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International
Journal
Of Modern Engineering Research (IJMER)

Survey and determination of probiotic activity of *Bacillus subtilis* Natto strain

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ABSTRACT:- *Bacillus subtilis natto strain is identified by method of 16S RNA sequence analysis. Through the survey of a number of beneficial properties, it is found that Bacillus subtilis natto strain is also a typical probiotic. This strain translates enzymes such as amylase, protease, cellulase, respectively 1.62cm, 1.80cm, 1.71cm and enzyme of nattokinase 72.5 FU/ml after being fermented for 24 hours of culture at 37^{0}C in a NB medium. Its tolerance of low pH (pH2) in the simulated gastric juice (SGJ) medium after 120 minntes ofincubation has a survival rate of 85.95% compared with the initial rate. Its tolerance 0.3% bile salt in simulated intestinal juice (SIJ) medium after 4 hours of incubation has a survival rate of 84% compared with the initial rate. Its adhesive ability of Bacillus subtilis natto with organic solvents such as xylene, chloroform , and ethyl acetate is 32.8%, 17.43% and 53.93%, respectively.*

Keywords:-*Bacillus subtilis natto, probiotic, nattokinase.*

I. INTRODUCTION

A long time ago, probiotic was known in products of functional foods and pharmaceuticals. Probiotics play an important role in promoting and maintaining human health as support in digestion, reducing intestinal disorders, absorptionof lactose, improving immune function, preventing inflammations, lowering cholesterol, and inhibiting harmful microorganisms in the intestines. To promote these beneficial effects, probiotic bacteria must be present with an appropriate survival cell density in products and maintain high survivability during human digestion. Through several studies, it is showed that *B.subtilis* strain is also a typical probiotic. First, the *B.subtilis as a* probiotic product in spores can be stored at room temperature in dry condition without affecting the survivability. The second advantage is that the spores can survive moving through a pH acid medium of gastric juice [1][2]. At present, not only abroad but also in Vietnam, many *B.subtilis* probiotic products have been licensed as functional foods. In this study, we use *B.subtilis* natto strain to be able to exploit the functions of probiotic of biomass.

II. MATERIALS AND METHODS

2.1. Microorganism strain and cultural medium

Bacillus subtilis nattoselected from a collection of HCMC University of Technology
Bacillus subtilis natto grows in the nutrition broth (NB) and nutrition agar (NA), pH 7.5, the temperature at 37^{0}C, the agitation of 150 rpm.

2.2. Research methods

2.2.1. Molecular biology method

Identification of *Bacillus subtilis* natto by determining some biological characteristics and 16S rRNA sequence analysis.

2.2.2. Biochemical method

2.2.2.1. Determine of digestive support enzymes activity (amylase, protease, cellulase)

Assessment hydrolysis capability of amylase, protease and cellulase according to agar diffusion method, supplemented by the corresponding substrate: 1% casein to test protease activity, 1% starch to test amylase activity and 0.5% Na-CMC to test cellulase activity. Enzyme activity is determined by the D - d (cm) index after 24 hours of growing in a culture medium (D: diameter of resolution ring, d: diameter of agar hole). If the fermented fluid with activities of amylase, protease and cellulase will create transparent rings around the agar containing enzyme. The area of starch that has not been hydrolyzed is green. The area of casein that has not been hydrolyzed is opaque. The area of cellulose that has not been hydrolyzed is yellowish brown [3].

2.2.2.2. Determination of nattokinase enzyme activity

Nattokinase activity is measured according to fibrin hydrolysis. Suck out 0.4ml fibrinogen solution and 1.4ml 0.72% borate buffer (pH 8.5) in vitro. Add 0.1ml 20U/ml thrombin solution. Then add 0.1ml enzyme solution, incubated at 37^0C. After 1 hour, add 2ml 0.2M trichloroacetic acid. Centrifuge 15000rpm for 10 minutes, transparent fluid intake. Measure the absorbance of transparent fluid at 275nm wavelength. One activity unit of nattokinase (FU- fibrinolytic units) is defined as the increase of 0.01 absorption of the reaction solution in a minute [4].

2.2.3. Microbiological methods

2.2.3.1. Survey of tolerance to low pH of *B.subtilis* natto in simulated gastric juice (SGJ) medium.

Acid tolerance of bacteria was assessed by density of survival cell, based on the method of Charter et al (1998). Centrifuge cultured B. *subtilis* natto after 24 hours of culture with 4000 rpm speed for 10 minutes. Collect biomass and wash 2 times with physiological saline. Implement an addition of 100µl cell suspension fluid into 10ml pH 2 adjusted SGJ fluid, incubated at 37^0C. Determination of B. *subtilis* natto bacteria [log (CFU/ml)] at the times of 0 minute, 30 minutes, 60 minutes, 90 minutes, 120 minutes [16].

2.2.3.2. Survey of bile salt tolerance of *B.subtilis* natto in simulated intestinal juice(SIJ) medium.

Survey the bile salt tolerance of *B. subtilis* natto bacteria in NB medium with addition 0.3% bile salts based on method of Dunne et al (2011). Centrifuge cultured fluid of *B. subtilis* natto after 24 hours of culture. Collect biomass, wash 2 times with physiological saline. Add 100µl cell suspension fluid into 10 ml of bile salt added NA solution reaching a concentration of 0.3% (SIJ). The cell density of bacterial cells of B.*subtilis* natto [log (CFU/ml)] is determined by spreading discs at the times of 0 hour, 1 hour, 2 hours, 3 hours, and 4 hours [17].

2.2.3.3. Survey of *B.subtilis* natto adhesive ability.

Survey of auto aggregation ability of B.subtilis natto

Biomass of bacterial cells collected after culturing is washed 2 times with pH 7.2 PBS buffer. Then it is in resuspension in PBS buffer. This resuspension fluid is incubated for 5 hours to facilitate the autoaggregation and deposition of the bacteria. After an hour,suck out his resuspension fluid and measure OD at 600nm wavelength. Percentage of autoaggregation of bacteria is calculated by the formula: 1 - (At/Ao) .100. In which: At: absorption at 1, 2, 3, 4, 5 hours and Ao: absorptionat 0 hour [18].

Survey of adhesive ability of B.subtilis natto with organic solvents

Biomass of bacterial cells collected after culturing is washed twice with KNO_3 0.1M (pH 6.2) solution and resuspended in this solution (Ao). Put 1ml solvent in turn into 3 test-tubes containing 3ml cell suspension. Vortex in 2 minutes and leave it alone 20 minutes at room temperature until the appearance of delamination, remove the solvent, measure OD at a wavelength of 600nm (At). Percentage of adhesion with solvent is calculated by the formula: 1 - (At/Ao) .100 [15][19].

III. RESULTS AND DISCUSSION

3.1. Results of preliminary identification from biological characteristics and molecular biology

The colony of B.*subtilis* natto bacteria on nutrient agar medium has a rounded form, serrated shape, rough surface.

B. subtilis natto bacteria cells are rod-shaped, the purple dye of gentian violet. This is consistent with the theoretical basis as positive Gram is capable of mobility, located separately or linked together into chains, relatively uniform in terms of size.

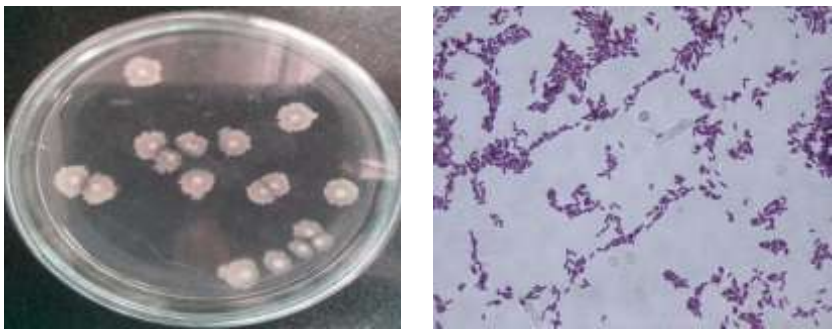

Figure 1.B.subtilis natto under the microscope and colony morphology

From the results of culture, examine macroscopically and microscopically, we assert that B.subtilis natto bacteria is pure.

The results of 16S rRNA sequence analysis proved that the bacterium that the work employed is named *Bacillus subtilis*.

TGGCTCAGGATGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAATGGATTAA
GAGCTTGCTCTTATGAAGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCCAT
AAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAACATTTTGAACCGCATG
GTTCGAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAGCT
AGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATC
GGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCT
TCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGT
CGTAAAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGG
TACCTAACCAGAAAGCCACGGCTAACTACGTG

Figure 2.B.subtilis natto of 16S rRNA sequence analysis

3.2. Determine the activity of digestive support enzymes

Follow the perforated agar method to test the digestive support enzyme activity.

Table I.Hydrolysis diameter of digestive support enzymes from B.subtilis natto

Enzyme	Amylase	Protease	Cellulase
D-d (cm)	*1.63*	*1.80*	*1.71*

The results in Table I show that fermented fluid is capable of hydrolysis starch, casein and CMC, demonstrating that B.*subtilis* natto bacteria is active with all three enzymes. Through the test, the diameters of resolution ring of all three enzymes of amylase, protease and cellulase are respectively 1.63cm, 1.80cm and 1.71cm. According to research by Sun Yan (2011) which used casein and Na-CMC for screening 20 strains of B.*subtilis* natto (NY1-NY10, NS1-NS10) there are protease and cellulase activities from natto of Japanese, Dalian and Beijing. The experimental results selected two strains with the highest protease activity (NY-1) and the highest cellulase activity (NS-1) respectively 44.12 ± 1.48 U/ml for 18 hours of fermentation and 60.94 ± 1.22 U/ml at 40 hours of fermentation [3].

B.subtilis natto bacteria can translation enzymes such as amylase, protease and cellulase help support digestive and potential as a probiotic used to produce biological preparations.

3.3. Determination of nattokinase enzyme activity

After culturing *B.subtilis* natto strain in NB medium at temperature of 37^0C, after 24 hours, quantitative analysis of nattokinase activity from culture medium is 72.5 FU/ml. According to research by Nguyen Anh Tuan et al (2015) which has optimized fermentation medium of B.*subtilis* natto bacteria by RSM-CCD experimental design to determine the highest nattokinase activity of 69.3 ± 0.2 FU/ml after 20 hours of fermentation at temperature of 37^0C and pH 7.5 [4].

Nattokinase is an extracellular enzyme, high fibrinolytic activity.

B.subtilis natto bacteria after 20 hours of fermentation at temperature of 37^0C and pH 7.5 for nattokinase activity of 72.5 FU / ml.

3.4. Survey result of some of probiotic activities of *B.subtilis* natto

3.4.1. Tolerance to low pH

The gastric juice tolerance is a characteristic that a probiotic bacteria strain must have. The number of bacteria will decline significantly by the harsh conditions of the gastric juice. According to research by Zhou (2009), the pH 2 value is considered the decision limit in the screening of probiotic microbial strains [5].To be able to pass the stomach, the probiotic strain must be able to withstand the harsh medium of gastric juice including digestive support enzymes and low pH. Therefore, we conduct a survey of survival capability of *B.subtilis* natto strain in the medium of pH 2 simulated gastric juice.

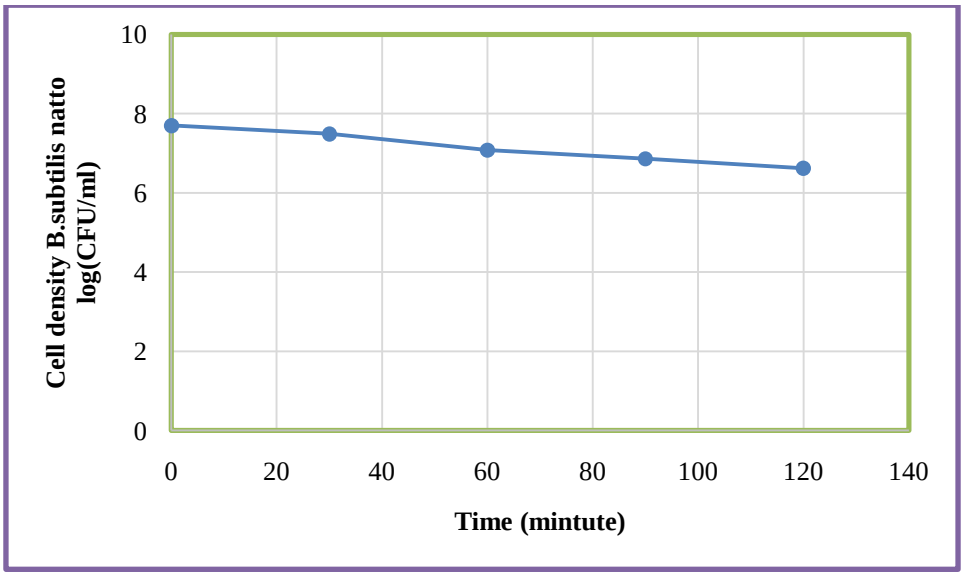

Figure 3.The survivability of B.subtilis natto in SGJ medium (PH 2)

Research results show that the simulated gastric juice medium at pH 2 at the time of 120 minutes, the survivability of B.*subtilis* natto is 85.95% compared with the initial rate. It is explained that medium pH is very important for the growth and development of many types of bacteria. The cations H^+ and anions OH^- are the two most active ions in all kinds of ions.

The slightest changes also affect the bacterial cells. When the medium around cells in the form of low pH leads intracellular pH tend to lower, the difference of membrane pH is significantly reduced and reduces the proton motive power. The result of this phenomenon that have led cells must form spores to be able to survive in this harsh medium.

Based on the experimental results we conclude that SGJ medium affects the survivability of *B.subtilis* natto bacteria. However, these bacteria are able to survive at pH 2 after 4 hours.

3.4.2. Bile salt tolerance

The survivability of bacteria entering the gastrointestinal tract that is affected by bile salts is important criteria for selection of probiotic bacteria. Because after moving through the stomach, the food will arrive towards small intestine. At the place where the bile salts secrete and engage primarily in the emulsion and lipid solution, playing an important role in the digestion of fats can cause the bile salts to affect the cell membrane of microorganisms. Probiotic is only effective and beneficial when they colonize and survive in the small intestine [6]. The average concentration of the bile salts present in the small intestine is 0.3% and the concentration of decision for screening probiotic strains [7].In addition, the processing time of simulation based on the storage period of food in the stomach from 2 hours to 3 hours [8]. Therefore, we survey the survivability of *B.subtilis* natto bacteria in artificial bile salt medium.

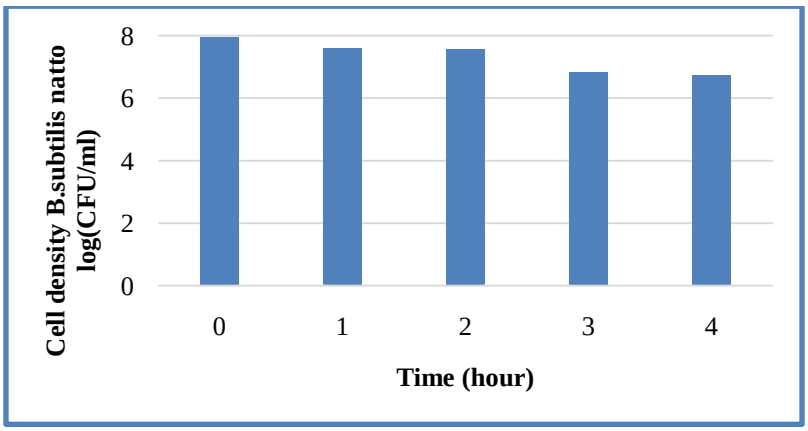

Figure 4.The survivability of B.subtilis natto in SIJ medium

Figure 4 shows *B.subtilis* natto bacteria could withstand 0.3% concentration of bile salts with a survival rate of over 84%. According to Pichereau, Hartke and Auffray (2000), the bacteria are capable of meeting the changes immediately around them by re-establishing the processes of metabolism, thereby increasing the survivability in harsh conditions. Tolerance is "encoded" in the cellular defense systems, created by the elements of physics and chemistry (salt stress, bile salts ...) in the medium, causing "stress" allows survival against "challenge" dose of the same element [9]. In addition, while living in the harsh medium, *Bacillus* will react by sporulation mechanism to be able to adapt to the medium conditions that are not favorable
Experimental results conclude that *B.subtilis* natto strains that are resistant to bile salt concentration is 0.3%.

3.4.3. Adhesive ability of *B.subtilis* natto

The probiotic strains must stick into the intestinal wall, well localized in the digestive tract and proliferate. The adhesive ability is considered an important requirement in order to prevent rapid excretion by the contraction of the stomach. There, at first they must have the adhesive ability to compete for the positional and nutrients with pathogenic microorganisms and maintain the balance of intestinal flora. Therefore, they have to survive when passing through the stomach - where there are pH acid and digestive supportive enzymes.

Survey of the adhesive ability of *B.subtilis* natto bacteria is conducted by surveying the autoaggregation ability and adhesive ability with solvent.

Table II.The adhesive ability of B.subtilis natto

Evaluation criteria	Adhesive ability (%)
Autoaggregation ability	*25.42*
Adhesive ability with xylene solvent	*32.80*
Adhesive ability with chloroform solvent	*17.43*
Adhesive ability with ethyl acetate solvent	*53.93*

In this table, the autoaggregation ability enables *B.subtilis* natto bacteria to stick together to form one large population, enhance vitality and the development of the strain according to relationship style of supporting the same species. The autoaggregation ability also relates to intestinal adhesion and increases the ability to store in the digestive tract. When surveying the autoaggregation ability of *B.subtilis* natto bacteria reaches 25.42% after 5 hours, therefore can be used as probiotics. Also surveying the autoaggregation ability of *Lactobacillus* and *Bifidobacterium,* Orlowsk and Beeleka (2006) found great variation in the autoaggregation ability of strains, 5 strains were surveyed in the laboratory with the results of the autoaggregation is 5.5%, 15%, 23%, 75% and 77% at room temperature [10]. According to research by Rahman (2008) about the autoaggregation ability of *Bifidobacterium* strains at 37^0C, the result is in the range of 0.9% - 13.2% [11].
In addition, the adhesive ability with solvent (MATS - Microbial adhesion to solvents) is determined by the method of Bellon Fontaine [15]. This is an indirect method to selectively study cell lines capable of high intestinal adhesion.

Results of *B.subtilis* natto adhesion to xylene is 32.8%, showing hydrophilic properties of the cell surface and is one potential probiotic strain. The hydrophilic properties of the surface of the bacterial cells may be due to the acidic or base compounds on the cell surface, or maybe both. Rahman (2008) has announced the adhesive ability with solvents of some of *Bifidobacterium* bacteria with very large fluctuations (14.4% - 97.3%) [11]. According to Pelletier (1997), survey results of adhesion to hexadecane (nonpolar solvent) of several strains of *L.casei subsp. casei, L.paracaei subsp. paracasei, L.rhamnosus* are 5.8% - 26.5% [12].
The high adhesive ability of bacterial cells to ethyl acetate can entail the opportunity to adhere to the polarizing elements with basicity in intestinal epithelium. Results of *B.subtilis* natto adhesion to ethyl acetate is 53.93%, showing the polarity and acidity of the cellsurface is low. According to the research of Maria (2007), the adhesive ability with ethyl acetate of some strains of *L.johnsonii, L.plantarum, L.paracasei, L.casei* between 0 - 79.2% [13].

The adhesion of *B.subtilis* natto bacterial cells to chloroform is 17.43%, demonstrating the basicity of the cell surface is low. Research by Provencio (2009) has announced the adhesive ability of L*actobacillus casei* with chloroform from 20-98% [14]. A long with that, Maria (2007) has recorded chloroform adhesion of certain strains of the species L.*johnsonii, L.plantarum, L.paracasei, L.casei* ranging from 11.6% - 100% [13].
Through the survey, adhesive ability of *B.subtilis* natto with xylene, chloroform and ethyl acetate is respectively 32.80%, 17.43% and 53.93%. The study results show that the potential strain is a probiotic typical of the adhesive ability forming biological barriers.

IV. CONCLUSION

The bacterial strain is identified as *Bacillus subtilis* natto. This strain translates digestive support enzymes as amylase, protease, cellulase, respectively 1.62cm, 1.80cm, 1.71cm and enzyme of nattokinase 72.5

FU/ml after 24 hours of culture in NB medium. *B.subtilis* natto is potentially probiotic when surveying some probiotic activities as tolerance to low pH (pH 2) on simulated gastric juice (SGJ) medium after 120 minutes of incubation has asurvival rate of 85.95% compared with the initial rate, tolerance of 0.3% bile salts in simulated intestinal juice (SIJ) medium after 4 hours of incubation has a survival rate of 84% compared with the initial rate, the adhesive ability of *Bacillus subtilis* natto with organic solvents such as xylene is 32.8%, chloroform is 17.43% and ethyl acetate is 53.93%. Therefore, *B.subtilis* natto has potential applications in functional foods and pharmaceutical products.

Acknowledgements

This review is funded by HoChiMinh University of Technology under grant number TNCS-2015-KTHH-05.

REFERENCES

[1]. Spinosa, M.R., Braccini, T., Ricca, E., De Felice, M., Morelli, L., Pozzi, G., and Oggioni, M.R., On the fate of ingested Bacillus spores, Research in Microbiology 151, 2000, 361-368.
[2]. Barbosa, T.M., Serra, C.R., La Ragione, R.M., Woodward, M.J., and Henriques, A.O., Screening forBacillus isolates in the broiler gastrointestinal tract, Applied and Environmental Microbiology 71, 2005, 968-978.
[3]. Sun Yan, Wang JiaQi, Screening of protease and cellulase producing Bacillus subtilis natto strain and its growth characteristics, Dongbei Nongye Daxue Xuebao Journal, 42(3), 2011, 39 – 43.
[4]. Nguyen Anh Tuan, et al., Determination the optimum fermentation in obtaining nattokinase by Bacillus subtilis natto, IJIAS, Vol.13, No.3, 2015, 663-668.
[5]. Zhou, J., et al., Analysis of the microflora in Tibetan Kefir grains using denaturing gradient gel electrophoresis, Food Microbiology, 20, 2009,598-602.
[6]. Havenaar R, Huis In't Veld MJH, Probiotics: a general view. In: Lactic acid bacteria in health and disease, Elsevier Applied Science Publishers, 1992.
[7]. Gilliland SE., and Speck ML., Deconjugation of bile acids by intestinal lactobacilli, Applied and Environmental Microbiology, 33, 1977, 15–18.
[8]. Zhou J., Liu X., Jiang H., Dong M., Analysis of the microflora in Tibetan Kefir grains using denaturing gradient gel elactrophoresis, Food microbiology, 26, 2009, 770-775.
[9]. Pichereau V., Hartke A. & Y. Auffray, Starvation and osmotic stress induced multiresistances. Influence of extracellular compounds,International Journal of Food Microbiology. 55, 2000, 19-25.
[10]. Orlowaski A., Bielecka M., Preliminary characteristics of Lactobacillus and Bifidobacterium strains as probiotic candidates, Pol.J.food Nutr. Sci, 15, 2006, 269-276.
[11]. Rahman MM., Woan-Sub Kim WS., Kumura H., Shimazaki KI., Autoaggregation and surface hydrophobicity of Bifidobacterium, World J Microbiol Biotechnol, 24, 2008, 1593-1598.
[12]. Pelletier C., Bouley C., Cayuela C., Bouttier S., Bourlioux P., Bellon-Fontaine MN., Cell surface characteristics of Lactobacillus casei subsp. casei, Lactobacillus paracasei subsp. paracasei and Lactobacillus rhamnosus strains, Appl Environ Microbiol, 63(5), 1997, 1725-1731.
[13]. Maria Guadalupe VP., Molecular and physiological studies on the functionality of probiotic Lactobacilli, Verlag Dr. Hut, 2007.
[14]. Provencio D., Lopis, Antolin, Torres, Monedero., Adhesion properties of lactobacillus casei strains to resected intestinal frahments and components of the extracellular matrix, Arch Microbiol, 191, 2009, 153 – 161.
[15]. Bellon Fontaine MN, Rault J, Van Oss CJ, Microbial adhesion to solvents: a novel method to determine the electron-donor/ electron-acceptor or Lewis acid-base properties of microbial cellsColloids Surf B Biointerfaces, 7, 1996, 47:53.
[16]. Charteris, W.P., Kelly,P.M., Morelli, L. and Collins, J.K., Development and application of an invitro methodology to determine the transit tolerance of potentially probiotic Lactobacillus and Bifidobacterium species in the upper human gastrointestinal tract, Journal of applied microbiology, 84, 1998, 750 – 768.
[17]. Dunne,C.,O`Mahony,L.,Murphy,L.,Thornton, G.,Morrissey,D.and O`Halloran, S.,Invitroselectioncriteriaforprobioticbacteriaofhuman origin:Correlationwithinvivofindings,Amer.J.Clin.Nutr.,73, 2001, S386-S392.
[18]. Del Re, B., Sgorbati, B., Miglioli, M. and Palenzona, D., Adhesion, autoaggregation and hydrophobicity of 13 strains of Bifidobacterium longum,Letters in Applied Microbiology, 31, 2000, 438 – 442.
[19]. Kos B., Suskovic J., Vukovic S., Simpraga M., Frece J., Matosic S., Adhesion and aggregation ability of probiotic strain Lactobacillus acidophilus M92", J Appl Microbiol, 94, 2003, 981-987.