fermented in a 50 mL centrifuge tube was weighed and centrifuged at 13000 g for 30 min at 4°C. Separated supernatant was drained, and the pellet was weighed. Water holding capacity was reported as the percentage (%) of pellet weight. Triplicates of test were done.

Probiotics properties

Acid tolerance:

Cells of overnight cultures were harvested and resuspended in 10 mL of M17 broth adjusted at pH 2, pH 3 and pH 6.5. The cultures were incubated at 42°C for 2 h. Viable cells number was determined at the end of incubation[16].

Bile salt tolerance

Cells pellet from overnight culture of LAB was resuspended in 10 mL of M17 broth adjusted at pH 2, pH 2.5, pH 6.5 and containing 0.3 (w/v) oxgall (dehydrate fresh bile, Pasteur institute, Alger's, Algeria). After incubation at 42°C for 2 h, cultures turbidity was monitored at OD_{650} nm, with determination of viable cells number. The control comprised M17 broth without oxgall[16].

Resistance to simulated gastrointestinal juice

The resistance to simulated gastrointestinal juice was verified as described by Viscoco Pinto et al[17]. The same conditions as those described by these authors were used. Resistance was assessed in terms of viable cells counts and enumerated after incubation at 42°C for 3h with pepsin (Sigma-Aldrich, Germany), bile salts (Bovine bile, Pasteur institute, Alger's, Algeria) and simulate duodenal secretion ($NaHCO_3$ 6.4 g/L, KCl 0.239 g/L, NaCl 1.28 g/L), reflecting time spent in stomach and small intestine[17]. Triplicates of test were done.

Inhibitory activity

The inhibitory activity was screened by the agar spot agar in M17 medium[18]. The indicator strains used were *Listeria monocytogenes (L)*, *Klebsiella oxytoca (K), Staphylococcus aureus (S)* (Stock cultures: Laboratory of Biotechnology, Environment and Health, university of Jijel, Algeria) *Escherichia coli* ATCC 25422 (*E*) and *Bacillus subtilis* ATCC21332 (*B*). Twenty milliliters of M17 broth was inoculated with each strain (v/9v) and incubated at 42°C for 18 h. After incubation, a cell-free solution was obtained by centrifuging the culture (6000 x g for 15 min), followed by filtration of the supernatant through a 0.2 µm pore size. A part of supernatants were neutralized with NaOH (5 N) to pH 6.5. The inhibitory activity of supernatants and neutral supernatants of cultures were tested. The diameters of inhibition zones were measured after incubation at 37°C for 24 h. Duplicates of test were done.

Hydrophobicity

Hydrophobicity was determined according to Iyer et al.[19] by measurement of partition of bacterial cells between organic n-hexane and xylene. Cell-surface hydrophobicity (% H) was calculated as follows:

Hydrophobicity (%) = $(OD_0 - OD)/ OD_0 \times 100$

Where OD_0 and OD are the optical densities before and after extraction with the organic solvent, respectively. Duplicates of each test were done.

Adherence of LAB to epithelial cell

The adhesion test was peformed using the method of Kos et al. [20] with minor modifications. Briefly, Segment of poultry ileum was washed with sterilized phosphate-buffer saline (PBS pH 7.2 : K_2HPO_4 1.21 g/L, KH_2PO_4 0.34 g/L, NaCl 8 g/L). It was held at 4°C for 30 min and then washed three times with PBS. The epithelial cell concentration was adjusted to 5×10^4 cells/mL. Cell pellet from overnight culture of LAB was resuspended in PBS (pH 7.2) and adjusted to 1×10^8 cells/mL. One mL of such bacteria suspension was mixed with 1 mL of the cell suspension of poultry ileum epithelial cells. The mixture was incubated at 37°C for 30 min. The adhesion was observed using phase contrast microscopy (magnification fold of 200) after stained with 0.5 % crystal violet for 5 min. Adhesion was scored positive if at least 10 bacteria per epithelial cell could be observed. Duplicates of experiment were done.

Coexistence test

Coexistence between *St. thermophilus* R_4 and *Lb. delbrueckii* subsp. *bulgaricus* and between *St. ther-*

mophilus R_{14} and a same commercial strain was tested using a cross-street method[21]. The strains were streaked perpendicularly and across each other on MRS agar plates[22]. After incubation in anaerobic jars at 42°C for 48h, the antagonism was recorded by apparition of inhibition zones. Triplicates of essay were done.

Statistical analysis

Statistical analysis was conducted using ANOVA analysis (StatBox logiciel, Grimmer Soft; version 6.4, France). A $P<0.05$ was considered statistically significant.

Results and Discussion

Technological properties

The evaluation of technological attributes should be an important consideration for the selection of strains for food applications. The milk inoculated with pure LAB strains coagulated after 6 hours. In this study *St. thermophilus* R_{14} was the fastest acid producing strain (Table I).

TableI I: Production of lactic acid by strains (g/L)

Strains	0h	3h	6h
St. thermophilus R_4	1.7 ± 0. 1	4.5 ± 0.2	6.2 ± 0.4
St. thermophilus R_{14}	1.7 ± 0. 1	4.7 ± 0.5	7.7 ± 0.6

The role of *St. thermophilus* in milk fermentation is due to its rapid conversion of lactose into lactic acid, causing a rapid decrease in pH and the production of metabolites important for their technological properties. After 6h of incubation the pH decrease from pH 6.6 (pH of milk) to pH 4.75 (sample with R_4) and pH 4.60 (sample with R_{14}). The values of pH are not so far from those reported by Moreira et al.[23] (3.76 – 4.39), who analyzed samples of milk fermented by different strains of *Lb. bulgaricus* and *St. thermophilus*.

As shown in Table II, the values of the diameter of the proteolysis zone ranged from 17 and 33 mm. The isolates were able to grow on YMA media where bacterial proteolytic activity led to clear zones. *St. thermophilus* R_4 showed the highest activity. We recommended this strain for cheese technology. Many *St. thermophilus* strains synthesize exopolysaccharides (EPS) that contribute to the desirable viscous texture and rheological properties of fermented milk products, yogurt in particular. *St. thermophilus* R_4 and R_{14} produced respectively 0.150 and 0.553g of EPS/EPS per liter and its appear that strain R_{14} produced the high quantity of EPS and we recommended it for yogurt production. The sensory characteristics of commercial yogurts have changed remarkably due to the consumers preferring a less acidic product with thick texture. This had led to the yogurt industry to select EPS producing strains. Uemura et al.[24] reported that capsular polysaccharides impart a ropy texture to yogurt. The yogurt bacteria and *Lb. delbrueckii* subsp. *bulgaricus* produce EPS with low concentrations attained 100 – 800 mg/L[25].

Table II: Proteolytic activity and EPS production by strains

Strains	Diameter of proteolysis zone (mm)	EPS production (g/L)
St. thermophilus R_4	33.00 ± 0.18	0.150
St. thermophilus R_{14}	17.00 ± 0.07	0.553

Syneresis of fermented milk was significantly different ($P< 0.05$). Syneresis obtained with strain coded R_4 (5.2 %) was higher than obtained with R_{14} (1.9 %) (Table III). The greater syneresis of fermented milk (R_4) may be explained by the higher proteolysis activity and the lower EPS production of the strain compared with the other strain R_{14}. Aryana et al.[26] reported that high syneresis can be explained by the greater titratable acidity and/or greater proteolysis. In our case, similar acidifying ability was obtained with the strains but strains coded R_4 showed the higher proteolysis activity.

TableI III: Syneresis and water holding capacity of fermented milk (%)

Strains	Syneresis (%)	Water holding capacity (%) 1day
St. thermophilus R_4	5.20 ± 0.50	21.20 ± 2.53
St. thermophilus R_{14}	1.90 ± 0.70	11.00 ± 1.50

Water holding capacity (WHC) obtained after 24 h was 21.20 % and 11.00 % in milk samples fermented by strains R_4 and R_{14} respectively (Table III). The difference was significant ($P < 0.05$) and WHC of fermented milk prepared with strain R_4 was greater compared to milk fermented by *St. thermophilus* R_{14} (+ 10.20 ± ± 1.03 %). Perhaps the difference in the WHC is related to the technological properties of the strains especially the proteolysis activity and the EPS production.

Probiotics properties

Resistance to acidity is among the *in vitro* test frequently suggested for the evaluation of the probiotic potential of an individual strain[27]. Results showed that *St. thermophilus* R_4 seem to have better acid tolerance. In general, strain R_{14} showed lower viability in M17 broth at pH 2.0 than at pH 3.0 and pH 6.5. Results of Table IV showed a progressive reduction in viability at pH 2.0, while a variable response at pH 3.0 was observed. In the study conducted by Iyer et al.[19], survival up to a log count of 7.4 at pH as low as 2.0 indicated good degree of acid tolerance of indigenous strains of dairy *St. thermophilus.* Vinderola and Reinheimer[28] observed better survival of *St. thermophilus* strains at pH 3.

Table IV. Acid tolerance of tested strains

Strains	Number of viable cells x10^8/mL					
	pH 2		**pH3**		**pH6.5**	
	0h	**2h**	**0h**	**2h**	**0h**	**2h**
St. thermophilus R_4	45.33	40.33	36.00	33.00	54.00	66.00
St. thermophilus R_{14}	30.33	22.00	42.33	38.33	29.00	31.33

The ability to survive the action of bile salts is an absolute need of probiotic bacteria, and it is generally included among the criteria used to select potential probiotic strains. It was reported that 0.3 % bile salt is considered to be a critical concentration for the selection of resistant strains[29]. From the results of the present study (Table V), it appears that *St. thermophilus* R_4 exhibited good resistance to low pH and bile salt since the number of cells was important on M-17 agar with 0.3 % of oxgall. In contrary *St. thermophilus* R_{14} showed bad resistance to bile salt. The results of study conducted by Iyer et al.[19] showed that both strains of *St. thermophilus* isolated from Indian fermented milk products proved to be quite resistant even in presence of 2 % bile salt.

At the end of time incubation in simulated gastrointestinal juice, strains showed decrease in cell counts (Table VI). The strains exhibited good resistance to these hostile conditions but *St. thermophilus* counts were partially affected by GIJ and decreased significantly after 3h of incubation ($P < 0.05$). The results showed that *St. thermophilus* R_4 has better survival rate. The study conducted by Pilar et al.[30] to evaluate the survivability of *St. thermophilus* along with three probiotic strains with regard to their resistance to simulated gastrointestinal stress showed that gastric emptying (above pH 3.0) release about 53 % of viable cells of *St. thermophilus* into the intestinal tract, and 23 % of them subsequently survive intestinal stress.

As shown in Table VII, *St. thermophilus* strains showed some antibacterial effect against the five pathogens assayed. *E. coli*, *K. oxytoca*, *L. monocytogenes* and *B. subtilis* proved to be the most sensitive pathogens, in contrary, *S. aureus* was resistant. The R_{14} strain showed the best inhibition activity, the diameters of inhibition zones were between 9 mm and 19 mm with a best activity against *E. coli* and *L. monocytogenes.* The supernatants of strains exhibited the best inhibitory activity. The diameters of inhibitions range from 13 mm to 33 mm. It was reported that production of certain metabolites by LAB such as lactic acid lowers the pH with a decisive role in inhibiting the development of pathogenic bacteria. Secretion of hydrogen peroxide is also an important factor and was identified as having inhibitory effect on pathogenic bacteria[31]. In our study, the inhibition activity of neutral supernatants became very lower and the diameters of inhibitions zones were no larger than 6mm, so, the antimicrobial activity of our supernatants is related to the production of lactic acid.

The results showed that tested strains have variable degree of hydrophobicity and they have more affinity for xylene (27.54 % and 29.76 %) followed by n-hexane (Table VIII). Similar results were obtained by Iyer et al.[19].

Table V. Bile salt tolerance at different pH of tested strains

Strains	Number of viable cells x 10^8/mL (0.3 % oxgall)					
	pH 2		**pH 2.5**		**pH 6.5**	
	0h	**2h**	**0h**	**2h**	**0h**	**2h**
St. thermophilus R_4	41.33	29.00	31.66	27.33	41.66	40.00
St. thermophilus R_{14}	30.33	14.00	26.33	16.00	31.33	29.33

Table VI: Resistance to simulated gastrointestinal juice of tested strains

Strains	Number of viable cells x 10^8/mL	
	0h	**3h**
St. thermophilus R_4	16.33 ± 0.09	14. 13 ± 0.12
St. thermophilus R_{14}	21.66 ± 0.08	16.16 ± 0.15

Table VII: Antibacterial activity of strains and supernatants

St. thermophilus and supernatants	Zone of inhibition (mm)				
	*E. coli ATCC*25422	*S. aureus*	*K.oxytoca*	*L. monocytogenes*	*B. subtilis* ATCC21332
R_4	2	–	1	1	1
S	2	–	2	2	2
NS	0	–	0	0	0
R_{14}	2	–	1	1	1
S	2	–	2	2	2
NS	0	–	0	0	0
S: Supernatant; NS:Neutral Supernatant (-):No inhibition (0): inhibition zones between 2 and 8mm (1): inhibition zones between 8 and 12mm (2): inhibition zones larger than 12mm					

Table VIII: Hydrophobicity and adherence capability of tested strains

Strains	Hydrophobicity (%)		Adhesion
	n-hexane	xylene	
St. thermophilus R_4	26.54 ± 3.45	27.54 ± 2.40	++
St. thermophilus R_{14}	28.35 ± 2.36	29.76 ± 1.23	+++
(++):Positive adhesion (+++): Excellent adhesion			

In another study, the hydrophobicity of *St. thermophilus* varied from 24 % to 98 % depending on their source[32]. The variation in hydrophobicity (in terms of the affinty to solvents) and among strains has been explained by the fact the adhesion depends upon the origin of strains as well as surface properties[19].

The ability to adhere to host intestinal mucosa is considered as an important selection criterion for LAB strains intended for probiotic use. In this study, *St. thermophilus* R_{14} showed the best adherence specificity to poultry ileum epithelial cells with efficiency of more than 15 bacteria per epithelial cell. Data showed that hydrophobicity property is generally thought to be correlated with bacteria adhesion[21]. In our study, strains showed lowest hydrophobicity and affinity to xylene and n-hexane but they adhered *in vitro* to poultry ileum epithelial cells in high number.

The compatibility assay was conducted to obtain a mixture of thermophilic starter used for yogurt production with the recognized species *St. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus*. The results of the cross-streak test showed the absence of antagonistic activity and confirmed the compatibility between the strains. So, we have two thermophilics starters *St. thermophilus* R_4 – *Lb. delbrueckii* subsp. *bulgaricus* and *St. thermophilus* R_{14} – *Lb. delbrueckii* subsp. *bulgaricus*. Timmerman et al.[33] reported that in the probiotic mixtures, the isolates with antagonism might cause the loss of viability of other strains and lead to diminished efficacy.

Conclusion

To conclude, the *St. thermophilus* strains were found *in vitro* to possess desirable technological traits and probiotic properties in terms of acid production, EPS production, proteolytic activity, bile salt tolerance, cell surface hydrophobicity, antibacterial activity and adherence to ileum epithelial cells. These strains with proven promising probiotic attributes are good candidates for further application in Algerian yogurt industry.

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Summary

Idoui T., Sifour M., Haddar HO.: The probiotic properties of autochthonous *Streptococcus thermophilus* isolated from traditional algerian foods

This study aimed to assessing the technological traits and probiotic potential of autochthonous *St. thermophilus* R_4 and R_{14} isolated from Algerian traditional fermented milk cold "Raib". The results showed that these local strains were fastest acid producing, have good proteolytic activity, have the ability to produce exopolysaccharides and exhibited good antagonistic activity.

The results of the probiotic properties showed that these strains were able to survive at pH 2.5 and 0.3 % bile with a good adherence to epithelial cells. These strains showed a good viable count in simulated gastrointestinal juice. The results of cross-streak test showed the absence of antagonistic activity and confirmed the compatibility between these strains and a commercial *Lb. delbrueckii* subsp *bulgaricus.* The tested strains were found in vitro possess desirable probiotic properties and they are good candidates for their application as probiotic starter in the food industry.

For the Algerian yogurt industry, potential *St. thermophilus* strains able to be used as starter in combination with other local *Lb. delbrueckii* subsp *bulgaricus* may be found.

Keywords: Probiotic, *Streptococcus thermophilus*, Autochthonous, traditional foods

Souhrn

Idoui T., Sifour M., Haddar HO.: Probiotické vlastnosti původní bakterie *Streptococcus thermophilus* izolované z tradičních alžírských potravin

Tato studie se zaobírá posouzením technologických vlastností a probiotického potenciálu původních bakterií *St. thermophilus* R_4 a R_{14} izolovaných z tradičního alžírského fermentovaného mléka nazývaného "Raib". Výsledky ukazují, že tyto lokální kmeny produkují kyselinu rychleji, mají dobrou proteolytickou aktivitu, produkují exopolysacharidy a jsou dobrými antagonisty. Výsledky probiotických vlastností ukazují, že tyto kmeny jsou schopné přežít v pH 2.5 a v 0.3 % žlučové kyselině s dobrou adherence na epiteliální buňky. Dále vykazují dobrou viabilitu v simulovaném gastrointestinálním prostředí. Výsledky "cross-streak" testu prokázaly nepřítomnost antagonistické aktivity a potvrdil kompatibilitu mezi těmito kmeny a komerčními *Lb. delbrueckii subsp bulgaricus*. Testované kmeny vykazují *in vitro* žádoucí probiotické vlastnosti a jsou vhodnými kandidáty pro jejich použití jako probiotika v potravinářském průmyslu.

Keywords: probiotika, *Streptococcus thermophilus*, tradiční potraviny

EFFECTS OF NATURAL SUBSTANCES ON MICROBIAL BIOFILMS

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Introduction

Biofilms often contaminate wide range of surfaces including living tissues and medical devices, industrial or potable water system piping or natural aquatic systems. The pathogenic or opportunistic pathogenic microorganisms in the form of biofilms cause very resistant infections or even fatal diseases. It is the reason of increased attention to this topic in many groups of researchers.

Biofilm definition

Microbial biofilm is an assemblage of microorganisms, which are irreversibly attached to biotic or abiotic surface or to each other and are enclosed in an extracellular matrix (ECM), which is composed of extracellular polymeric substances (EPS).[1] Biofilms may be single-species such as those involved in endocarditis but more common are multi-species biofilms such as biofilm in dental plaque etc.[2] The ability of biofilm formation in bacteria or yeast has been already known for some time, but recently this ability was discovered also in surface-associated filamentous fungi such as *Aspergillus fumigatus*, *Aspergillus niger*, *Trichoderma* spp. etc.[3] The solid-liquid interface is most frequent and best understood interface for the attachment and growth of microorganisms.[4] It was shown that microorganisms in biofilm have different behaviour than planktonic cells. They are up to 1000 times more resistant and also the profile of gene transcription is distinct from that of planktonic cells.[5]

Biofilm forming

Biofilm development (Fig. 1) includes generally five defined basic steps (adsorption, adhesion, microcolony formation, mature biofilm and dispersal).[3]

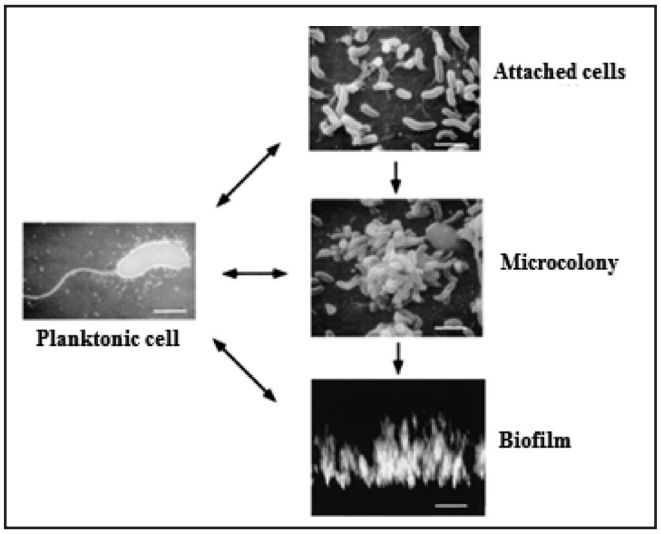

Fig. 1: Steps in biofilm formation by *Vibrio cholerae*; the planktonic bacterium bar = 1 μm the attached cells and microcolony bar = 2 μm, and the biofilm bar = 10 μm[5]

Many details are known about the first stages (microbial attachment and initial biofilm formation). The effects of the substratum, conditioning films forming on the substratum, hydrodynamics of the aqueous medium, characteristics of the medium, and

various properties of the cell surface are important parameters for proper modelling of the cell attachment.[4] The ability of microbial adhesion depends on the surface properties. The surface materials may be either hydrophobic such as Teflon (DuPont), various plastics, latex, and silicone or highly charged hydrophilic such as glass and various metals. Other important property of surface materials is roughness. Some of them are quite rough and textured (e.g., water pipes) while others are much smoother (e.g., silicone or Teflon catheters).[6] The cell surface properties are also very important parameters of the cell attachment. Microbial cells can overcome the repulsive forces of the substratum by using flagella, pili, fimbriae, or glycocalyx, which facilitate the cell attachment on the surface.[6]

After initial adhesion, the irreversibly attached cells (i.e. those not removed from surface by gentle rinsing) begin to proliferate, form microcolonies and produce the extracellular polymers whose composition is different and very specific for each existing biofilm.[6]

The next stage is the maturation of biofilm, when three-dimensional structure is developed and the cells in biofilm are interlaced and encased by extracellular polymeric matrix. At last some of the cells are released from biofilm and they can start a new cycle.[6]

Biofilm structure

The architecture of biofilm is constantly changing, because it is affected by external and also by internal processes. Biofilms are heterogeneous and unique communities but share some structural attributes that are universal. The microcolonies consist of microbial cells that are separated from other microcolonies by interstitial voids (water channels). These water channels allow the flow of liquids, and thus diffusion of nutrients and oxygen to cells within the biofilm. On the contrary, they allow secretion of extracellular or waste products to the environment. The biofilm structure may also include some particles of nonmicrobial components from the host or environment (e.g., erythrocytes and fibrin). Due to close proximity of cells arranged in the biofilm there is suitable environment for gene exchange and quorum sensing (QS).[2]

Extracellular Polymeric Matrix

Every extracellular polymeric matrix has at least a little different chemical and physical properties, but the general composition is similar. The main compounds are polysaccharides, proteins, extracellular DNA, etc. Another important trait of ECM is high hydration enabled by the incorporation of large amounts of water into its structure by hydrogen bonding. The production of EPS and development of ECM increases with biofilm maturation and its most important role is the protection of biofilm against antimicrobial agents, probably due to ability of direct binding to these substances.[2,4]

Quorum sensing (QS)

Quorum sensing is a complex mechanism of intercellular communication in biofilm, which serves for the regulation of collective behaviour of cells in biofilm. It is mediated by small diffusible signal molecules – autoinducers (most commonly N-acyl homoserine lactones (AHLs)) secreted by the microorganisms. This mechanism can regulate the secretion of virulence factors, biofilm forming, bioluminescence, etc. Pathogenic bacteria, yeasts and fungi use this mechanism to survive the host immune response during infection phase.[7,8]

Biofilm in nature

Microorganisms living in nature primarily prefer to be in a form of biofilm.[4] Almost 99 % of bacteria in nature can exist in this form. They attach to either abiotic surfaces such as soil and aquatic systems and wide range of medical or industrial devices, or biotic surfaces such as tooth enamel, heart valves, or the lung, and middle ear.[6]

It is already known that biofilm formation plays an important role in many kinds of diseases such as endocarditis, osteomyelitis, dental caries, middle ear infections, medical device-related infections, ocular implant infections, and chronic lung infections in cystic fibrosis patients, etc.[2] The most common bacteria isolated from medical devices belong to the group of coagulase-negative staphylococci, further *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Enterococcus faecalis, Klebsiella pneumoniae, Pseudomonas aeruginosa* (Fig. 2, a)*, Escherichia coli, Bacteroides species*, *Enterococcus species*, etc. The representatives of yeast are for example *Candida albicans, Candida parapsilosis* (Fig. 2, b)*, Candida tropicalis* and *Trichosporon cutaneum* (Fig. 2, c) and also moulds can be isolated from these materials, especially *A. fumigatus* (Fig. 2, d).[6]

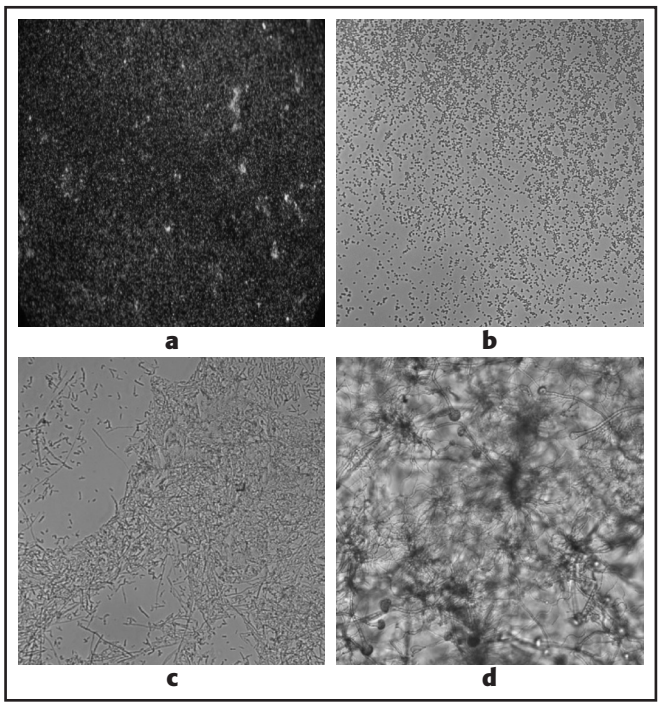

Fig. 2: Examples of microbial biofilms; **a** – *P. aeruginosa* biofilm, zoom 400x, **b** – *C. parapsilosis* biofilm, photographed by automatic microscope Cellavista, objective 20x, **c** – *T. cutaneum* biofilm, photographed by automatic microscope Cellavista, objective 20x, **d** – *A. fumigatus* biofilm, photographed by automatic microscope Cellavista, objective 20x

How to fight against biofilm?

Microbial community of biofilm is able to survive 10 – 1000 times higher concentrations of antimicrobial substances than planktonic cells and is much more resistant to phagocytosis. Therefore it is extremely difficult to destroy it and remove from infected tissues. The surgical removal and replacement of the infected tissue or medical device is often the only possible solution for a lot of patients. Currently, the finding of new possibilities is the aim of study for many groups of researchers. Generally there are two possible ways to fight against biofilm: the first one is the eradication of biofilm and the second one is the prevention of infection by using agents which help to avoid microbial adhesion.[2]

Eradication of mature biofilm

Because microorganisms in biofilm are much more resistant than planktonic cells, it is necessary to use the higher concentrations of antibiotics. Unfortunately it is not the best solution as microorganisms begin to develop the multiresistance and it is not friendly for the environment and often not applicable in human medicine. Therefore scientists try to find new strategies, for example the inhibition of QS, when the communication is not allowed among the microorganisms which leads to biofilm inhibition. Further mechanical removal by a friction is possible, but it is often painful for the patient. Another opportunity is enzymatic removal by dispersin B. However, it is much more appropriate to try to prevent the biofilm formation on the surface.[8]

Prevention of biofilm forming

For the avoidance of biofilm formation it is possible to prepare anti-adhesive materials, which preclude the microbial adhesion, or antimicrobial materials, which kill the cells before they can adhere to the surface. Anti-adhesive materials preparation originate by the modification of their surface, especially by changing of roughness, surface energy or immobilization of anti-adhesive compounds, such as poly(ethylene glycol) or polysaccharides. The surface of the antimicrobial materials is modified by the incorporation or covalent binding of antibiotics, quaternary ammonium, and silver, copper, etc.[8]

The new promising method for the prevention of biofilm formation is the use of natural products, which have antimicrobial activity and no microbial resistance to them is developed.[8]

Effects of natural substances

Natural substances may help with the treatment of biofilm infections. We can obtain them from microorganisms, marine organisms, plants, and also animals.[9] But the most commonly studied are the natural substances produced by microorganisms or obtained from plants.

Microbial natural products

Microorganisms are one of the most important producers of natural substances.[9] Representatives of these products are QS inhibitors. QS inhibitors are competitive antagonists of AHLs without signalling activity, which bind to the same receptor site as AHLs and disturb the intercellular communication in biofilm. The examples of these inhibitors are riboflavin and lumichrome.[9]

Biosurfactants are natural surface active compounds, which have the ability to change interfacial tension and thus negatively affect microbial adhesion. Most common producers of biosurfactants are bacteria and fungi. Glycolipids, lipoproteins, polysaccharides, or proteins can have these properties. Recently, there is a great interest in biosurfactants, which are called rhamnolipids (amphipathic anionic glycolipids composed of rhamnose and b-hydroxy-fatty acids), which are produced by *P. aeruginosa, Acinetobacter calcoaceticus, Enterobacter asburie*, etc.[10] Another biosurfactants are for example surfactin and iturin A (lipopeptides), which are produced by *Bacillus subtilis*, putisolvins (cyclic lipopeptides), produced by *Pseudomonas putida*, viscosin and massetolide A (also cyclic lipopeptides), produced by *Pseudomonas fluorescens*, etc.[9]

Plants

Plants have been used as a natural medicine since ancient times. They provide the wealth of natural products useable as whole plants, their parts or extracts. Many of the substances obtained from plants have antimicrobial or even antibiofilm activity.[9]

For example, natural polyphenols belong among these substances. They are contained in green tea (i.e., pigallocatechin-3-gallate) and fruits such as blackberries, raspberries, strawberries and cranberries (i.e., ellagic acid). Polyphenols can act as antagonists of AHLs. Proanthocyanidin in red wine is also polyphenol with antiadhesion and antibiofilm activity.[9]

Flavonoids are also very efficient natural products with antibiofilm activity. The most effective flavonoid is baicalein, which is obtained from *Oroxylum indicum* or the roots of *Scutellaria baicalensis*. Further, baicalin (the glucuronide of baicalein), naringenin, kaempferol, quercetin, and apigenin have the ability to inhibit biofilm formation.[9]

Furanocoumarines (i.e., bergamottin and dihydroxybergamottin) contained in grapefruit juice are potent inhibitors of G- bacteria autoinducers. Aesculetin present in *Aesculus hippocastanum* (horse chestnut) is also able to prevent biofilm formation.[9]

Very interesting possibility is the use of commonly used spices, which are able to prevent microbial adhesion. Examples include the cinnamaldehyde (flavor essence from cinnamon trees), which has been shown to reduce the production of EPS. Further, vanilla and curcumin can be used as bacterial QS inhibitors, peppermint has just weak antibiofilm activity, capsaicin (the compound of chilli and pepper) has a negative effect on microbial adhesion and biofilm formation, and the compounds of garlic have the prophylactic properties, etc.[9]

Among the terpenic compounds belongs the essential oil from *Satureja hortensis* with antibiofilm effects and also pentacyclic triterpene boswellic acid, which is obtained from *Boswellia serrata*. This biologically active substance has ability to prevent biofilm formation or even to eradicate mature biofilm.[9]

Similarly the stilbenes (i.e. resveratrol) have the potential antibiofilm activity.[11]

Conclusion

Biofilm infections are a big complication especially in today's medicine. The finding of new solutions is necessary for better and more efficient treatment of diseases originated from biofilm formation. The use of natural substances obtained from microorganisms, plants or some other living organisms is promising way for the future research in this area. There is a wealth of substances with potential or proven antimicrobial or even antibiofilm activity which can be tested for the finding of new possibilities of the treatment of biofilm-based diseases.

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Summary

Kvasničková E., Pádrová K., Masák J.: Effects of natural substances on microbial biofilms

Microbial biofilms are communities of microorganisms, which are irreversibly attached to a surface, interface or to each other and are embedded in an extracellular polymeric matrix. They cause wide range of human infections and contaminate medical instruments and industrial devices. Because microorganisms in biofilm are up to 1000 times more resistant than planktonic cells, the prevention or eradication of biofilm is extremely difficult. It is the reason of increased attention to this topic in many groups of researchers. Higher concentrations of antibiotics are not solution because microorganisms soon develop multiresistance. Therefore the use of natural substances represents new promising method for the treatment of biofilm infections. They can be used in low concentrations and no resistance against them is developed. Recently many natural substances with antibacterial, antifungal or antibiofilm activities were isolated from microorganisms or plants. Examples of these substances are groups of polyphenols, flavonoids, stilbenes, terpenes, commonly used spices, biosurfactants and many others. In this review the possible application of selected natural substances is described.
Key words: biofilm, natural substances, prevention, eradication

Souhrn

Kvasničková E., Pádrová K., Masák J.: Vliv přírodních látek na mikrobiální biofilmy

Mikrobiální biofilmy jsou společenství mikroorganismů, která jsou nevratně přichycena k povrchu, rozhraní či k sobě navzájem a jsou obalena v extracelulární polymerní matrix. Tato mikrobiální uskupení jsou zodpovědná za širokou škálu onemocnění a kontaminace lékařských nástrojů či průmyslových zařízení. Protože mikroorganismy žijící ve formě biofimu jsou až tisíckrát odolnější než planktonické buňky, prevence vzniku či eradikace existujících biofilmů je velice obtížná. To je důvod zvýšeného zájmu mnoha vědeckých skupin o studium dané problematiky. Použití vyšších koncentrací antibiotik není vhodným řešením, protože mikroorganismy si vůči nim velmi snadno vytváří multirezistenci. Proto je velice aktuální snaha o využití přírodních látek pro léčbu biofilmových infekcí. Přírodní látky mohou být často použity v nižších koncentracích a u mikroorganismů vůči nim nevzniká rezistence. V nedávné době byla u mnoha přírodních látek produkovaných mikroorganismy či izolovaných z rostlin experimentálně potvrzena jejich antimikrobiální, antifungální či dokonce antibiofilmová aktivita. Jako příklady lze uvést polyfenoly, terpeny, flavonoidy, stilbeny, některé složky běžně používaného koření, biosurfaktanty a mnoho dalších. Tento přehledový článek pojednává o možných aplikacích některých z nich.
Klíčová slova: biofilm, přírodní látky, prevence, eradikace

SURVIVAL OF FREE AND MICROENCAPSULATED *Lactobacillus plantarum* G1 IN AN *IN VITRO* SIMULATED GIT SYSTEM IN PRESENCE OF DIFFERENT BEVERAGES

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Introduction

In recent years, people are increasingly interested in foods which offer a balanced diet and promote human health. This is why the demand on functional foods is growing steadily. Probiotics are one of the main components of functional foods present generally in products in two forms: supplements and foods[1,2].

Nowadays, it is well known that probiotics are microorganisms belonging most commonly to lactic acid bacteria like *Lactobacillus*, *Bifidobacterium* and some other species such as *Saccharomyces* and *Streptococcus*[3]. In addition, several studies have shown that probiotics possess many beneficial effects including the prevention or treatment of intestinal infections, irritable bowel syndrome, improvement of the digestibility of food products...[4,5]. However, in order to exert its beneficial effects, probiotics must be present at a minimum level of 10^6 CFU/g of food product or 10^7 CFU/g at point of delivery or be eaten in sufficient amounts to yield a daily intake of 10^8 CFU/g [6]. Furthermore, they must also survive during their passage through digestive tract, resist gastric acid and bile salts to arrive viable and in sufficient amounts to intestine[7]. For this purpose, microencapsulation appears to be an ideal tool for bacterial cell protection for which many studies proved the protective role against adverse conditions to which probiotic can be confronted[8].

Several researchers reported the role of microencapsulation in improving cell viability of probiotic bacteria in conditions simulating the gastrointestinal tract [9,10,11], others studied the viability of microencapsulated probiotic cells in food matrixes (as probiotic carriers)[1,12,13], but no report (or little) is found on the use of microencapsulated cells in presence of both the ingested food and the gastrointestinal conditions simultaneously. The aim of this work is therefore to study the viability of free and microencapsulated probiotic *Lb. plantarum* using sodium alginate (2 %) as a matrix in a system simulating the gastrointestinal tract functions and in presence of three beverages: green tea, black coffee and orange juice that are commonly and highly consumed by Algerian population and worldwide.

Materials and methods

Bacterial strain

Lb. plantarum G1 was previously isolated from chicken crop and identified by 16S rRNA technique (Accession number: KC965107). The strain was grown on MRS medium (Institut Pasteur, Algiers).

Microencapsulation procedure

Alginate (2 % w/v) capsules containing the *Lb. plantarum* cells were prepared by dissolving 2 g of sodium alginate (Louis Francois) in 80 mL distilled water under constant stirring and heating at 80ºC. The solution was autoclaved and cooled to 40ºC to which 20 mL of a freshly prepared cell suspension was added. The mixture was injected into cold 0.05M $CaCl_2$ cross-linking solution for 1 hour [14].

Viability of free and microencapsulated Lb. plantarum under in vitro simulated human gastrointestinal tract (SGI) conditions

In order to study the viability of free and microencapsulated *Lb. plantarum* in simulated human gastro-intestinal tract (GIT) and to evaluate the effect of some commonly consumed beverages on this probiotic bacteria, three beverages were prepared as recommended by the manufacturer instructions for this study: black coffee solution "*NESCAFÉ Gold®*" «Nestlé», green tea "*EsSahraa®*», orange juice «*Rouiba®*" and distilled water.

The tests were performed in sterile Erlenmeyer flasks (250 mL), free and microencapsulated cells were exposed to simulated gastro-intestinal tract *in vitro.* At the beginning, either free or microencapsulated *Lb. plantarum* cells were treated in simulated gastric conditions by inoculating 50 mL of each of the three sterile HCl-acidified beverages (pH 2.0) with freshly prepared probiotic cells (the initial concentration was approximately 10^{14} CFU/mL); the flasks were incubated for 90min at 37 ± 1°C in a shaking water bath at 50 rpm. Subsequently, the cells were incubated for 150 min under intestinal conditions by introducing a mixture of 1 % pancreatin, 0.3 % bile salts, 0.5 % NaCl, aseptically to the beverages (pH was adjusted to 6.8 using NaOH). The same temperature was kept. However, shaking was adjusted to 150 rpm. Samples of 1 mL (for free cells) or 5 beads (for microencapsulated cells) were taken, at 0 min (the beginning), 90 min (after stomach conditions), and 240 min (after the whole reaction) for the determination of total viable counts [15]. The microencapsulated cells were released after dissolving of five beads in 2 mL of 1M phosphate buffer (pH 7.0), followed by shaking for 15min using a vortex.

Total viable counts of *Lb. plantarum* were determined by a pour plate method using MRS agar after serial dilutions in normal saline. Plates were incubated at 37°C for 48 h; the results were expressed as log CFU/mL.

The test was repeated three times and the average of the resulting counts was plotted.

Results and discussion

In order to simulate the gastro-intestinal tract *in vitro*, we have designed a model that is presented in Figure 1. A digestion time of 4h was used according to many *in vitro* models including that proposed by Pacheco and Toro (2010)[15]. The delivery time is relatively short to simulate a fast transit, suitable to liquid foods like beverages. It should be mentioned that the slight anaerobic conditions found in the intestine were not considered in this model. Evidently, *in vivo* conditions can never be completely simulated; several factors influence results of *in vitro* digestion, like food characteristics, enzyme activity, stress and digestion time [16].

In this study, the initial count of probiotic cells was in the range of 10^{14} CFU/mL in order to maximize the number of surviving cells after the whole treatment. At the end of the reaction, viable count revealed that the applied conditions did not totally inhibit the growth of the cells, suggesting that the proposed model is appropriate for simulating the GIT.

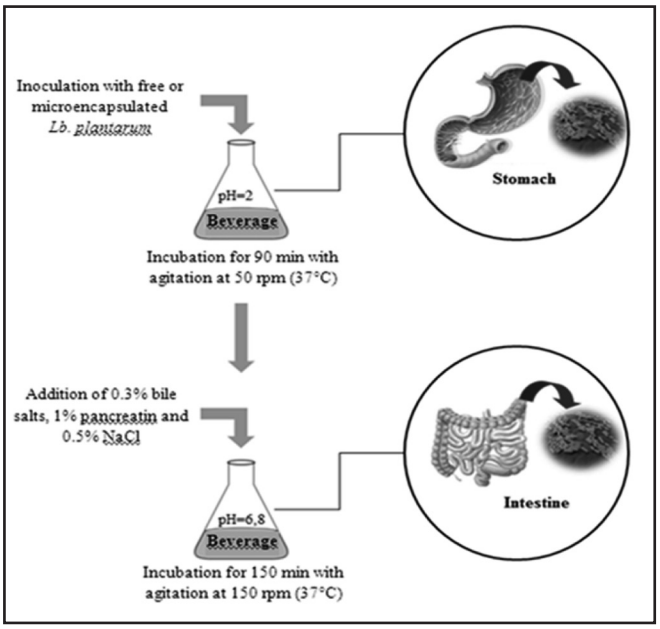

Fig. 1: Schematic representation of the laboratory designed simulated GIT

The initial beads resulting from the microencapsulation of *Lb. plantarum* cells within alginate were spherical, relatively uniform in size (2.5 mm), weighing approximately (0.0133 g). After their exposure to SGI conditions, the beads incubated in water remained stable. However, the diameter and the weight of beads incubated in coffee and tea have decreased to 2 mm and 0.006 g, respectively. In contrast, the diameter and the weight of beads incubated in orange juice have increased to 2.7 mm and 0.0157 g, respectively (Table I).

The decrease in the size and weight of the beads incubated in coffee and tea can be probably due to disintegration of the gel in the presence of excess monovalent ions, and harsh chemical environment; especially when we know that alginate may undergo a reduction in its molecular weight when it is exposed to low pH [17]. Moreover, the volume of beads incubated in orange juice increased, and this could be explained by the formation of hydrophobic alginate in acidic conditions leading to bead swelling, thus particles are most stable [13].

Counts of free and microencapsulated *Lb. plantarum* in distilled water after exposure to SGI conditions are shown in Figure 2a. After 90min in the solution simulating the stomach (pH 2.0), 10.38 log CFU/mL of the microencapsulated cells kept their viability, however, the count of free cells after the same treatment was 9.17 log CFU/mL. When both free and encapsulated cells were exposed to intestine fluid (digestive enzymes and bile salts), 7.04 log CFU/mL of free cells and 10.28 log CFU/mL of microencapsulated cells survived in these conditions; this means that viability of microencapsulated cells was not highly affected by intestine treatment. The same results were found by Todorov and coworkers (2012); they have indicated that the protection given by sodium alginate coats is more evident in conditions simulating intestine than in conditions simulating the stomach conditions. They also mentioned that microencapsulated *Bifidobacterium bifidum* and *Lb. acidophilus* viability decreased after exposure to 2 % (w/v) and 4 % bile salts[10].

When microencapsulated *Lb. acidophilus* CSCC 2400 and *Lb. acidophilus* CSCC 2409 were subjected to low pH and high bile salts concentration there was a significant increase in viable cells counts compared to the free cells[18]. The study of the viability of encapsulated *Lb. casei* NCDC-298 in alginate at low pH and high bile salt concentration showed that survival of encapsulated *Lb. casei* was better compared to free cells[19].

Survival of free and microencapsulated *Lb. plantarum* in SGI conditions in the presence of green tea is shown in Figure 2b. The number of free cells decreased from 14.89 log CFU/mL to 9.39 log CFU/mL after the treatment in gastric conditions, however, after the incubation in simulated intestinal fluid it reached 6.69 log CFU/mL. On the other hand, the microen-

Table I: Physical characteristics of alginate beads containing *Lb. plantarum,* before and after digestion

	Initial characteristics	Beads incubated in SGI conditions with			
		Distilled water	***Green tea***	***Black coffee***	***Orange juice***
Diameter (mm)	2.5	2.5	2	2	2.7
Form	spherical	spherical	spherical	spherical	spherical
Weight (g)	0.0133	0.0133	0.006	0.006	0.0157

capsulated cells decreased to reach 10.77 log CFU/mL after the incubation in acidic conditions, and 9.07 log CFU/mL after being incubated in intestinal conditions. This indicated that the viability of *Lb. plantarum* was slightly improved using microencapsulation in acidic and intestinal simulated conditions.

Results of the effect of black coffee ingestion on the viability of *Lb. plantarum* (Figure 2c) indicated that the number of free cells decreased from 14.9 log CFU/mL to 8.3 log CFU/mL after the incubation in acidic conditions, while it reached 6.47 log CFU/mL after the incubation in intestine like conditions. However, better results were recorded with microencapsulated cells, since 9.47 log CFU/mL survived the intestine like environment.

Comparison of the results presented above indicated that the cell viability in water was higher than those in green tea and black coffee, while the cell viability in the GIT-like conditions supplemented with black coffee was lower for free cells. Some authors mentioned that the matrix food might protect the lactic acid bacteria from the acid pH present in the human stomach. As a result, probiotic delivery will be enhanced[15]; in contrast, our results on green tea and black coffee effect reflected a negative action on cell viability.

In order to explain the loss of cell viability, the composition of the two beverages as well as the effect of these constituents on the studied strain should be known. The most important constituents of green tea include multiple catechin components like: epigallocatchin-3-gallate, epicatchins-3-gallate, epicatchin and epigallocatechin[20]. Furthermore, coffee includes: flavonoids, caffeic acid, ferulic acid, nicotinic acid, trigonelline, quinolinic acid, tannic acid, pyrogallic acid and caffeine. It is important to note that some of these constituents are known as polyphenol compounds, which are associated generally with antioxidative and anti-in-flammatory activities[21].

It was reported that growth of *Lb. plantarum* was significantly reduced in presence of 1 g/L *p*-coumaric acid (phenolic compound) and the inhibitory activity increased in presence of NaCl. Furthermore, when *Lb. plantarum* cells are exposed to ripe olives, the inhibitory effect of diffused phenolic compounds on these bacteria was significant only when it is associated with NaCl, moreover; the bactericidal effect of phenolic compounds is related to the alteration of cell wall and cytoplasmic membrane, which will possibly lead to destruction of cell envelope[22].

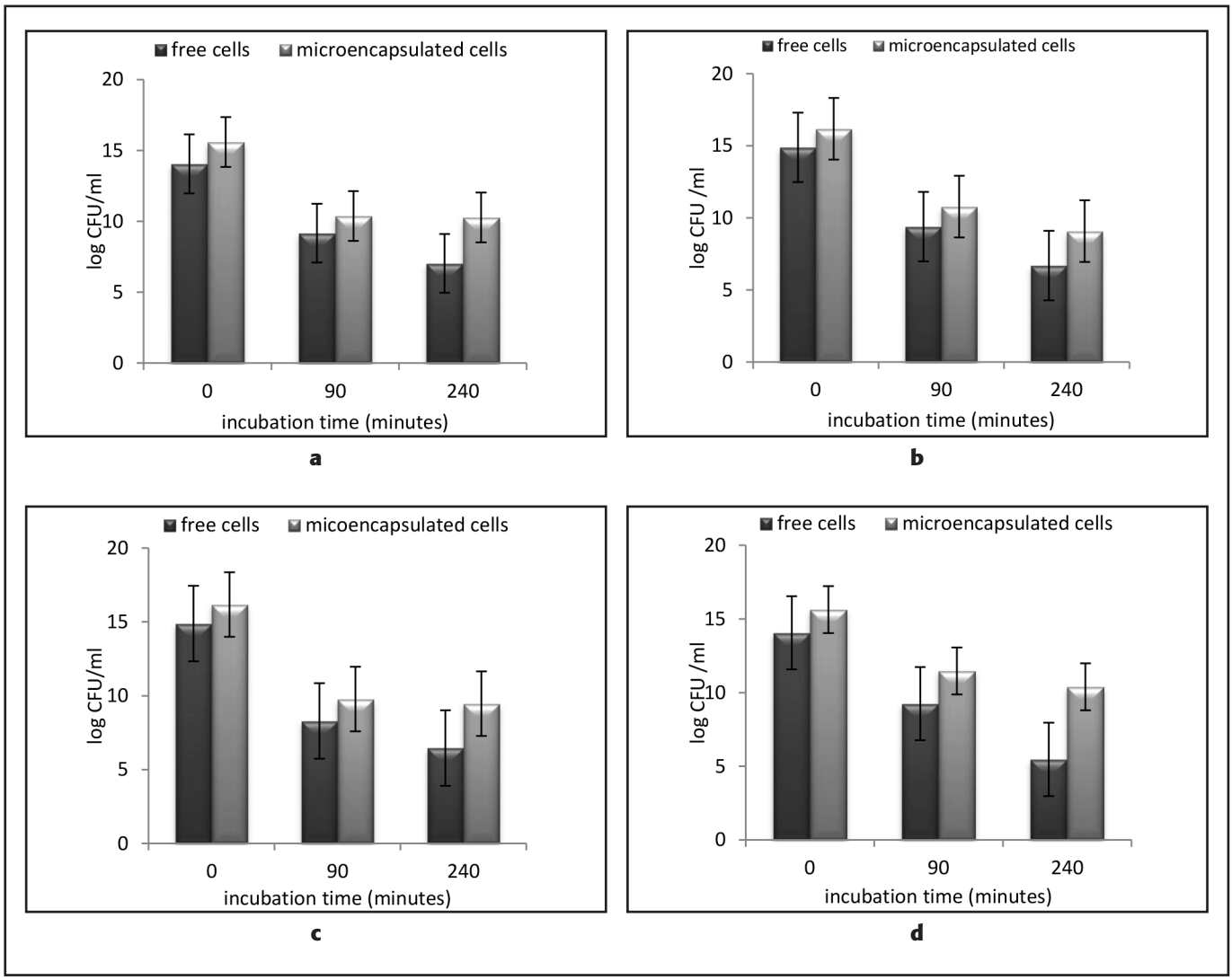

Fig. 2: Viability of free and microencapsulated *Lb. plantarum* in SGI conditions with **a**: distilled water, **b**: green tea; **c**: black coffee and **d**: orange juice

Rozés and Peres (1998) reported the effects of phenolic compounds on the fatty acid composition of *Lb. plantarum* membranes. They described that increasing amounts of caffeic and ferulic acids induced a gradual increase in the amounts of myristic, palmitoleic, stearic and 9, 10-methylenehexadecanoic acid with a concomitant decrease of lactobacilli acid. On the other hand, the addition of tannins induced an increase in the lactobacillic acid level at the expense of vaccenic acid content. Their results suggested that, in presence of acidic phenols, the fatty acid composition is altered in terms of what occurs in response to low temperature or high alcohol concentration[23].

Overall, the decrease in viability of cells during the gastric conditions may be due to certain compounds of tea and coffee like polyphenols, which possess antimicrobial activity and also to cell stress caused under high acidity. Furthermore, the loss of viability during intestinal conditions can be explained probably by the polyphenol compounds and NaCl combination effect.

The effect of the ingestion of orange juice on the viability of free and microencapsulated *Lb. plantarum* is shown in Figure 2d. The initial number of about 14.06 log CFU/mL for free cells was reduced to 9.25 log CFU/mL after the treatment in acidic conditions and it was decreased more to reach 5.47 log CFU/mL after the treatment in intestinal conditions. Results obtained in the case of microencapsulated cells were more interesting because 11.74 log CFU/mL resisted gastric conditions, which correspond to 3×10^{11} CFU/mL. In addition, the loss of viability during intestinal conditions was not important since the number of encapsulated cells decreased to 10.39 log CFU/mL.

In reality, orange juice is a source of bioactive compounds consisting of: vitamin C, sugar, organic acids, dietary fibers, carotenoids and phenolic compounds, which include phenolic acids and flavanones [13; 24]. There are very little studies regarding the effect of ingestion of orange juice on viability of probiotics in human GIT. However, several researches have shown that the main factor influencing the survival of probiotics in fruit juice is the pH as well as the level of organic acids, dietary fibers, proteins, total phenol and oxygen[24]; other studies have demonstrated that the presence of glucose may improve the survival of Lactobacilli in acidic conditions. On the other hand, literatures have shown that a low pH during bacterial growth induces an acid tolerance, which may protect probiotic bacteria not only from acid pH but also from other stresses such as heat, osmotic or oxidative stress[25].

Based on the results of the experiment, the loss of viability for free *Lb. plantarum* during gastric conditions is more important than in the case of microencapsulated one, which is probably due to death of cells under high acidity. Nualkaekul and co-workers (2011) reported that when the cells are present in the environment of low external pH, the energy consumption that is required for maintenance of the intracellular pH is increased. As a result, other crucial cellular functions are depressed of ATP and the cells cannot survive[26]. Furthermore, the action of bile salts on viability of free and encapsulated cells when *Lb. plantarum* is ingested with orange juice is less harmful than gastric conditions. The lower decrease in cell count may be explained by the effect of phenolic compounds of orange juice in the presence of NaCl.

Conclusion

Based on the presented results, the viability of *Lb. plantarum* in SGI conditions and in the presence of coffee, green tea and orange juice was improved by microencapsulation, as a acceptable count of bacteria was obtained after the overall reaction of digestion, suggesting that a satisfactory amount of live bacteria could be successfully delivered to the lower intestine to exert its action.

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Summary
Ouled-Haddar H., Sifour M., Bouridane H., Merabet B., Yakoubi R., Boudergui A.: Survival of free and microencapsulated *Lactobacillus plantarum* g1 in an *in vitro* simulated git system in presence of different beverages
In recent years, people are increasingly interested in foods which offer a balanced diet and promote human health. This is why the demand on functional foods is growing steadily. Many recent studies are focusing on the beneficial effect of probiotics, which are one of the main components of functional foods as well as improving their capacity to survive during passage through digestive tract to arrive viable and in sufficient amounts to colon. Therefore, the conception of an efficient, low-cost system for the delivery of probiotic bacteria is highly attracting food science researchers. In the current paper, microencapsulation within sodium alginate is tested for its capacity to improve *Lb. plantarum* G1 viability in an *in vitro* simulated gastrointestinal system designed in the laboratory. Incubation was realized in presence of three beverages that are commonly and highly consumed by Algerian population and worldwide they include black coffee, green tea and orange juice, the objective was to contribute in developing recommendations to properly consume probiotics,
The results indicated that introducing probiotics simultaneously with one of the studied beverages resulted in a decrease in probiotic viability, however, microencapsulation within alginate beads improved its viability even at the end of the gastrointestinal simulated transit, suggesting that probiotics, when encapsulated, can reach the lower intestine. The cell viable counts for black coffee, green tea and orange juice were 9.47, 9.07 and 10.39 log CFU/mL, respectively.
Keywords: Microencapsulation, Probiotics, *Lactobacillus plantarum*, Simulated GIT

Souhrn
Ouled-Haddar H., Sifour M., Bouridane H., Merabet B., Yakoubi R., Boudergui A.: Přežití volné a mikroenkapsulované bakterie *Lactobacillus plantarum* G1 v různých nápojích v *in vitro* simulovaném gastrointestinálním systému
V posledních letech se lidé stále více zajímají o potraviny, které nabízejí vyváženou stravu a podporují lidské zdraví. To je důvod, proč poptávka po funkčních potravinách neustále roste. Mnoho studií se zaměřuje na stadium příznivého vlivu probiotik, které jsou jednou z hlavních součástí funkčních potravin, jakož i na zlepšení schopnosti probiotik přežít během průchodu trávicím traktem poskytování v životaschopné podobě až do tlustého střeva. Proto koncepce efektivního, nízkonákladového systému pro transport probiotických bakterií přitahuje stále větší pozornost odborníků. V tomto článku je testována mikroenkapsulace do alginátu sodného s cílem zlepšit životaschopnost *Lb plantarum* G1 v *in vitro* simulovaném žaludečním a střevním systému. Inkubace probíhala v přítomnosti tří nápojů, které jsou hojně konzumované v alžírské populaci a na celém světě – černá káva, zelený čaj a pomerančový džus. Cílem bylo přispět doporučením pro řádnou konzumaci probiotik. Výsledky ukázaly, že požití probiotika současně s jedním ze studovaných nápojů vedlo ke snížení životaschopnosti probiotických bakterií. Mikroenkapsulace do alginátu ovšem zlepšila jejich životaschopnost, dokonce i na konci gastrointestinálního systému. Viabilita bakterií v černé kávě, zeleném čaji a pomerančovém džusu byla 9.47, 9.07 a 10.39 log CFU/mL, resp.
Klíčová slova: mikroenkapsulace, probiotika, *Lactobacillus plantarum*, simulace gastrointestinálního traktu

PROBIOTIC PROPERTIES OF FREE AND ENCAPSULATED CELLS OF A BACTERIOCINOGENIC *Lactobacillus curvatus* G6 OF HUMAN ORIGIN

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Introduction

Probiotics are defined as 'live microorganisms, which when ingested in sufficient number, confer a health benefit on the host and may have a positive effect in the prevention and treatment of specific diseases'[1, 2, 3]. Probiotic bacteria should be safe for consumption, reach the intestines alive in large numbers and should maintain the balance of the intestinal flora by altering favorably the gut environment in such a manner that the growth of friendly beneficial bacteria are promoted and harmful disease causing organisms are inhibited[4]. Some preparations of probiotics have been used to prevent diarrhea induced by antibiotics. Studies have shown the effects of probiotics on a variety of gastrointestinal disorders, including inflammatory bowel disease, the irritable bowel syndrome and vaginal infections[5].

Beneficial effects depend on the ability of the probiotic strains to maintain viability in the food during shelf-life and to survive the natural defenses of the host and multiply in the gastrointestinal tract (GIT)[6]. Many factors such as acidity, oxygen content, bile acid of the GIT, heat treatment and long period of storage affect the survival of probiotics in food, fermented products and in the gastrointestinal tract of the host. Several methods have been used to enhance the viability of probiotics, including selection of resistant

strains, stress adaptation, incorporation of micronutrients, and microencapsulation in food matrices[7, 8]. The encapsulation is defined as the technology for packaging solids, liquid, or gaseous materials in capsules to protect the microencapsulated materials from the surrounding environment, or conversely to protect the environment from the active ingredient, while nutrients and products can migrate through the semi-permeable membrane of the capsule. Polysaccharides like alginate, gellan, *k*-carrageenan and starch are the most commonly used materials in microencapsulation of bifidobacteria and lactobacilli[7, 9-11].

The main objective of this work is the study of probiotic properties of a bacteriocinogenic *Lactobacillus curvatus* G6 of human origin and the effect of microencapsulation of this strain in sodium alginate on some probiotic and technological skills.

Materiel and Methods

Bacterial strains and culture conditions

Lb. curvatus G6 used in this study was previously isolated from newborn feces and was considered as bacteriocinogenic bacteria [12]. The isolate was cultivated on MRS broth (Institut PASTEUR, ALGIERS) and incubated at 37°C without shaking. All the experiments are repeated in triplicates.

Microencapsulation procedure

The *Lb. curvatus* G6 isolate was cultivated in MRS broth at 37°C for 20 h, cells were harvested by centrifugation. The pellet was washed with normal saline then resuspended in 5 mL of normal saline. Encapsulation was realized according to the method of Boyaval et al.[13]. Five mL of bacterial suspension were mixed with 45 mL of 2 % alginate solution previously sterilized by autoclaving and homogenized using a magnetic stirrer. The mixture was introduced into a sterile syringe and was injected through a needle into previously autoclaved 100 mL of $CaCl_2$ solution (0.05M) supplemented with 0.1 % (v/v) Tween 80. The resulting beads were separated from $CaCl_2$ solution after 30 min and then washed two times with sterile distilled water. The beads were conserved in normal saline at 4°C for further utilization.

Evaluation of probiotics skills of *Lb. curvatus* G6 *in vitro*

Tolerance to acid

The tolerance of free and encapsulated cells to acid conditions was realized according to Ding and Shah [14]. MRS broth (pH 2.0) was inoculated with $45x10^{12}$ CFU of encapsulated cells per mL of medium and $11x10^{12}$ CFU of free cells of *Lb. curvatus* G6 per mL of medium and then incubated at 37°C for 2 h without shaking. One mL of the free culture and appropriate number of beads was collected at 1 h intervals (0 h, 1 h, 2 h). Encapsulated cells were liberated from microcapsules after dissolving a number of beads in 2 mL of phosphate buffer 1M (pH 7.0), followed by shaking for 15 min using a vortex. Viable count was carried out on MRS agar after appropriate serial dilution in normal saline and the plates were incubated at 37°C for 48 h. The bacterial cells were enumerated as CFU/mL and the results were expressed in percent viability.

Tolerance to bile salts

The tolerance of the free and encapsulated cells to bile salts was realized according to Ding and Shah [14]. MRS broth containing 0.3 % of bile salts (pH 5.8) was inoculated with $42x10^{12}$ CFU of encapsulated cells and $8x10^{12}$ CFU of free cells of *Lb. curvatus* G6 per mL of medium and then incubated at 37°C for 8h. A viable count on MRS agar was carried out after 0, 4 and 8 h of incubation. Plates were incubated at 37°C for 48 h. Encapsulated cells were liberated from microcapsules as described before. The bacterial cells were enumerated as CFU/mL and results were expressed in percent viability.

Survival of Lb. curvatus G6 in simulated intestinal fluid

Simulation of an experimental intestinal fluid (SIF) was prepared by dissolving 9 g/L of NaCl, 10 g/L of pancreatin, 10 g/L of trypsin and 3 g/L of bile salts in distilled water then pH was adjusted to 6.5 [15]. One mL of free cells culture and appropriate number of beads were incubated at 37°C in MRS broth containing the intestinal fluid for 3 h. Three samples were collected at 0 h, 1.5 h and 3 h and the appropriate dilutions were made. Cell release, viable count and cell viability were realized as described before.

Effect of temperature and cold storage on the viability of Lb. curvatus G6 in vitro

The viability of free and encapsulated *Lb. curvatus* G6 in different temperatures was evaluated by incubating free and encapsulated cells at different temperatures (25°C, 40°C, 50°C and 60°C) for 20 min [16]. Cell viability was estimated before and after the incubation period. The viability of *Lb. curvatus* G6 under cold storage was evaluated by conserving 5 mL of a suspension of free and encapsulated cells in 25 mL normal saline at 4 ± 1°C for 2 weeks [17]. Aliquots of 1 mL were taken each week to determine the total number of viable cells.

Hydrophobicity test

The ability of *Lb. curvatus* G6 to adhere to hydrocarbons was determined as follows: The bacterial pellet of a fresh culture was harvested by centrifugation (12000g for 15min at 20°C), washed two times with urea-magnesium sulfate buffer, and resuspended in 1.2 mL of the same buffer. The initial absorbance of the suspension at 450 nm (OD_{450i}) was adjusted to approximately 1. Then 0.6 mL of xylene was added slowly to 3 mL of bacterial suspension and incubated at 37°C for 10 min, the mixture was vortexed for 2 min. After 15 min, the aqueous phase was recovered and the final absorbance was measured at the same wavelength (OD_{450f}). The percentage of cell surface hydrophobicity was calculated by the following equation[18]:

$$\textbf{Hydrophobicity}\ \% = \frac{(\mathbf{OD_{450i} - OD_{450f}}) \times \mathbf{100}}{\mathbf{OD_{450i}}}$$

Autoagregation and coagregation

Autoagregation was determined by the method described by Todorov et al. [19]. The bacterial cells of an overnight culture was harvested by centrifugation (7000 g, 10 min, 20°C), washed, resuspended in sterile normal saline (0.85 % NaCl) and the initial optical density at 660 nm (OD_0) of the bacterial suspension was adjusted to 0.3. After 60 min of incubation at 37°C, the cell suspension was centrifuged (300 g, 2 min, 20°C), and the supernatant optical density (OD_{60}) was measured at the same wavelength. The experiment was performed in duplicate. The autoagregation was determined using the following equation:

$$\textbf{Autoagregation}\ \% = \frac{(\mathbf{OD_0} - \mathbf{OD_{60}}) \times \mathbf{100}}{\mathbf{OD_0}}$$

Co-aggregation of *Lb. curvatus* G6 with the following strains: *Lb. plantarum* F12, *Lb. casei* ssp. *tolerans* G4, *Lb. plantarum*, Methycilin resistant *Staphyococcus aureus* (MRSA), *Listeria monocytogenes* was determined in a similar manner as autoagregation according to Todorov et al. [19]; the bacterial pellet of fresh cultures of the strains was harvested by centrifugation (7000 g for 10 min at 20°C), washed, resuspended in sterile saline (0.85 % NaCl), and then the initial absorbance at 660 nm (OD_i) of bacterial suspensions was adjusted to 0.3. A volume of 500 µl of the suspensions of *Lb. curvatus* G6 was mixed with an equal volume of each strain, incubated at 37°C for 60 min, then harvested by centrifugation (300 g, 2 min, 20°C), and the absorbance of the supernatants (OD_f) were determined at 660nm. Coaggregation was determined using the following equation:

$$\textbf{Coagregation}\% = \frac{(\mathbf{OD_i} - \mathbf{OD_f}) \times \mathbf{100}}{\mathbf{OD_i}}$$

Results and discussion

Aspect and size of beads

Microencapsulation of *Lb. curvatus* G6 was realized using 2 % soduim alginate, beads prepared using the extrusion technique have a spherical form with a diameter of about 3 mm. The number of cells in each bead was determined after lysis of beads and counting the viable cells on MRS agar, the average of cell number was about $15x10^{11}$ CFU/bead.

Evaluation of the probiotics skills in vitro

Effect of acid conditions on the survival of free and encapsulated Lb. curvatus G6

In order to compare the tolerance of free and encapsulated *Lb. curvatus* G6 cells to acid conditions, about $11x10^{12}$ CFU/mL of free cells and $45x10^{13}$ CFU/mL of encapsulated cells were incubated at pH 2.0 for 2 h. The obtained results are presented in Table I. After 1 h of incubation, a significant loss in free cells of *Lb. curvatus* G6 was observed and the number reached 10^9 CFU/mL, which represents a survival rate of 69 %, the decrease of viable cells continue to reach approximately 46 % after 2h of incubation in acidic conditions. Under the same conditions the number of encapsulated cells also decreased and reached $36x10^{10}$ CFU/mL, which represent a rate of 79 % after 1 h incubation, the decline continues and the number of cells reached $83x10^6$ CFU/mL with a survival rate of 54 % after 2 h of incubation. From these results, it appears that microencapsulation with 2 % sodium alginate improved the viability of the *Lb. curvatus* G6 strain against acidic conditions (pH 2.0) by increasing the survival rate by about 8 %, the experiment was repeated in triplicates.

The obtained results are similar to those reported by several authors which have tested the viability of probiotic bacteria in acidic conditions. Ouled Haddar et al. [11] showed an increase of 14 % in the survival of *Lb. curvatus* B431 isolated from traditional butter encapsulated in 2 % sodium alginate. Similarly, Todorov et al. [6] reported a survival rate of 85.71 % and 40.47 % for encapsulated and free cells of *Lb. plantarum* ST16Pa, respectively; this study was carried out at pH 1.6 for 3 h. In addition, the survival of *Lb. acidophilus* LA1 exposed for 2h at pH 2.0 was increased by 27 % when encapsulated in sodium alginate [20]. In another study, encapsulation in 2 % sodium alginate increases the survival of *Lb. acidophilus* by 15.9 % and *Bifidobacterium* sp. by 16.7 % compared to free cells [21]. Kneifel et al. [22] and Lourens-Hattingh and Viljoen [23] reported that microencapsulation has been successfully used to increase the survival of probiotic microorganisms in fermented products with high acidity such as yogurt.

Table I: Effect of acidity, bile salts and simulated intestinal fluid on the survival of free and encapsulated *Lb. curvatus* G6

Incubation conditions	Incubation time (h)	Survival rate (%)	
		Free cells	Encapsulated cells
Incubation at pH 2.0	0	100	100
	1	69	79
	2	46	54
incubation with 0.3 % bile salt	0	100	100
	4	78	82.
	8	47	56
incubation with simulated intestinal fluid	0	100	100
	1.5	56	69
	3	27	45

Tolerance of free and encapsulated Lb. curvatus G6 to bile salts

Bile salts are one of the barriers to be bypassed by probiotic bacteria to reach their site of action, therefore the tolerance of the strain *Lb. curvatus* G6 in the free and encapsulated forms to bile salts at pH 5.8 was evaluated and the results are illustrated in Table I. From these results, a considerable reduction in the number of free cells (from $8x10^{12}$ CFU/mL to 11x109 CFU/mL) was noted after incubation for 4 h in the bile salts and the survival rate was 78 %. This reduction was continued after 8 h of incubation, the cell number reached 106 CFU/mL with a survival rate of 46.5 %. The number of viable encapsulated cells was reduced after 4 h of exposure to bile salts from 42x1013 CFU/mL to 11x1011 CFU/mL (82.43 % of cells survived) and after 8h of incubation, a decrease was observed and cell number reached 15x107 CFU/mL (56 %). Based on the obtained results, microencapsulation improved cell viability of *Lb. curvatus* G6 in the presence of bile salts (0.3 %) by about 9.5 %.

The survival of the probiotic bacteria *Lb. plantarum* and *Bifidobacterium lactis* Bi-07 was increased approximately 2-fold when they were encapsulated in 3 % sodium alginate [14]. In another study, Chandramouli et al. reported that the encapsulation of *Lb. acidophilus* in 2 % sodium alginate increased significantly the viability of cells in 1 % bile salts [24]. On the other hand, Trindade and Grosso reported that the encapsulation of *Lb. acidophilus* and *Bifidobacterium bifidum* in sodium alginate was not effective in protecting the cells incubated with 2 % and 4 % bile salts [25].

Survival of free and encapsulated Lb. curvatus G6 in simulated intestinal fluid

The results of the effect of SIF on the viability of free and encapsulated *Lb. curvatus* G6 are illustrated in Table I. The number of free cells ($13x10^{11}$ CFU/mL) decreased significantly after 1.5 h of incubation in the presence of pancreatic enzymes to reach $7x10^{6}$ CFU/mL, which represents a survival rate of 56 %, the decrease continues after 3 h of incubation, the number of cells reached $2x10^{3}$ CFU/mL with a survival rate of about 27 %. For the encapsulated cells, the initial number $20x10^{12}$ CFU/mL was reduced after 1.5 h incubation under the same conditions and reached $20x10^{8}$ CFU/mL with a rate of 69 %, after 3 h incubation the number decreases and reach $11x10^{5}$ CFU/mL, which represents a rate of 45 %. These results showed that microencapsulation increased the viability of *Lb. curvatus* G6 cells against the adverse conditions of simulating intestinal fluid by 18 % comparing with free cells. It is important to mention that the effect of simulated intestinal fluid (SIF) on the viability of encapsulated cells noted after only 3h of exposure is lower than that obtained after 8h of exposure to bile salts alone. These results are similar to those obtained by Woraharn et al. which studied the survival of the *Lb. plantarum* CMU-FP002 strain under the same conditions used in this study; they obtained a survival rate of approximately 60 % for 2 % alginate beads [15]. Similarly, Gbassi reported the following survival rate 46 %, 49.01 % and 47.52 % for three strains encapsulated in sodium alginate, *Lb. plantarum* 800, *Lb. plantarum* CIPA 159 and *Lb. plantarum* 299v, respectively, the isolates were incubated in presence of 10 g/L of pancreatin, 3 g/L of bile salts at pH 6.0 [26].

Evaluation of technological skills of Lb. curvatus G6 in vitro

Effect of heat treatment on free and encapsulated cells

The results of heat resistance of free and encapsulated *Lb. curvatus* G6 at different temperatures are shown in Figure 1. Initially, the same number of free and encapsulated cells ($62x10^{11}$ CFU/mL) was used. A significant reduction in free *Lb. curvatus* G6 cells viability in function of temperature increase was noted, 81 % ($21x10^{9}$ CFU/mL), 63 % (10^{8} CFU/mL) and 5 % ($5x10^{5}$ CFU/mL) of the cells survived after exposure to 40°C, 50°C, and 60°C, respectively. While, the encapsulated cells are more resistant and present the following survival rate: 93 % ($82x10^{10}$ CFU/mL), 75 % ($44x10^{8}$ CFU/mL), and 66 % ($26x10^{7}$ CFU/mL) after exposure to 40°C, 50°C, and 60°C, respectively. According to the obtained results, we noted that there was an important protection of *Lb. curvatus* G6 cells by microencapsulation, the viability of cells was improved by 12 %, 13 % and 21 % at 40°C, 50°C, and 60°C, respectively.

Pasteurization is one of heat treatment applied to various food products in order to eliminate harmful microorganisms and pathogens responsible for deterioration of food in order to prolong its shelf life and food safety. It is therefore important to protect beneficial organisms against heat treatments. It was reported that microencapsulation improved the viability of *Lb. acidophilus* LA1 incubated at different temperatures; a survival rates of 9.2 % and 84 % for the free and encapsulated cells, respectively after incubation for 30 s at 72°C was observed[20]. The survival rate of free and encapsulated *Lb. acidophilus* ATCC 43121 after exposure for 30 min at 65°C was 77 % and 90 %, respectively[27]. Ding and Shah also showed that the microencapsulation protects effectively probiotic strains against heat treatment (65°C) [28].

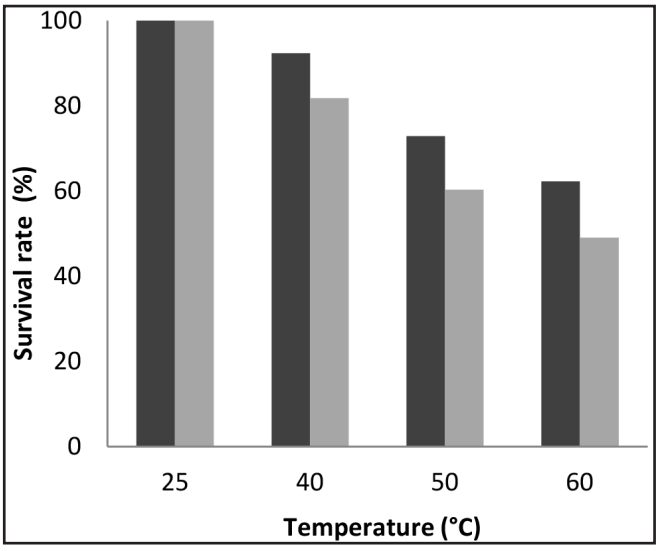

Fig. 1: Effect of temperature on the survival of free and encapsulated *Lb. curvatus* G6 after 20 min of incubation. ■ Encapsulated cells, ■ Free cells.

Effect of cold storage on free and encapsulated cells

Temperature is a critical factor affecting the viability of probiotic microorganisms during the storage period. Therefore the storage of free and encapsulated *Lb. curvatus* G6 at 4°C was conducted to evaluate the efficacy of encapsulation to protect bacterial cells in the period of storage. The obtained results are shown in Figure 2. A remarkable reduction in the survival of free cells has been shown (about 85 % after a week and only 69 % after two weeks) during the period of storage. However, encapsulated cells conserved their viability, with a survival rate of 93 % and 88 % after one and two weeks, respectively. From the obtained results, we can say that microencapsulation effectively protects *Lb. curvatus* G6 cells against cold storage conditions and with an improvement rate of about 18.07 %.

It was reported that microencapsulation in 2 % sodium alginate considerably protect *Lb. acidophilus* strain stored at 5°C for 8 days with a survival rate of 95 % compared with *B. lactis* which present a survival rate of about 34 % [29]. In another study, Woraharn et al. showed that 75 % and 96. % of encapsulated *Lb. plantarum* CMUF-002 cells survived after 2 and 8 weeks of incubation at 4°C, respectively [15]. Furthermore, the viability of free and encapsulated *Lb. acidophilus* DD910 incubated at 4°C for 7 weeks was checked, and the recorded survival rate were 47 % and 73 % for the free and encapsulated cells, respectively[30].

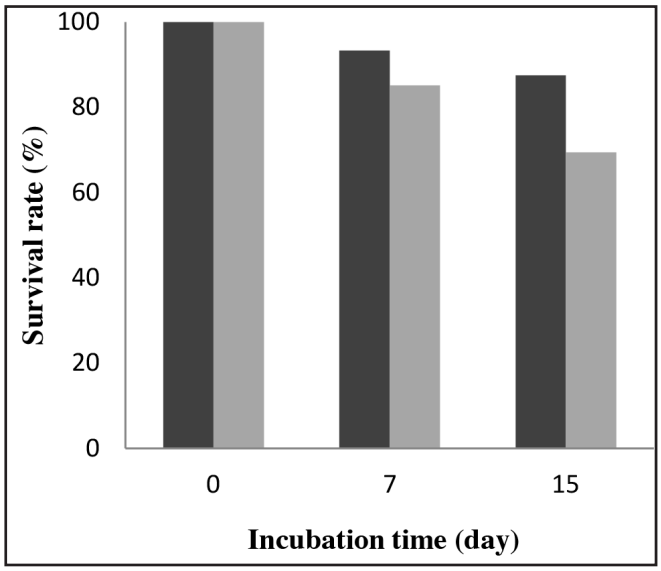

Fig. 2: Effect of cold storage (4°C) on the viability of free and encapsulated *Lb. curvatus* G6. ■Encapsulated cells, ■ Free cells.

Surface hydrophobicity

To highlight the presence of hydrophobic interactions type, hydrophobicity test was carried out using xylene. According to the results, a low hydrophobicity (4.4 %) of the strain *Lb. curvatus* G6 for xylene was found. This result is similar to that reported by Todorov et al. who evaluated the hydrophobicity of *Enterococcus mundtii* using n-hexadecane as a hydrocarbon and with a percentage of 5.57 % [19]. However, Todorov et al. reported in another study, that hydrophobicity recorded for *Lb. plantarum* ST16Pa was 68.7 % [6]. This result suggest that the adhesion of *Lb. curvatus* G6 to epithelial cells is not attributed to hydrophobic interactions, which is one of the properties that facilitate the first contact between the microorganisms and the intestinal cells of the host. Hydrophobicity may help cells to adhere to epithelial cells but is not a prerequisite condition for strong adhesion to intestinal cells [19]. Therefore, the adhesion of *Lb. curvatus* G6 involve other types of interactions involving other factors such as surface proteins, adhesins or pili, these require further research to determine the ways by which occurs the adhesion

Autoaggregation and coaggregation

Aggregation between bacteria is an essential mechanism for biofilms formation and it is one of the mechanisms of inhibition of pathogenic microorganisms, for this reason it can be considered as a criterion for selection of potentially probiotic strains. The results of the assay are illustrated in Figure 3. The value of autoagregation observed for *Lb. curvatus* G6 was 45 %. Different values have been obtained for coaggregation of *Lb. curvatus* G6, the highest value of coagregation was observed for *Lb. casei* ssp. *tolerans* G4 (84 %) and the lowest one was recorded for *Listeria monocytogenes* and MRSA (0 %). The autoagregation level reported for *Lb. curvatus* G6 is higher than the level reported for *Lb. plantarum* ST16Pa[6]. Todorov et al. reported in another study different level for *Enterococcus mundtii* ST4V coaggregation with different strains of *Lactobacillus* and *Listeria* [19]. Some lactobacilli are capable to autoagregate but can also coagregate with pathogenic microorganisms. They create a particular microenvironment around the pathogen with a higher concentration of inhibitory substances directed against it. The molecules involved in the aggregation are located on the surface of the cells (lipoteichoic acids, proteins or carbohydrates), or they were secreted by the cells (soluble proteins or peptides)[31].

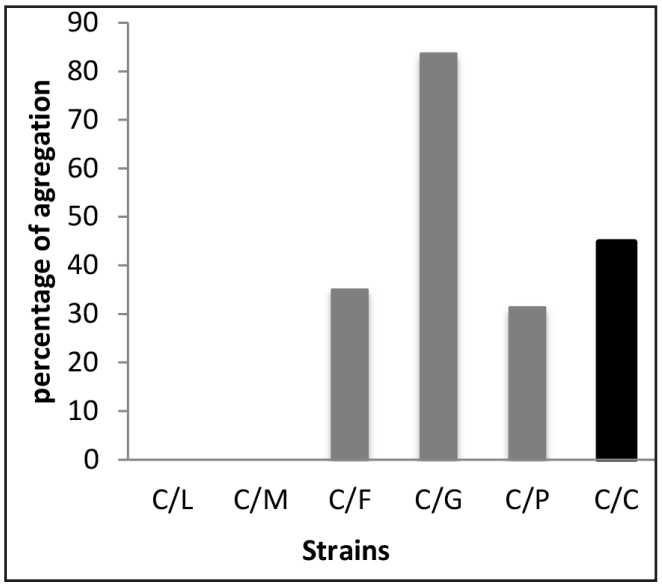

Fig. 3: Percentage of autoagregation and coagregation of *Lb. curvatus* G6 with different strain. Autoagregation: black, coagregation: gray, C: *Lb. curvatus* G6, L: *Listeria monocytogenes,* M: MRSA, F: *Lb. plantarum* F12, G: *Lb. casei* ssp. *tolerans* G4, P: *Lb. plantarum*

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Summary

Sifour M., Ouled-Haddar H., Gharbi N., Graidia G.: Probiotic properties of free and encapsulated cells of a bacteriocinogenic *Lactobacillus curvatus* G6 of human origin

This study was conducted to confirm the protective effect of microencapsulation of a bacteriocinogenic probiotic lactic acid bacteria *Lb. curvatus* G6 in 2 % sodium alginate gel to maintain its viability during food product processing and storage and against the hostile gastrointestinal tract conditions. The tolerance of *Lb. curvatus* G6 to acidic pH, bile salts and pancreatic enzymes was evaluated. In addition, the effect of cold storage and heat treatment on viability was also tested. Results showed that the strain is resistant to low pH, bile salts, digestion by pancreatic enzymes, cold storage and heat treatment, particularly when encapsulated in sodium alginate

Keywords: Alginate, encapsulation, *Lactobacillus curvatus*, probiotic bacteria

Souhrn

Sifour M., Ouled-Haddar H., Gharbi N., Graidia G.: Probiotické vlastnosti volných a enkapsulovaných bakteriocinogenních buněk *Lactobacillus curvatus G6* izolovaných z lidského zažívacího traktu

Úkolem této studie bylo potvrdit protektivní efekt mikroenkapsulace bakteriocinogenních probiotických bakterií mléčného kvašení *Lb. curvatus* G6 ve 2 % gelu alginátu sodného k zachování jejich viability v průběhu technologického zpracování, skladování a v podmínkách zažívacího traktu. Byla prokázána tolerance *Lb. curvatus* G6 v prostředí kyselého pH, solí žlučových kyselin, pankreatických enzymů, při snížené i zvýšené teplotě.

Keywords: Alginát, enkapsulace, *Lactobacillus curvatus*, probiotika

EVALUATION OF AUTOCHTHONOUS SELECTED YEASTS FROM GRAPES AND CELLAR IN WINEMAKING OF AGLIANICO VINE

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Introduction

Aglianico cultivar is a red grape variety of Greek origin autochthonous of Irpinia, a small district of Campania region in Southern Italy characterized by an established wine industry. Wine production following the oldest way is obtained with the spontaneous fermentation, carried out by the indigenous yeasts present on the surface of the grape and on the cellar equipments.[1,2] The grape native microflora responsible for the early stages of the grape must fermentation are mainly composed by non-*Saccharomyces* yeasts with a low fermentative power. As the alcoholic fermentation proceeds, the fermentative yeasts such as *Saccharomyces cerevisiae* become predominant and replace the non-*Saccharomyces* ones, during the mid-to-final phases of fermentation. The *Saccharomyces* "sensu stricto" strains, named "winery" yeasts are mainly present on the winery equipments surfaces. Each winery is characterized by its own fermentative microflora originated by selection in a very long time among the species "resident" in the cellar. Recently, to plan the winemaking process and standardize the quality of the product, the utilization of microbial starter composed by selected *S. cerevisiae* yeasts has been suggested. However, this procedure inhibits the growth of the non-*Saccharomyces* strains that greatly contribute to the wine quality.[3] In fact, in the first fermentation-phase these species utilize sufficient sugars and amino acids from the grape juice, and generate enough amounts of end-products to have an imprint on wine character. In particular, they are involved in the determination of the colour and aromatic complexity of the wine, which are important determinants of the product typicality. Several researchers have promoted the utilization of such selected native yeasts in order to get mixed fermentation inocula.[4] In addition, several studies revealed that significant positive differences in the qualitative and quantitative volatile compounds composition of the wines have been obtained with a guided fermentations compared to those produced with spontaneous fermentation.[5,6] The aim of this work was the selection of new combinations of yeast strains isolated from the autochthonous microflora of Aglianico grapes and wine cellar, located in Irpinia, and their use in winemaking process to improve the organoleptic and sensory characteristic of the wine thus preserving the peculiarities of this typical regional product.

Materials and Methods

Chemicals

The culture media for the isolation, the reactivation and the growth of different yeast strains were purchased from Oxoid (Hampshire, UK). Chemical reagents and solvents are all of analytical grade and were from SIGMA-Aldrich (St. Louis, MO, USA).

Isolation and molecular identification of yeasts

To isolate different yeasts populations, serial dilutions of Aglianico grape samples, previously homogenized in sterile Ringer solution, were plated on two different solid agar media: WL nutrient agar and Lysine-agar.[7,8] On WL medium, yeast species have been distinguished by different colony morphologies and colours. The isolation of non-*Saccharomyces* species has been carried out on Lysine medium in which *Saccharomyces* spp. cannot grow.[9] The yeast species identification was performed by the analysis of the D1/D2 domain of 26S rDNA sequence. The genetic region was PCR amplified directly from individual yeast colonies, as described by Arroyo-Lopez et al.[10] The standard primers utilized were those commonly referred to as NL1 and NL4 in the literature.[11]

Determination of the yeast fermentative power

One hundred mL of pasteurized must (100°C for 30 min) was inoculated with a 1 % (v/v) microbial biomass suspension (24 h growth). The must fermentation tests were carried out in shake-flasks incubated at 28°C and 200 rpm on an orbital shaker (New Brunswick Scientific Co., Inc., USA). The weight loss caused by CO_2 production was determined every day until a constant weight has been reached and maintained for three days. The fermentative vigor was expressed as g of CO_2 per 100 mL of must.

Yeasts selection for the resistance to sulphur dioxide

The tests were carried out on Aglianico must, pasteurized for 30 min and inoculated with a 1 % of microbial biomass suspension (24 h growth). Potassium metabisulfite (MBK) was used as the source of sulphur dioxide and the yeasts resistance at concentrations of 100 and 250 mg/L was tested. The tests were performed at 28°C for 7 days.[12]

Growth of yeast strains

The selected yeast strains were inoculated in 100 mL YPD liquid medium. The growth was carried out at 28°C in shake-flasks at 200 rpm on an orbital shaker for 24 h. The obtained yeast suspension was used to inoculate (1 % v/v) 10 L of YPD liquid medium

in a pilot plant fermenter. The obtained biomass was stored at 4°C.

Winemaking process

Aglianico grapes were harvested at 18.4°C Brix, destemmed, pressed and then fermented with their skins. MBK was added at a final concentration of 100 mg/L to the juice and, after the addition of 20 mg/L of Trenolin® Rouge DF pectolytic enzyme (Erbslöh Geisenheim AG, Geisenheim, Germany), it was warmed at 18°C for 18 h. The must was then divided into two 30 L tanks for fermentation heated up to 26°C. One tank was inoculated with the starter ST1 containing *S. cerevisiae* FLOSW4 and *H. uvarum* AGSW15 (3 x x 10^6 CFU/mL) and the other tank was inoculated, as control, with 0.15 g/L (about 3.2 x10^{10} CFU/g) of dry *S. cerevisiae 254D-ICV, Lalvin (*Lallemand-Italia), starter ST5, previously rehydrated according to manufacturer's instructions. At the beginning of the fermentation a mobilisator and a nutrient (0.2 g/L Vita*Drive*®, Vitamon® Combi from Erbslöh) were added. Moreover, in the middle of fermentation, another aliquot of the nutrient, at the same concentration, was added. The fermentation process was completed in 15 days and when the residual sugars concentration reached a value smaller than 2 g/L, 60 mg/L of MBK was added. The wine was decanted with aeration for 3 days followed by 10 days without aeration.

Sample preparation of HS-SPME-GC analysis

The SPME fiber (PDMS-100um, polydimethylsiloxane) was conditioned according to the manufacturer's recommendations prior to its first use. To a 20 mL Headspace vial 5 mL of wine samples was added, along with 3 g of NaCl and octan-3-ol in hydro-alcoholic solution (1/1, v/v) at 100 µg/L as an internal standard. The solution was homogenized with a vortex shaker and then loaded onto a Gerstel autosampling device. The program consisted of swirling the vial at 250 rpm for 5 min at 40°C, then inserting the fiber into the headspace for 30 min at 40°C as the solution was swirled again, then transferring the fiber to the injector for desorption at 240°C for 30 min.[13]

Gas Cromatography-Mass Spectrometry

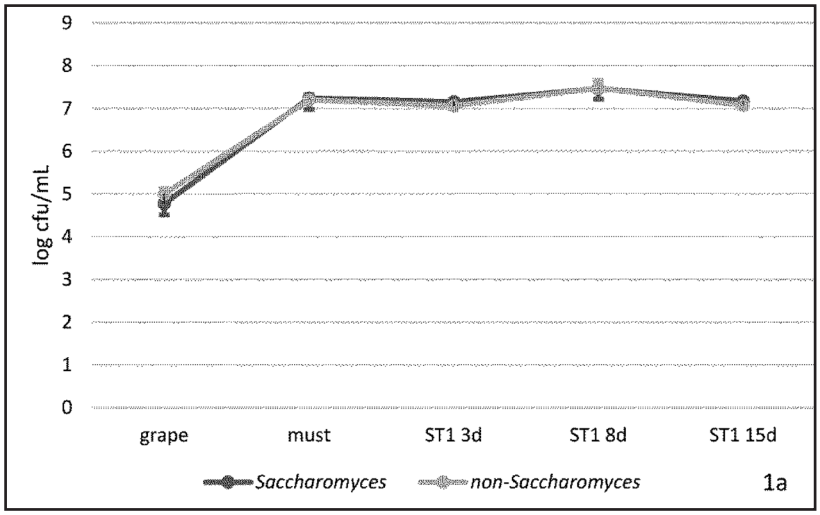
Gas chromatography analyses were carried out using a 7890 Agilent GC system coupled to an Agilent 5975 inert quadrupole mass spectrometer equipped with a Gerstel MPS2 autosampler. The capillary column employed was a HP-Innowax (Agilent technologies) (30 m x 0,25 mm id. 0,50 µm film thickness) and the carrier gas was helium. Splitless injections were used. The initial oven temperature was set to 40°C for 1 min. The temperature was increased in four steps: 40 – 60°C at 2°C/min; 60 – 150°C at 3°C/min, 150 – 200°C at 10°C/min and 200 – 240°C at 25°C/min; the final temperature was maintained for 7 min. The injector, the quadrupole, the source and the transfer line temperature were maintained at 240°C, 150°C, 230°C and 200°C, respectively. Electron ionization mass spectra in full-scan mode were recorded at 70eV electron energy in the range 40 – 300 amu. Peaks were identified using both the NIST 98 and Wiley libraries. Quantification was performed by using the relative concentration in µg/L of the internal standard, calculated as the ratio between each compound area and the internal standard area. The samples were analyzed in triplicate and blank runs were made by using an empty vial every two analysis.[14]

Results and Discussion

Microbiological analysis

We have previously reported[15] that the microbiological analysis of Aglianico grapes revealed the presence of the yeast species mainly belonging to the genera *Hanseniaspora, Kloeckera, Metchnikowia, Candida* and *Saccharomyces* according to Beltran et al.[16]. On the other hand, the yeast strains identified as *Saccharomyces* spp. were the most represented components in the cellar microflora as reported by Ciani et al.[17]. Moreover, some yeast belonging to *Hanseniaspora*, *Kloeckera*, *Metchnikowia* genera were found to be predominant in the must with MBK and in the first days of the fermentative process according to Hierro et al and Fleet. [18,2]

Technological screenings involving the resistance to sulphur dioxide, the alcohologenic power and fermentative power analyses (data not shown), conducted with the isolated yeasts in this study pointed to two strains, FLOSW4 and AGSW15, belonging to *Saccharomyces* and non-*Saccharomyces* spp*.,* respectively, which showed the best performance among the isolates. These two strains have been identified as *Saccharomyces cerevisiae* and *Hanseniaspora uvarum* by the analysis of the D1/D2 domain of 26S rDNA sequence. The experimental winemaking processes carried out with the Aglianico grape must inoculated with the mixed culture of the isolated yeast strains showed that these autochtonous yeasts were able to trigger and complete the alcoholic fermentation leaving a sugar residue smaller than 2 g/L. The Figure 1 shows the kinetics of fermentations obtained by employing of autochtonous starter (ST1) and commercial yeast (ST5), used as control. The growth curves of autochtonous starter, ST1 (Figure 1a), showed that native *S. cerevisiae* yeast (FLOSW4) and the non-*Saccharomyces* yeast (*H. uvarum* AGSW15) are able to start and complete the winemaking process. After 3 days, the load of yeast population was higher than 10*7 cfu/mL and was able to grow over time. Instead, in Figure 1b, with regard to the ST5 commercial starter, we can note that the *S. cerevisiae* strain was able to lead the fermentation process but on the other hand, it has not been able to inhibit the native non-*Saccharomyces* yeasts growth, that remained during all winemaking process, as already reported.[4,19] The data obtained from the trials showed that the autochtonous *S. cerevisiae* yeast has a similar behavior as the commercial *S. cerevisiae* strain to pilot the alcoholic fermentation. Finally, it has been shown to have the ability to modulate the growth of non-*Saccharomyces* yeast as reported previously.[20]



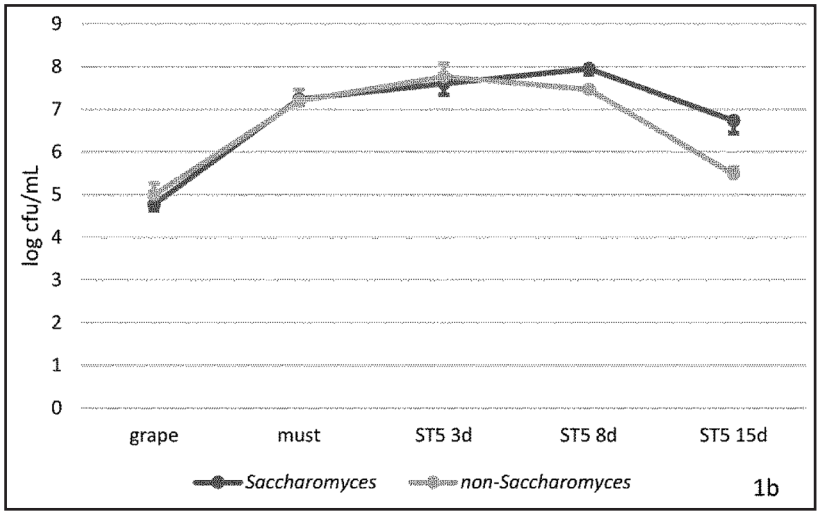


Fig. 1: Kinetics of must Aglianico of trials fermentation with autochtonous starter ST1 (*H. uvarum* + *S. cerevisiae*) and commercial yeast ST5 (*S. cerevisiae* 254D-ICV). The values are the means of three determinations for each sample, the vertical bars represent standard deviation (RSD<5 %)

Volatile compounds detected by SPME-GC/MS analysis

The data about the volatile compounds identified in Aglianico wine samples by SPME-GC/MS, reported in Table 2, are in general agreement with those cited in literature.[21,22] In particular, according to Romano et al.[23], it is to underlined that the differences in the volatile molecules composition of wine samples obtained by utilizing different yeast species appear to be quantitative rather than qualitative. Ethyl esters of straight-chain fatty acids and acetates of higher alcohols are the dominating esters in wine samples and they are formed during the alcoholic fermentation process. The aroma compounds such as isoamyl acetate, ethyl caproate, ethyl caprylate, ethyl caprate and diethyl succinate were detected as the major esters. Also, other types of acetates such as ethyl acetate, hexyl acetate and phenylethyl acetate were identified. Hexanoic, octanoic, decanoic acids and dodecanoic acid, which also contribute to the aroma of the wine, were detected as the most abundant acids. In the alcohols group, in addition to ethanol, isobutyl alcohol, 1-butanol, isoamyl alcohol, 1-hexanol, 1-pentanol-4-methyl, 1-pentanol-3-methyl, (Z)-3-hexen-1-ol, (E)-3-hexen--1-ol and 2-phenylethanol were found. Terpenes that play an important role in the aroma composition of wine have often been used to differentiate different grape cultivar.[24] Specifically, among the compounds of this class, linalool, limonene, alfa terpinene, neryl acetate, α-terpineol, geraniol, nerolidol, β-citronellol, 2,3-dihydrofarnesol and farnesol were identified in the aAglianico wine samples. Furthermore, the volatile phenol, 4-ethylphenol and 4-ethylguaiacol were also revealed. Among the esters, ethyl caproate, ethyl caprate and ethyl caprylate, which are responsible for the fruity, green apple and soap flavor, appeared to be present at higher concentration especially when the autochthonous starter (ST1) was utilized. Also the amount of diethylsuccinate, which mainly contributes to create the body of the wine[25] appeared to be present in more elevated amounts in wines obtained by using the ST1 starter. Among terpenes, in particular the 2,3-dihydrofarnesol was at higher levels in the experimental wine respect control one. This sesquiterpenic compound, known for its antioxidant, anti-inflammatory, antibacterial and anti-cancer properties, may arise directly from grape and/ or rearrangement reactions during the winemaking process and/or aging processes., As this kind of hydrophobic analytes present in several fruits and vegetables has been proposed to promote long and medium-term health beneficial effects it can be hypothesized that also the experimental Aglianico wine could have these healthy properties.[26]

Regarding the total alcohols, their amount was higher in wines produced by ST1 compared to that obtained by commercial yeast (ST5).

On the other hand in the class of phenols the 4-ethylguaiacol and the 4-ethylphenol were more abundant in ST1 wine. These compounds may negatively affect the quality of the wine producing unpleasant odors. Specifically, the presence of ethylphenols (4-ethylphenol and 4-ethylguaiacol) in red wine produces "phenolic" and animal odor. In the wine ST1 the 4-ethylguaiacol was at a higher level respect to the corresponding olfactory threshold. Moreover the experimental Aglianico wine, could have beneficial effect on health, because it contained the 2,3-dihydrofarnesol compound, belonging to sesquiterpenoids class. This class has a known beneficial effect on health.[26]

Conclusions

The fermentation carried out with the new starter showed positive differences compared to commercial yeasts. The results demonstrated that the two native selected strains successfully dominated the winemaking process and the Aglianico wine obtained showed a higher amount of the esters, responsible for the fruity and green apple flavor that enhance the aromatic complexity and strenghth the "terroir".

Table I: Quantification of volatile compounds identified in the wine produced by ST1 *(H. uvarum + S. cerevisiae*) and commercial yeast ST5 (*S. cerevisiae* 254D-ICV)

	ST5 (µg/L ± SD)	ST1(µg/L ± SD)		ST5 (µg / L ± SD)	ST1(µg/L ± SD)
Esters and Acetates			2,3-butandiol	60,98 ± 0,68	28,51 ± 0,39
ethyl acetate	2410,1 ± 26,9	2744,1 ± 38,0	1-octanol	17,2 ± 0,2	26,9 ± 0,4
ethyl propanoate	45,5 ± 0,5	30,8 ± 0,4	1-nonanol	2,3 ± 0,0	26,5 ± 0,4
ethyl isobutyrate	43,2 ± 0,5	27,5 ± 0,4	1-decanol	19,4 ± 0,2	22,0 ± 0,3
isobutyl acetate	25,0 ± 0,3	25,9 ± 0,4	benzyl alcohol	10,1 ± 0,1	11,5 ± 0,2
ethyl butyrate	125,1 ± 1,4	134,3 ± 1,9	2-pheneylethanol	1531,2 ± 17,1	1406,4 ± 19,5
ethyl, 2-methylbutanoate	21,0 ± 0,2	23,7 ± 0,3	methionol	8,9 ± 0,1	6,8 ± 0,1
ethyl isovalerate	20,3 ± 0,2	24,8 ± 0,3	*Total alcohols and thiols*	*31805,9 ± 354,5*	*32397,4 ± 448,7*
isoamylacetate	853,4 ± 9,5	842,7 ± 11,7	**Aldehydes and ketones**		
ethyl caproate	1165,6 ± 13,0	1736,5 ± 24,1	acetaldehyde	6,2 ± 0,1	ND
butylisovalerate	5,5 ± 0,1	5,8 ± 0,1	2,3-butanedione	26,6 ± 0,3	5,2 ± 0,1
hexyl acetate	23,9 ± 0,3	20,2 ± 0,3	*Total aldehydes,ketones*	*32,8 ± 0,4*	*5,2 ± 0,1*
ethyl heptanoate	22,6 ± 0,3	40,9 ± 0,6	**Acids**		
ethyl lactate	234,6 ± 2,6	369,8 ± 5,1	acetic acid	263,5 ± 2,9	293,7 ± 4,1
methyl octanoate	14,3 ± 0,2	14,5 ± 0,2	butanoic acid	2,7 ± 0,0	ND
ethyl caprylate	3664,0 ± 40,8	5915,9 ± 81,9	butanoic acid ,2-methyl	27,4 ± 0,3	20,5 ± 0,3
isoamyl caprylate	35,4 ± 0,4	61,6 ± 0,9	hexanoic acid	47,1 ± 0,5	23,4 ± 0,3
ethyl nonanoate	34,3 ± 0,4	36,7 ± 0,5	octanoic acid	286,2 ± 3,2	377,5 ± 5,2
ethyl 2-hydroxy caproate	15,9 ± 0,2	31,1 ± 0,4	nonanoic acid	6,8 ± 0,1	6,5 ± 0,1
isoamyl lactate	12,4 ± 0,1	7,6 ± 0,1	decanoic acid	14,1 ± 0,2	162,3 ± 2,3
methyl decanoate	10,2 ± 0,1	8,0 ± 0,1	dodecanoic acid	38,8 ± 0,4	13,9 ± 0,2
ethyl caprate	2020,2 ± 22,5	3349,2 ± 46,4	*Total acids*	*686,5 ± 7,7*	*897,7 ± 12,4*
ethyl benzoate	3,7 ± 0,0	ND	**Volatile phenols**		
diethyl succinate	566,4 ± 6,3	625,3 ± 8,7	phenol 4-ethyl-3-methyl	8,4 ± 0,1	ND
ethyl 9-decenoate	20,2 ± 0,2	58,3 ± 0,8	phenol, 2-methoxy-4-methyl	11,4 ± 0,1	3,8 ± 0,1
methyl salicylate	6,6 ± 0,1	5,0 ± 0,1	4-ethyl guaicol	9,3 ± 0,1	57,5 ± 0,8
ethyl phenylacetate	20,5 ± 0,2	27,4 ± 0,4	phenol 4-ethyl	38,5 ± 0,4	155,5 ± 2,2
diisobutylsuccinate	17,2 ± 0,2	13,4 ± 0,2	*Total volatile phenols*	*67,6 ± 0,8*	*216,8 ± 3,0*
ethyl 4-hydroxybutanoate	24,7 ± 0,3	25,6 ± 0,4	**Terpenes**		
phenethyl acetate	70,2 ± 0,8	46,5 ± 0,6	limonene	16,8 ± 0,2	15,3 ± 0,2
ethyl dodecanoate	164,6 ± 1,8	672,1 ± 9,3	α terpinene	10,6 ± 0,1	4,5 ± 0,1
ethyl hydrocinnamate	143,3 ± 1,6	2,6 ± 0,0	neyl acetate	9,5 ± 0,1	ND
Total esters and acetates	*11865,1 ± 132,6*	*16927,6 ± 235,4*	linalolo	40,5 ± 0,5	22,5 ± 0,3
Alcohols and thiols			βcitronellol	29,1 ± 0,3	35,7 ± 0,5
ethanol	19776,6 ± 220,4	20198,1 ± 279,7	α terpineol	9,8 ± 0,1	4,0 ± 0,1
isobutyl alcohol	802,5 ± 9,0	699,3 ± 9,7	geraniol	6,5 ± 0,1	4,1 ± 0,1
1-butanol	20,6 ± 0,2	13,5 ± 0,2	nerolidol	69,7 ± 0,8	10,0 ± 0,1
isoamyl alcohol	9211,3 ± 102,7	9588,3 ± 132,8	2,3-dihydrofarnesol	10,7 ± 0,1	197,0 ± 2,7
1-pentanol,4-methyl	5,7 ± 0,1	7,4 ± 0,1	farnesol	14,1 ± 0,2	19,9 ± 0,3
1-pentanol,3-methyl	16,6 ± 0,2	21,5 ± 0,3	*Total terpenes*	*217,4 ± 2,4*	*313,0 ± 4,3*
1-hexanol	308,8 ± 3,4	330,3 ± 4,6	**Lactones**		
3-hexen-1-ol (E)	6,9 ± 0,1	6,1 ± 0,1	butyrolactone	16,8 ± 0,2	14,2 ± 0,2
3-hexen-1-ol (Z)	2,5 ± 0,0	2,1 ± 0,0	γ n-amylbutyrolactone	1,3 ± 0,0	2,7 ± 0,0
1-octen-3-ol	4,6 ± 0,1	2,3 ± 0,0	*Total lactones*	*18,1 ± 0,2*	*16,9 ± 0,2*

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Summary

Boscaino F., Sorrentino A., Ionata E., La Cara F., Volpe MG.: Avaluation of Autochthonous selected yeasts from grapes and cellar in winemaking of Aglianico vine

Aglianico cultivar is an ancient red grape variety native of Campania region of the Southern of Italy. Until a few decades ago, wine was produced by natural fermentation carried out by autochthonous yeasts present on the grapes and in the cellar. The grapes epiphytic microflora is prevalently composed of apiculate yeasts with a poor fermentative power and by oxidative yeasts, belonging to non-*Saccharomyces* group. On the other hand, most of the yeast strains present in the cellar belong to *Saccharomyces* "sensu stricto". The aim of this work was the selection of new combinations of yeast strains isolated from the autochthonous microflora of Aglianico grapes and wine cellar, located in the Irpinian area, and their use in winemaking process to improve the organoleptic and sensory peculiarities of the wine obtained. The yeasts isolated from grapes and cellar were characterized by morphological, biochemical, and technological analysis. They belonged mainly to the genus *Saccharomyces, Hanseniaspora*, Kloeckera, *Rhodotorula, Metschnikowia* and *Candida*. Among these yeasts the two strains (AGSW15 and FLOSW4) that showed the best winemaking performance were selected and identified as *H. uvarum* and *S. cerevisiae* by 26S rDNA D1/D2 region sequence analysis. These yeasts, when utilized in cellar for semi-industrial-scale fermentations, successfully dominate the alcoholic fermentation and contributed to the improvement of the wine organoleptic qualities as assessed by the flavor profile obtained through the SPME-GC/MS technique. These results clearly suggest the utilization of these selected strains in autochthonous fermentation starter to preserve the peculiarities of this typical regional wine.

Keywords: Aglianico cultivar, autochthonous yeasts, starter, volatile compounds

Souhrn

Boscaino F., Sorrentino A., Ionata E., La Cara F., Volpe MG.: Hodnocení vybraných přirozeně se vyskytujících kvasinek z hroznů a sklepa při výrobě vína Aglianico

Kultivar Aglianico je velmi stará odrůda červeného vína pocházející z regionu Campania v jižní Italii. Do nedávna bylo víno vyráběno fermentací pomocí kvasinek vyskytujících se na hroznech a ve sklepě. Mikroflora ulpívající na hroznech je složena především z kvasinek se slabými fermetačními a oxidačními schopnostmi, které nepatří do skupiny *Saccharomyces*. Naproti tomu většina kmenů kvasinek přítomných ve sklepě patří mezi sacharomycety. Úkolem této práce bylo vybrat nové kombinace kmenů kvasinek izolovaných z přirozeně se vyskytující mikroflory hroznů Aglianico a vinného sklepa lokalizovaného v irpinské oblasti a tyto kombinace kvasinek využít ke zlepšení organoleptických a sensorických vlastností takto připravených vín. Kvasinky izolované z hroznů i sklepa byly charakterizovány po stránce morfologické, biochemické a technické. Tyto kvasinky patří zejména mezi rody *Saccharomyces, Hanseniaspora, Kloeckera, Rhodotorula, Metschnikowia and Candida.* Mezi těmito kvasinkami měly nejlepší vlastnosti při přípravě vína dva kmeny (AGSW15 a FLOSW4), identifikované jako *H. uvarum a S. cerevisiae* na základě sekvenční analýzy úseku D1/D2 26S rDNA. Tyto kvasinky, použité při poloprovozní fermentaci, úspěšně dominovaly v průběhu alkoholické fermentace a ve zlepšení organoleptických vlastností potvrzených technikou SPME-GC/MS. Dosažené výsledky podporují použití vybraných kmenů kvasinek k zahájení fermentace umožňující zachovat zvláštnosti charakteristické pro toto typicky regionální víno.

Klíčová slova: Kultivar Aglianico, autochtonní kvasinky

ISOLATION AND IDENTIFICATION OF NEW CHITINOLYTIC FUNGUS *Petromyces alliaceus* H5

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Introduction

Chitin, a β-(1,4)-linked polymer of N-acetyl-D-glucosamine, is one of the most abundant naturally occurring polysaccharides. Chitin occurs in combination with other polymers, such as proteins. In nature, it is found in two crystalline forms. The α-form has antiparallel chitin microfibrils with strong intermolecular hydrogen bonding and is the most abundant chitin in nature, found in shrimps and crabs. β-chitin has parallel chitin chains and occurs in squid pens. Chitin is widely distributed in nature, particularly as a structural polysaccharide in fungal cell walls, the exoskeletons of arthropods, the outer shells of crustaceans, and nematodes. Approximately 75 % of the total weight of shellfish, such as shrimp, crab, and krill, is considered waste. Chitin comprises 20–58 % of the dry weight of this waste [1].

Some of the most common chitin-containing organisms that represent over a half of the living organisms on Earth are the insects. Insects are a class of invertebrates within the arthropod phylum that have a chitinous exoskeleton. The diversity helps them to invade in all environments. Their uncontrolled spread causes serious economical losses in agriculture each year all over the world. These losses account for 40 % of potential production [2] and despite the increasing use of pesticides, crop losses have remained relatively constant [3].

In addition to agriculture, the insects cause major damages in other fields. While success in struggle with malaria in Europe and America by distraction of essential habitats that brought to endangering of some animal species, problems with insects as hosts of many human diseases are still unsolved. Much more, during the last years NASA released several reports for their intension in developing of "Bug off" coatings for airplanes. Small insects that hit plane's wings during takeoff and landing, cause significant increase in fuel consumption throughout all the period of flight. This leads to the question – could we control the insect population?

Substantial for insect survival and distribution in the environment is their chitinous cuticle that prevents them from different external influences. It plays supportive role as exoskeleton, as well as a protective shield against physical, mechanical and chemical factors. Lysis of chitinous cuticle especially in larval stages causes lethal ending of the insects. Enzymes that degrade this insoluble polymer are chitinolytic enzymes or chitinases [4].

Chitinases are glycoside hydrolases that cataa lyze the decomposition of chitin. They hydrolyze the β-1,4-glycosidic bonds between the N-acetyl-D--glucosamine residues that comprise a chitin chain. Complete enzymatic hydrolysis of chitin to free N-acetylglucosamine is performed by a chitinolytic system consisting of a diverse group of enzymes that catalyze the hydrolytic polymerization 1.

Chitinases are produced by microorganisms, fungi, insects, plants, animals and even humans. Many bacp terial strains that produce chitinases were isolated, purified and characterized in the past two decades from *Serratia*[5], *Pseudomonas*[6], *Bacillus*[7,8],*Chromobacterium*[9], *Paenibacillus*[10], *Erwinia*[11] and *Vibrio*[12], as well as *Streptomyces*[13]. Fungal strains *Trichoderma harzianum* and *Aspergillus niger* also become perspective chitinase producing species[14,15]. Many plant and animal chitinases are also described[16,17]. Chitinases were found in human blood serum – chitotriosidase and acidic mammalian chitinase[18,19]. Contrary to this, a very small number of chitinases is still commercially available. Therefore, the future of chitinases is close related to the increase in their production.

Since the works of Agostino Bassi, Pasteur, LeConte and Metchnikoff, who postulated the idea for using of microorganisms for insect biocontrol, the attention has been focused on chemical pesticides. In the last decades, the global understanding of disadvantages of chemical compounds has stimulated the research interest in exploration of new biofriendly methods by using some fungi against the insects. Many fungal strains was characterized as perspective producers of bioinsecticides, and over 170 products were developed from species of *Beauveria, Metarhizium, Lecanicillium* and *Isaria*[20].

In this study we present identification and a primary screening for chitinolytic activity of new fungal strains. In regard to chitinase production, the isolate *Petromyces alliaceus* H5 showed promising results.

Materials and methods

Isolates

All fungal isolates used in this study were from the collection of "Biodinamika" Ltd., Plovdiv, Bulgaria, and possessed biological activity against plant pathogens and insects, and microorganisms isolated from soils and substrates, enriched with chitin and chitosan.

Isolates from genus *Trichoderma* – Tr1, 2, 3, 4, 5 and 22 have been isolated from soil and decomposing

organic matter, and had biological activity against phytopathogenic fungi.

Isolates H1, 2, 5, 6, 7 and Cs1 have been isolated from the body of dead insects.

Isolates CH2, CHM1, CHH, CHO, Ch1, Cs and M1 were isolated from soil enriched with chitin and chitosan. CHO – from a plant growth substrate, containing peat perlite: sand: compost in the ratio of 2:1:1:1, amended with low molecular weight chitosan (<200: cP); CH2 – with a middle molecular weight chitosan (200 – 400 cP); CHH and SHM1 – with a high molecular weight chitosan (>400 cP). Isolates M1, Cs1 and Ch1 have been isolated from soil enriched with chitin (Yantai Shang Tai Trading Co Ltd, Yantai, China).

Screening of chitinolytic isolates

After collecting the isolates, they were tested for chitinolytic activity using agar diffusion assay on a solid selective medium, supplemented with colloidal chitin. The solid selective medium had the following composition (g/L): KH_2PO – 0.7; K_2HPO_4 – 0.3; NaCl – 2.0; $MgSO_4 * 7H_2O$ – 0.5; colloidal chitin – 5.0; agar – 20.0. pH was corrected to 5.5 – 6.0, after that prepared medium was autoclaved for 20 min at 121°C.

Colloidal chitin was prepared by Saadoun methodology [21] as 20 g of chitin (Sigma C7177) were added to 200 mL of concentrate hydrochloric acid (Merck 1.00317). The mixture was stired periodically for 1h and then 1 L of cold (4 – 6°C) distillated water was added. Stirring continued for another 10 min., and mixture was filtered true filter paper under vacuum. The filter cake was resuspended in 1L of water and filtered again till pH was not less than 4.0. Prepared medium was transferred in Petri dishes and inoculated with 0.1 mL suspension.

Isolates, which showed intensive growth or ability to hydrolyze the chitin by forming zones of clearance, were chosen for further investigations.

Cultivation of isolates

The next stage, an investigation of the biosynthetic process of isolates that showed ability to hydrolyze the chitin outside of the growing zone was carried out. The active fungal isolates presented fast growth, which covered the zone of clearance. For further analyses, the following fungi were chosen: Tr1, Tr2, Tr3, Tr4, Tr5, Tr22, H1, H2, H5, H6, H7, CH2, CHM1, CHH, CHO, M1, CH1 and Cs1. The ability of the chosen isolates to produce chitinolytic enzymes was observed in a liquid culture medium with the same composition, but without addition of agar. This medium was additionally supplemented with high molecular chitosan in a concentration of 5g/L. The cultivation of the strains was performed in 200 mL of medium in 500 mL flasks, at orbital rotary shaker at 180 min^{-1} at 28°C for 96 h. Cultivation was performed in a laboratory bioreactor with 1L of medium, and samples were taken at every 24 h, for a period of 144 h.

Chitinolytic activity assay

Chitinolytic activity was determined by the amount of reducing ends after enzyme hydrolysis using dinitrosalicylic acid (DNSA) method[22]. For this purpose, 1 mL of the crude enzyme (preliminary separated from mycelium for 10min at 10 000 min^{-1}) was mixed with 1 mL of 0.5 % colloidal chitin suspended in 0.1 M citrate buffer (pH 5.5). Reaction tubes were tempered for 45 min at 40°C. Enzyme reaction was stopped by addition of 3 mL DNSA, subsequently heated in boiling water bath for 5 min. After cooling down to room temperature, it was centrifuged at 3000 rpm for 15 min. Optical density of the samples was measured at λ = 575 nm at Spectroquant® Pharo 300 spectrophotometer (Merck). One international unit (IU) of chitinase activity was defined as the volume (mL) of enzyme required to produce 1 µmol of N-acetyl-D--glucosamine (NAG) for 1 h.

Molecular and taxonomic identification of the new fungal strain H5

DNA was extracted from samples using a bead beating protocol. PCR amplification was performed in Mastercycler Gradient thermal cycler (Eppendorf) using PuRe Taq Ready-To-GoTM PCR Beads (Amersho am Biosciences, Piscataway, NJ, USA) with universal primers as follow:

– PF-forward
(5'-AGGGATGTATTTATTAGATAAAAAATCAA-3')
– PF-reverse *(5'- CGCAGTAGTTAGTCTTCAGTAAATC-3').*

The obtained PCR products were purified by using GFXTM PCR DNA and Gel Band Purification Kits (Amersham Biosciences). Sequencing of amplified fragments was performed on ABI Prism 310 Genetic Analyzer by using BigDye® Terminator Kit version 3.1**.** The raw data from Genetic Analyzer were edited by Sequence scanner version 1.0 software (Applied Biosystems, Foster City, CA, USA). Sequences were compared to those available in databases by use of the network service to determine their approximate phylogenetic affiliations. The comparison of our data with NCBI Gene Bank Data Base reference sequences was conducted by Blast program.

Phylogenetic analysis of rDNA sequences were performed by using Clustal W version 1.7. The nucleotide sequences obtained in this investigation will be deposited in the NCBI nucleotide sequence databases. As previous results have some preliminary character, more certain definition was achieved with further analyses by sequencing of the internal transcribed spacer (ITS) regions, which are the first common DNA-barcode for Fungi[23]. It was performed at Leibniz Institute DSMZ – German Collection of Microorganisms and Cell Cultures.

Results and discussion

Screening for chitinolytic isolates and cultivation.

As mentioned above, the isolates with intensive growth or ability to hydrolyze chitin by forming zones of clearance, were chosen for further investigations.

As seen in Figure 1, isolates H1, H2, H5, H7, CH2, CHM1, CHH, CHO and M1 possessed significant chitis nolytic activity. Highest activity showed strains H5, CH2 and CHM1.

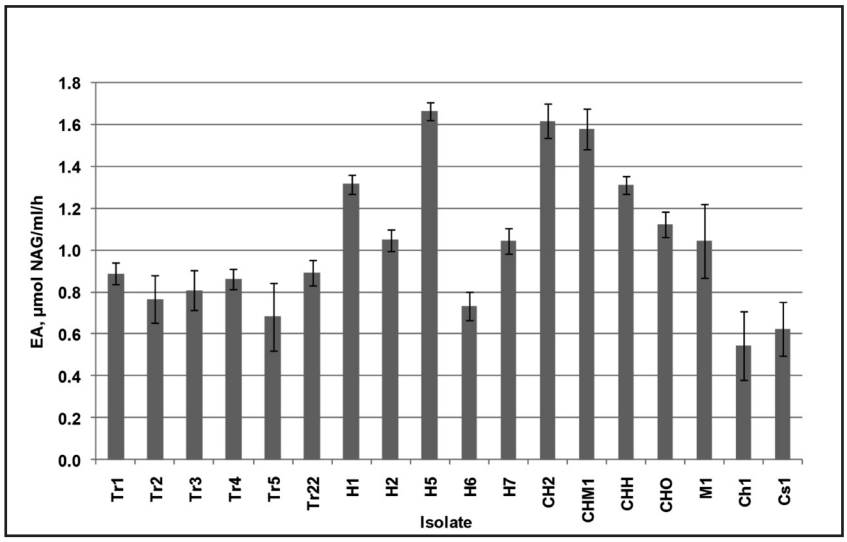

Fig. 1: Chitinolytic activity of isolates in a liquid medium on 96th h at 28°C

The investigation of the dynamics of enzyme synthesis started with cultivation of fungal isolate (designated H5), which showed most pronounced activity. Quantity of the reducing substances in the cultural medium and chitinolytic activity were determined on every 24th hour for a period of 144 hours. The data obtained during the performed analyses are shown on Figure 2.

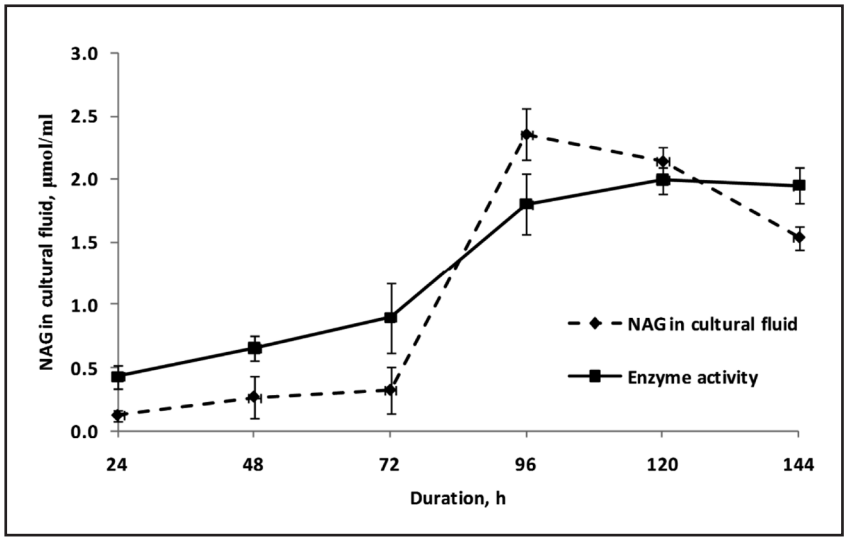

Fig. 2: Dynamics of enzyme activity (EA) and reducing sugars (NAG) in culture medium during the cultivation of isolate H5 in a bioreactor.

Dynamics of chitinase biosynthesis exposed strong dependence between chitinase activity and concentration of reducing sugars in the cultural medium (Fig. 2.). The enzyme synthesis was increasing between 24th h and 120th h. The producing level was highest between 72nd h and 96th h. The highest activity was achieved on 120th h, after which a low reduction was observed. The produced reducing substances in the culture medium followed the rising of the enzyme activity during the cultivation. The highest level of the reducing substances accumulation coincided with the highest level of enzyme activity rising. Isolate H5 revealed chitinase activity 1.66 ± 0.04 µmol NAG/mL/h, which was adequate to other citied fungal isolates [14]. Inducible character of chitinases, along with hard degradable substrate – chitin, probably was the reason for late (72nd h) biosynthesis of the enzyme. Important correlation of enzyme activity and concentration of reducing sugars between 72nd and 96th h, and between 120th – 144th h was observed. That suggested for probability of existence of hydrolysis' product regulation on chitinase biosynthesis of the fungal isolate H5. After this period of increasing, their quantity in the culture medium decreased very fast.

Production of enzymes with chitinase activity in fungi is described also by other authors.

Lima *et al.* (1997) [24] described the high capacity of six unidentified *Trichoderma* spp. isolates to produce chitinolytic enzymes and β-1,3-glucanases, and presented direct evidence for the hydrolytic action of crude enzymes and of one purified chitinase on the cell walls of *Sclerotium rolfsii* and *R. solani*.

Agrawal and Kotasthane (2012)[25] also reported for chitinase activity of *Trichoderma* isolates. They described the screening for chitinase activity using two different chitin sources (colloidal chitin derived from *Rhizoctonia* cell wall and commercial chitin) on solid media supplemented with Bromo cresol purple (pH indicator dye). Screened *Trichoderma* isolates were also assessed spectrophotometrically for N-acetyl-β--D-glucosamine (for total chitinolytic activity) and p-nitrophenol (for exochitinase activity) from colloidal chitin supplemented in broth. Released N-acetyl-β–D--glucosamine ranged from 37.67 to 174.33 mg/mL and 37.67 to 327.67 mg/mL and p-nitrophenol (pNP) ranged from 0.17 to 35.78 X 10-3 U/mL and 0.62 to 32.6 X X 10-3 U/mL) respectively with *Rhizoctonia* cell wall and commercial chitin derived colloidal chitin supplemented broth.

The production of chitinases is well studied also in members of fungal genus *Aspergillus*. For instance, Sherief *et al.* (1991) [26] reported for the ability of *Aspergillus carneus* strain isolated from soil, to decay the chitin at relevant conditions (pH 4.5 and 40°C), when the maximum chitinase activity was reached at 10 g chitin/L as a carbon source.

Escott *et al.* (1998) [27] described the induction of chitinolytic enzymes by *Aspergillus fumigatus*, which is a ubiquitous saprophyte and an important opportunistic fungal pathogen of humans. The authors have incubated *A. fumigatus* NCPF 2140 in growth medium containing 1 % colloidal chitin as sole carbon source. On the 72nd h after *A. fumigatus* was inoculated into the medium, they have observed an induction of extracellular chitinolytic activity of 1.5 µmol N-acetylglucosamine released min-1 (mg protein)-1, which is much lower than our H5 isolate.

Molecular and taxonomic identification of the new isolated strain H5

The obtained nucleotide sequences were paired accordingly and the 4 combined sequences were further analyzed for establishing of taxonomic affiliation. The nucleotide sequencing of 18S rDNA-fragment (681 bp) showed that the strain H5 appeared to be closely related to *Aspergillus flavus* strains since the observed identity was 100 %. It was deposited in Bulgarian National Bank of industrial microorganisms and cell cultures (NBIMCC 8735).

In order to have higher resolution, the internal transcribed spacer (ITS) fragment was PCR amplified and sequenced. The sequence was assembled from two independent cycle-sequencing reactions and sequencing runs (both using ITS4 primer), and edited using Sequencher software (GeneCodes). Isolate H5 showed 100 % (507/507bp) identity/homology to the fungi *Petromyces alliaceus* NHRL 35084 (EU021 603), which was anamorphic synonym to *Aspergillus alliaceus* Thom & Church (MB 256402).

At present, there is little information about identification of *Petromyces alliaceus* by sequence-based and morphological methods. Balajee *et al.* (2007) [28] reported for a clinical case, in which the authors managed to isolate *Petromyces alliaceus* from a patient with invasive pulmonary aspergillosis. They have used PCR amplification and nucleotide sequencing of the ITS and 28S ribosomal regions to identify the rare isolate *Petromyces alliaceus.*

Morphological characterization of H5 strain

Petromyces alliaceus Malloch and Cain is the only known sexually reproducing fungus classified in *Aspergillus* section *Flavi*[29]. Based on morphology of the sexual structures of H5 isolate – absence of sporulation; indehiscent ascocarps containing asci and ascospores formed within the pseudoparenchymatous matrix of stromata [30], it was additionally confirmed that the strain belongs to genus *Petromyces*.

Conclusion

A new fungal producer of chitinase *Petromyces alliaceus* H5 was isolated and identified. Chitinolytic microorganisms may find many biotechnological applications in various natural environments. Their application is not limited only to degradation (utilization) of the waste containing chitin. Numerous studies demonstrated the possibility of using them in biological control for production of chitinolytic enzymes with activity against some fungal phytopathogens. Chitinases are enzymes, which may find wide and effective application in different self-cleaning surfaces, especially in the planes, where sticking of insects to the wings during the flight cause significant increasing in fuel consumption. Due to all these reasons, studies on *Petromyces alliaceus* H5 as a promising producer of chitinase and its potential applications have to continue in future.

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Summary

Draganova D., Valcheva I., Stoykov Y., Tumbarski Y., Krastanov A.: Isolation and identification of new chitinolytic fungus *Petromyces alliaceus* H5

It is believed that chitinases play important physiological roles in filamentous fungi since chitin is one of the major cell wall components in these organisms. In this study we present a screening and identification of a new fungal isolate that could be used for the production of chitinase. For this purpose samples from soil, as well as from dead insects were collected and processed for further investigation. Eighteen isolates possessing chitinolytic activity on synthetic medium with colloidal chitin as sole carbon source were segregated. The one yielding highest chitinase activity was identified (by sequencing of the rDNA ITS fragment) as a previously unreported chitinolytic strain of *Petromyces alliaceus*. Subsequent cultivation in bioreactor yields a chitinase activity adequate to other reported in literature. The producing level was highest between 72nd h and 96th h and highest chitinase activity was achieved in 120th h of cultivation.
Keywords: chitin, chitinase, chitinolytic fungi, *Petromyces alliaceus*

Souhrn

Draganova D., Valcheva I., Stoykov Y., Tumbarski Y., Krastanov A.: Izolace a identifikace nové chitinolytické houby *Petromyces alliaceus* H5

Má se za to, že chitinasy hrají důležitou fyziologickou roli u vláknitých hub, protože chitin je jednou z hlavních složek buněčné stěny v těchto organismech. V této studii jsme provedli screening a následně identifikovali nový izolát, který by mohl být použit pro produkci chitinasy. Bylo vybráno osmnáct izolátů (z půdy a z mrtvého hmyzu) vykazujících chitinolytickou aktivitu na syntetickém médiu s koloidním chitinem jako jediným zdrojem uhlíku. Jeden, vykazující nejvyšší chitinasovou aktivitu byl identfikován (sekvenaci rDNA) jako kmen *Petromyces alliaceus*, u kterého dosud nebyla chitinolytická aktivita popsána. Následná kultivace v bioreaktoru poskytovala výtěžky chitinasy srovnatelné s literaturou. Nejvyšší produkce biomasy byla sledována mezi 72 a 96 hodinou a nejvyšší chitinasové aktivity bylo dosaženo po 120 hodinách kultivace.
Klíčová slova: chitin, chitinasa, chitinolytické houby, *Petromyces alliaceus*

RHAMNOLIPID BIOSURFACTANTS: PRODUCTION, CHARACTERIZATION AND APPLICATIONS

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Introduction

Surfactants are surface-active compounds capable of reducing surface and interfacial tension between liquids, solids and gases. Nowadays, the majority of commercially available surfactants is of a chemical origin[1]. These usually non-biodegradable compounds are often toxic, may bio-accumulate in livings and the process of their production, particularly by-products, can be hazardous to the environment[2]. Chemical surfactants play an important role in modern industry. Surfactants are used in detergents, cosmetics, food, manufacturing processes, as anti-static agents, lubricants and many other applications. The worldwide surfactants production achieved over 17-19 million tons in 2000; since this time, the surfactant production has gradually increased[3]. At present, chemical surfactants are still the main choice in the selection for surface active compounds in most chemical and commercial applications. Although, chemical and biological surfactants have similar surface activity, the chemical surfactants are less expensive; they are obtained as by-products of the petrochemical industry[4]. On the other hand, in recent years, many environmental regulations have come into force, thus biosurfactants gained increasing attention, because they are environmentally friendly[2].

Biosurfactants

Biosurfactants are a structurally diverse group of amphipathic compounds with both hydrophilic and hydrophobic moieties, synthesized by microorganisms. These compounds are categorized mainly by their chemical composition and microbial origin[5]. Their structure usually includes hydrophilic moiety represented by amino acids, peptides, mono-, di-, polysaccharides; and hydrophobic moiety consisting of unsaturated, saturated, fatty acids. Accordingly, the major classes of biosurfactants include glycolipids, lipopetides, lipoproteins, phospholipids, fatty acids, polymeric surfactants, and particulate surfactants[5].

The majority of known biosurfactants are glycolipids. They are composed of carbohydrates in combination with long-chain aliphatic acids or hydroxyaliphatic acids. The best known glycolipids are rhamnolipids, trehalosolipids, and sophorolipids[5].

Rhamnolipid classification

Rhamnolipids, in which one or two molecules of rhamnose are linked through the beta glycosidic bond to one or two molecules of β-hydroxy-decanoic acids, are the most studied glycolipids[5,6]. The first isolated rhamnolipid congeners were determined as R1 – R4,

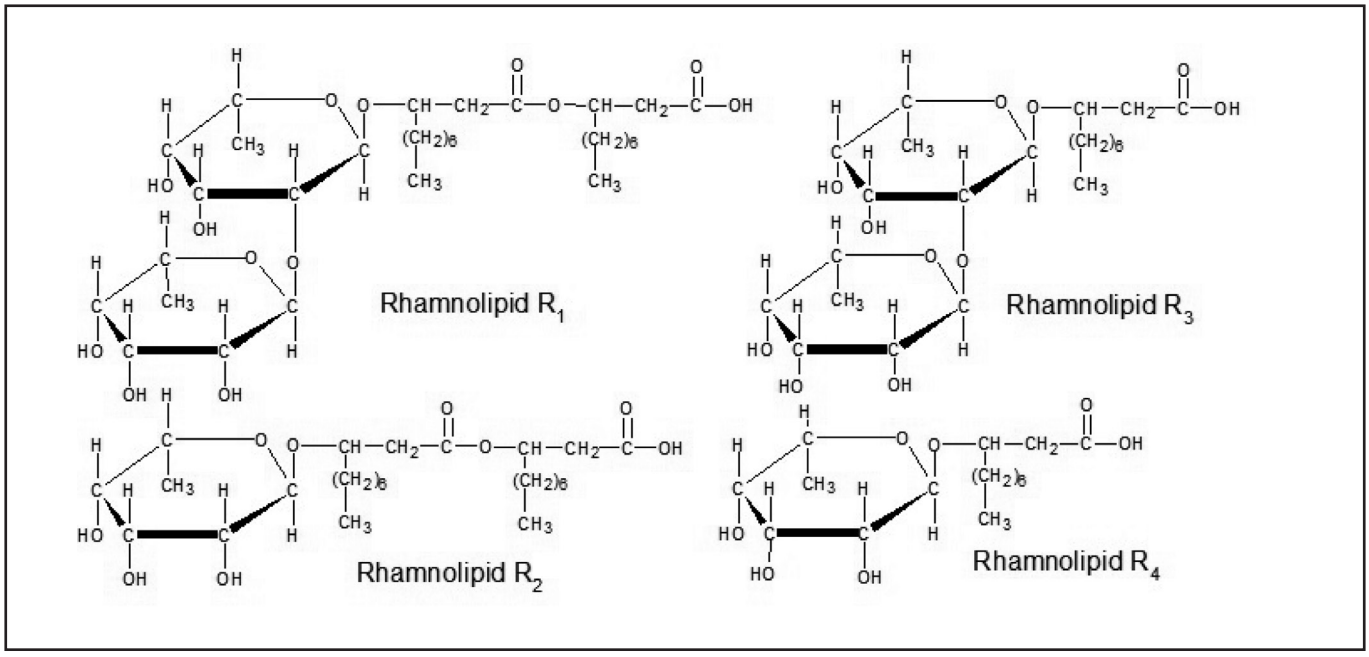

Fig. 1: Structures of the first described rhamnolipid congeners [7].

where R1 is α-L-rhamnopyranosyl-α-L-rhamnopyranosyl-β-hydroxydecanoyl-β-hydroxydecanoate (Rha--Rha-C_{10}-C_{10}), R2 is α-L-rhamnopyranosyl-β-hydroxydecanoyl-β-hydroxydecanoate (Rha-C_{10}-C_{10}), R3 is α-L-rhamnopyranosyl-α-L-rhamnopyranosyl-β-hydroxydecanoate (Rha-Rha-C_{10}), a R4 is – *α-L*-rhamnopyranosyl-β-hydroxydecanoate[5] (Fig. 1). At present, many other rhamnolipid congeners are known. Usually number of carbons in fatty acids ranges from 8 to12 atoms, but there are some references about rhamnolipids with fatty acids with 18 or even 22 carbon atoms[1].

Rhamnolipid production

Production of glycolipids containing rhamnose was first described by Jarvis and Johnson (1949)[8]. They observed production of this glycolipid by pathogenic bacterium *Pseudomonas aeruginosa*. Later studies have reported various rhamnolipid producers, including *Pseudomonas putida*, *Pseudomonas chlororaphis*, *Burkholderia pseudomallei*[6], *Burkholderia thailandensis*, *Pantoea stewartii, Acinetobacter calcoaceticus, Enterobacter asburiae, Enterobacter hormaechei, Nocardioides* sp. and *Pseudoxanthomonas* sp.[9].

In general, rhamnolipids are produced during the late stationary phase of growth[10], as a mixture of rhamnolipid congeners in a different ratio[3]. The quantity and quality of produced rhamnolipids are affected by two factors: composition of culture medium (sources of carbon, nitrogen, phosphorous, iron, and multivalent ions)[1,10] and cultivation conditions (pH aeration, temperature, stirring)[3].

There is evidence that to obtain high rhamnolipid concentration, it is necessary to expose the production cells to stress conditions (usually limitation by nitrogen and multivalent ions)[11]. The production of rhamnolipids under growth limiting conditions is characterized by a rapid increase in the rhamnolipids level[5]. One of a number of potential strategies for the production of high amount of rhamnolipids employs resting cells. Resting cells (stationary phase of growth) obtained under optimal growth conditions are subsequently used at high densities to produce rhamnolipids under the stress/limiting conditions[12]. This method could be applied to rhamnolipid production using free resting or immobilized cells. As documented with *Pseudomonas aeruginosa* DSM2874, the rhamnolipid yield coefficient ($Y_{p/x}$) increased more than fivefold (from 0.61 to 3.30) with the resting cells compared to cell propagated under nitrogen-limiting cultivation[13].

Physico-chemical properties

Rhamnolipids are surface active compounds with high efficacy. They are able to reduce the surface tension of water from 72 mN/m to 30 mN/m at concentration 10 – 200 mg/L. The addition of rhamnolipids to non-miscible water/oil system could reduce the interfacial tension from 43 mN/m to values below 1 mN/m and lower[7]. These properties make rhamnolipids the promising emulsifying agent of non-miscible mixtures. In addition, they contribute to stabilizing oil/water emulsions. Emulsions of *n*-alkanes (C10-C18) and aromatic compounds, crude oil, kerosene, coconut oil and olive oil in aqueous system can be well stabilized by rhamnolipids. These emulsions show only 5 – 25 % loss of stability after 24 h[7].

Surface active properties of rhamnolipids could be used at extreme salinity, temperature, and pH; in contrast to chemical surfactants. Chemical surfactants are deactivated by 2-3 % solution of salts[14]; rhamnolipids are not affected by sodium chloride concentrations of up to 6 %[15]. For example rhamnolipid from *Pseudomonas aeruginosa* MA01 is relatively stable and active even in the 10 % solution of sodium chloride[16]. Rhamnolipids are also stable at high temperature, as demonstrated by the temperature stability test of rhamnolipids produced by strain *Pseudomonas aeruginosa* MAO1. They can be exposed to 100°C for up to 75 min or autoclaving at 121°C for 45 min without reduction

of surface activity[16]. In addition, rhamnolipids show very good surface activity over a wide range of pH of 2 to 13. In the test of pH stability of rhamnolipids produced by strain *Pseudomonas aeruginosa* Bs 20, the maximum surface activity was reached at pH 7 to 8. Slight reduction in surface activity was observed at pH values less than 6 and more significant reduction in surface activity was observed at higher pH values (pH≥9)[15]. Decrease of surface activity at low pH values is caused by rhamnolipid precipitation[17].

Potential rhamnolipid applications

Rhamnolipids can be commercially produced at concentration up to 100 g/L. At these yield rates, rhamnolipid cost reaches 20 USD/kg (when the production volume is 20 m^3)[7]. It is worth noting, however, that common chemical surfactants cost 1 – 3 USD/kg[18]. Although rhamnolipids are still more expensive than chemical surfactants, rhamnolipids become more popular in applications demanding the use of environmentally friendly agents[7].

These applications involve environmental pollution treatment such as bioremediations of organic pollution, heavy-metal-contaminated sites, enhanced oil recovery, oil spill treatment[1]. In addition rhamnolipids can be used in the food, cosmetic, pharmaceutical, agriculture, and chemical industries[9].

Bioremediation of hydrocarbons

Rhamnolipids can be used to enhance bioremediation of many organic pollutants in soils, including chlorinated hydrocarbons, alkanes, paraffins, and polycyclic aromatic compounds[4]. They enhance the bioremediation by two mechanisms: 1. Rhamnolipids increase the solubility of hydrophobic substrate and 2. they increase the hydrophobicity of the bioremediation cells, which results in their better adhesion to the hydrophobic substrate[18]. In the case of *in-situ* bioremediation, the interaction of rhamnolipid with microbial cell is favorable. Only low levels of rhamnolipids are required to alter cell surface. For the increase in hydrocarbon solubility higher rhamnolipid levels are required. On the other hand, an increase in solubility of hydrophobic pollutant supports its mobilization and can subsequently cause spreading of the contaminated plume[18]. Besides to soil remediation, rhamnolipids can be applied in sea water remediation due to their salinity resistance[15].

Rhamnolipids can stimulate uptake of substrate (pollutant) by cells, but this influence differs for different microorganism species. Rhamnolipid can enhance the degradation of *n*-hexane by *Pseudomonas aeruginosa* whereas degradation by *Rhodococcus erythropolis* is suppressed[19].

Heavy metal removal

Several studies investigated removal of heavy metals by using rhamnolipids. Rhamnolipids are able to form complexes with Pb^{2+}, Zn^{2+} and Cd^{2+} ions. For example, the maximal apparent binding capacity for Cd^{2+} is 0.2 mol Cd^{2+}/mol rhamnolipid, with constant of stability cadmium/rhamnolipid (logK = – 2.47) which is higher than cadmium/sediment and cadmium/humic acid[7]. That research was confirmed by Juwarkar et al. (2007). They investigated the removing of lead and cadmium from contaminated soil. More than 88 % cadmium and 92 % of lead was removed when 0,1 % rhamnolipid solution was applied for up to 36 h. Moreover the use of the rhamnolipid solution led to decrease in soil toxicity and allowed natural microbial activity to take place[20].

Because the removal of heavy metals by rhamnolipids is affected by number of parameters (physical state of soil, physical state and concentration of metal, and composition and concentration of rhamnolipid) and these researches were set up under laboratory conditions, field applications would require individual approach to find a cost-effective strategy[20].

The rhamnolipids R1 and R2 produced by *Pseudomonas aeruginosa* are able to effectively remove copper from poor ore. Up to 53.4 % of copper was extracted from mining residues using 5 % rhamnolipid[21]. Addition of 1 % NaOH significantly enhances removing of copper from mining residues. The changes in copper removal efficacy under different conditions presumably pertain to the change in rhamnolipid structure[21]

Production of fine chemicals

Rhamnolipids are source of stereospecific L-rhamnose, which is commercially used in the synthesis of high-quality flavor compounds and as a basic material. Rhamnose can be obtained from quercitrin from oak bark, naringinin from citrus peels, or rutin. Rhamnose can be also obtained from rhamnose-containing polysaccharides produced by plants or microorganisms. Processing rhamnose from these materials is difficult. Plants sources are bulky and generate many wastes. Rhamnose in polysaccharides must be separated from other sugar components. Rhamnolipids produced by *Pseudomonas aeruginosa* are a good alternative. After easy separation from cells they can be hydrolyzed to produce mixture of L -rhamnose and β-hydroxy-fatty acids[18].

Cosmetics and health care

Many surfactants are used in cosmetics and health care. They are used in insect repellents, antacids, acne pads, antidandruff products, contact lens solutions, hair color and care products, deodorants, antiperspirants, nail care products, lipstick, eye shadows, mascara, toothpaste, lubricated condoms, baby products, antiseptics, shaving and depilatory products, foot care products, and moisturizers. In general, biosurfactants dispose of many advantages over chemical surfactants, including low irritancy or anti-irritancy effects and compatibility with skin. For example, rhamnolipids are used as cosmetics additives in Japan[18].

Rhamnolipids can be also used in pharmaceuticals as an emulsifying agents to enhance drug delivery, to release sputum from lungs or as auxiliary agents in vaccination[7,22]. The rhamnolipid ($Rha_2C_{10}C_{10}$ and $Rha_2C_{10}C_{12}$) produced by *Pseudomonas aeruginosa* B189 isolated from a milk factory exhibited significant inhibition of growth of human breast cancer cell lines (MCF-7). Moreover, ointment from rhamnolipids (0.1 %) can be used as treatment of a decubitus ulcer[9].

Agriculture

Rhamnolipids can be used in agriculture as environmentally friendly pesticides. They have zoosporicidal activity against *Pythium, Phytophora,* and *Plasmopara* species at concentration ranging from 5 to 30 mg/L. The proposed mechanism of the rhamnolipid action, similar to chemical surfactants, is that the rhamnolipid intercalates and disrupts the plasma membrane, although this mechanism was not established[2]. This observation has led to the development of a rhamnolipid-containing biofungicide used to prevent crop contamination by pathogenic fungi. This product was non-mutagenic and showed very low acute toxicity to mammal cells. It has been approved for direct use on vegetables, legumes and fruit crops. In addition, rhamnolipid also has protection activity against the fungus *Botrytis cinerea* in grapevines. Rhamnolipids inhibit spore germination and mycelium growth[9].

Food industry

Rhamnolipids can be potentially used in food industry as emulsifying agents to keep stability of baked and ice products, where they form emulsion of natural oils. They can be also used as stabilizers of fats to prevent sizzle during frying. The addition of rhamnolipids to dough can enhance stabilization, texture forming, and conservation of baked products[23]. The combination of rhamnolipids, nisin and natamycin increases the shelf life and inhibits microbial growth in UHT soymilk, salad dressings and cottage cheese[9]. Antiadhesive and antimicrobial properties of rhamnolipids can be used in protection of food products during production. The conditioning of surfaces (silicon rubber) by rhamnolipids decreased adhesion of *Streptococcus salivarius* and *Candida tropicalis* by 66 %. The adhesion of *Streptococcus aureus, Streptococcus epidermis, Streptococcus salivarius* and *Candida tropicalis* decreased upon treatment with rhamnolipid by 48 %[9].

Petrochemical industry

Due to their physicochemical properties (resistance to wide range of pH, salinity, and temperature), rhamnolipids can find an application in oil recovery from reservoirs under extreme conditions (high pressure, 85°C, extreme pH, high salinity)[15]. A new trend – microbial enhance oil recovery (MEOR) – takes advantage of microbial production of surface active compounds, and is considered as the most cost-effective strategy. As poor oil recovery from wells is due to low permeability of the rocks forming the reservoir or to the high viscosity of the crude oil, the ability of biosurfactants to reduce the oil/water interfacial tension and to form stable emulsions can improve the process efficacy[19].

Conclusion

Because of their interesting properties, rhamnolipids could have potential applications in the future. Disadvantages are their high production cost, and in many cases the main microbial producer, *P. aeruginosa,* is an opportunistic human pathogen. Rhamnolipids can be used in wide field of environmental applications, which may benefit from low-toxicity and biodegradability of these compounds. Due to their emulsifying, antimicrobial properties, and mammal cells friendly character, rhamnolipids can be further used in cosmetics, health care, and food industry. The future research should focus on process optimization to reduce the production costs and allow wider use of rhamnolipids to the other potential applications.

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Summary

Ježdík R., Kvasničková E., Masák J.: Rhamnolipid biosurfactants: production, characterization and applications

Rhamnolipids are important surface active compounds of microbial origin, which could be used in many areas. Although, at present are rhamnolipids more expensive than chemical surfactants, they slowly substitute chemical surfactants in applications with demand on using of non-toxic and biodegradable agents.

Keywords: Biosurfactant, Rhamnolipid, *Pseudomonas aeruginosa*

Souhrn

Ježdík R., Kvasničková E., Masák J.: Rhamnolipidové biosurfaktanty: produkce, characterizace a apilikace

Rhamnolipidy patří mezi významné povrchově aktivní látky mikrobiálního původu a mohou být použity v mnoha aplikacích. Přestože je produkce rhamnolipidů nákladnější, než syntéza chemických surfaktantů, jsou chemické surfaktanty postupně nahrazovány rhamnolipidy v aplikacích kladoucí důraz na netoxická a biologicky odbouratelná agens.

Klíčová slova: Biosurfaktant, rhamnolipid, *Pseudomonas aeruginosa*

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