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Effects of Detergents, Ions, and Organic Solvents on the Activity of Four *Bacillus clausii* Pectinases

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Abstract

Four different pectinolytic enzyme activity essays were employed to detect different pectinases of *Bacillus clausii* from an extracellular protein cocktail. The mixture was obtained by ethanol precipitation. Dinitrosalicylic acid (DNS) and sodium hydroxide (NaOH) titrimetric methods were used for the pectinase assays. Each of the essays produced a positive result, indicating the presence of pectate lyase (PGL), pectin lyase (PL), pectin methyl esterase (PME), and polygalacturonase (PG). Activities of these enzymes were then assessed at differing concentrations of NaCl, detergents (SDS and tweens), ion chelators, and organic solvents. Results, presented as histograms, were the indication of original findings, as most of them were new to the relevant literature.

Keywords: *Bacillus clausii*, ionic strength tolerance, organic solvent tolerance, pectinase activity, salt tolerance.

1. Introduction

The walls of growing plant cells are called primary cell walls and about 90% are composed of polysaccharides [1]. The plant cell wall is a barrier that protects plants against microbial infections. This barrier contains cellulose, hemicellulose, lignin and pectin polysaccharides. Pectin has a heterogeneous structure and gives rigidity to the cell wall by cross-linking polysaccharides [2]. At the same time, these bonds are thought to limit cell growth and reduce biodegradability [3].

Depolymerisation and esterification of polysaccharides in the cell wall is carried out by extracellular enzymes. Pectinases are a group of enzymes involved in the breakdown of pectin, a complex polysaccharide found in the primary cell wall and in the middle lamellae of higher plant tissues. After pectin is broken down by pectinases, it first enters the periplasmic space and then the cytoplasm [4]. Pectic degradation products regulate the physiological responses and gene expression of plants [1]. Phytopathogenic species such as *Erwinia* cause softening and rotting, especially in plant roots [2]. This explains the phytotoxicity mechanism of pectic fragments [1].

Aspergillus, *Erwinia*, and *Penicillium* species were mainly used in pectinase production. In the last fifteen years, *Bacillus*, *Fusarium*, *Kluyveromyces*, *Rhizopus*, and *Saccharomyces* have also been studied. Selection of microbial sources for pectinase production is generally based on the culture conditions, the number and type of pectinase produced, the pH, and thermal stability of the enzymes [5].

In today's fruit juice production, pectinases play a key role in the breakdown of cell walls, colour compounds, and aroma essences. Thus, thanks to pectinases, it has become possible to obtain high quality concentrated products. In addition, pectinases are used effectively in many other industrial areas such as textile, fibre processing, paper making, waste water treatment, animal feed preparation, tea and coffee production, and oil extraction [6-8]. It is estimated that microbial pectinases have a 25% share in the world food enzyme market [9].

This study was performed to gain an insight into the catalytic capacity of crude alkaline pectinolytic enzymes of *Bacillus clausii* in harsh environment where organic solvents, detergents or high concentrations of ions were present.

2. Material and Methods

2.1 Isolation and identification of *Bacillus clausii*

For the bacterial source, Koyulhisar (Sivas, Turkey) apple orchard soil samples were used. The isolates obtained were identified at the species level by partial protein- (mass spectrometry) and 16S rRNA gene sequence analysis [10,11].

2.1.1 Identification of bacteria by mass spectrometry

Colonies grown in Horikoshi-I agar at 37^{o}C by 24 h incubation were used in the analysis. The colonies grown in the plate were treated with the solution used in the MALDI-TOF device and compared with the partial protein profiles in the library of the device and the original spectrum results were recorded [11].

2.1.2 16S rRNA gene sequencing

PCR- and DNA sequencing conditions have been described in an earlier article [11].

2.2 Screening of pectinase activity

Soil samples were inoculated onto two separate agar media, containing polygalacturonic acid and pectin. Agar plates were incubated for three days at 37^{o}C. Gram iodine solution was dropped onto individual colonies and waited for 5 minutes. Zone formation was examined. As another method, 1% CTAB solution was dropped and zone formation was examined. Zones around the colony indicate the presence of pectinase activity.

2.3 Preparation of crude enzyme

Liquid Horikoshi-I medium was inoculated with bacteria and an overnight culture was obtained at 37^{o}C. After the incubation, cells were pelleted by centrifugation for 10 min at 5,000 rpm at +4^{o}C. Onto the culture supernatant 0.5, 0.6, 0.75, 1.0, 1.25, 1.75 or 2 volumes cold 95% ethanol were added and total protein content was precipitated cold ethanol and kept at -20oC overnight. Then the proteins were collected by centrifugation at 5000 rpm at +4^{o}C for 15 minutes. Ethanol was removed at room temperature.

2.4 Enzyme identification

Polygalacturonase (PG) activity was assayed by DNS method [12,13]. Enzyme activity was calculated as µmol galacturonic acid released per minute.
Pectin lyase (PL) assay relied on the absorbance of double bonds formed in the pectin substrate at 235 nm. The activity was calculated as the change of absorbance measured at 235 nm per minute [14].

Pectate lyase (PGL) activity was determined by reading the absorbance of double bonds formed in the pectin substrate at 235 nm. The activity was calculated as the change of absorbance measured at 235 nm per minute [15].

Pectin methylesterase (PME) activity was determined by monitoring and recording the pH change. The activity was calculated by measuring the amount of NaOH required to keep the pH constant during the reaction [13].

3. Results and Discussion

3.1 Identification of Pectinase-producing Bacteria

Fifty colonies isolated from the soil were grown in selective medium containing pectin and polygalacturonic acid.

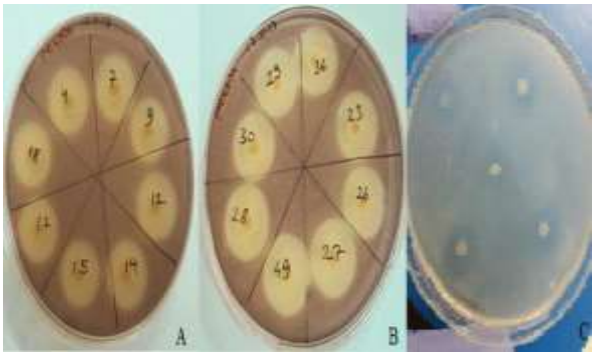

Figure 1. Detection of enzyme activity in the medium containing pectin.

After incubation (3 days at 37^{o}C), 1% CTAB and iodine solution were dropped onto the colonies. About a third of the colonies (16:50) formed zones on both media (Figure 1,2).

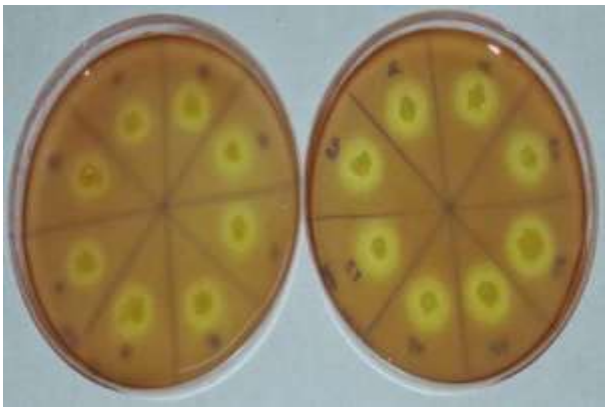

Figure 2. Detection of enzyme activity in media containing PGA.

Zones were visualised with iodine solution in the PGA medium containing agar plates. Zone formation was observed in all of the isolates.

3.2 Isolate identification

Morphological and partial biochemical characterization showed that the isolates were Gram-positive, catalase-negative, and rod-shaped bacteria (results not shown). One of the strains was selected for further studies.

Partial protein analysis was performed by MALDI-TOF mass spectrometry in the Department of Microbiology, Faculty of Medicine, Cumhuriyet University, and it was determined that all the isolates were *Bacillus clausii.*
A phylogenetic analysis based on 16S ribosomal RNA (rRNA) gene sequence comparison also showed that this strain belonged to *B. clausii*. The rRNA gene sequence was submitted to GenBank (Accession number: MT524963, GenBank).

3.3 Enzyme activity measurement
3.3.1 Effect of ionic strength

Effect of sodium chloride was investigated at differing NaCl concentration and under optimum pH and temperature points (Figure 3).

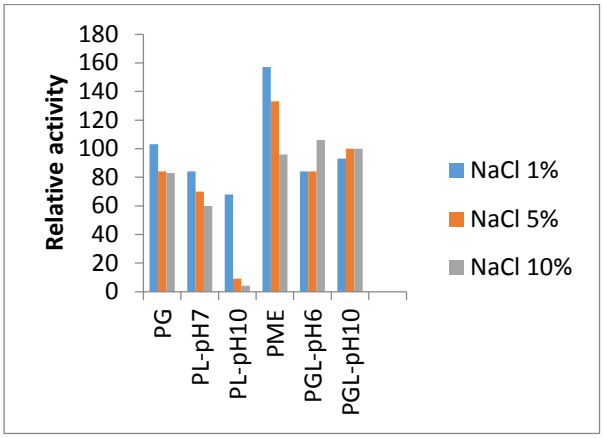

Figure 3. Effects of ionic strength.

Polygalacturonase (PG): 1% PGA and enzyme solutions containing NaCl in different concentrations were prepared. Activity measurements were performed under optimum conditions. Media without NaCl was used as a control group. The activity in the medium containing NaCl (1%) was determined to be the same as the control group. At 5% and 10% NaCl concentrations only 7% activity loss was observed (Figure 3).

Pectin lyase (PL): It was observed that there was 84% activity in the environment containing 1% NaCl at pH7, 70% activity in the environment containing 5% NaCl, and 60% activity in the environment containing 10% NaCl. At pH10, 68% activity was observed in the sample containing 1% NaCl, whereas in reactions containing 5% and 10% NaCl, the activity almost disappeared.

Pectate lyase (PGL): PGL activity was measured at pH6 and pH10 in reactions containing different NaCl concentrations. Although the activity was somewhat reduced in the sample containing 1% NaCl at both pH, values approaching the optimum activity were recorded in increasing NaCl concentrations (Figure 3).

Pectin methylesterase (PME): PME activity was measured in optimum conditions with different NaCl conditions. The activity was determined to be 157% in 1% NaCl, 133% in 5% NaCl and 96% in 10% NaCl (Figure 3).

3.3.2 Effect of organic solvents

Activities were measured in reaction media containing different organic solvents and under optimum conditions (Figure 4).

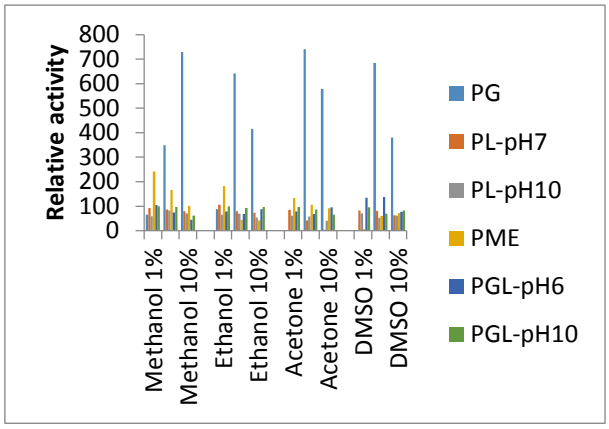

Figure 4. Effects of organic solvents.

Polygalacturonase (PG): 1%, 5% and 10% (v / v) methanol, ethanol, acetone and DMSO were added to the reaction medium to measure activity under optimum conditions. The group without organic solvent was used as a control. At a concentration of one percent, no significant loss of activity was observed in media containing methanol and ethanol, but no activity was recorded in media containing acetone and DMSO. In the medium containing five percent and ten percent organic solvents, an increase in activity was observed in all solvents compared to the control group (Figure 4).

Pectin lyase (PL): Since there were two different optimum pHs for PL, the effect of organic solvents on both pHs was investigated. It was determined that in the reactions containing 1% ethanol at pH 7, the relative activity increased by 6%. Loss of activity was observed in all other organic solvents at higher concentrations. It was shown that the enzyme was almost completely inhibited at a concentration of 10% of acetone (Fig. 4). In samples containing 1% and 10% methanol at pH10, 35% and 30% activity losses were observed, while 17% activity loss was observed in the environment containing 5% methanol. (Figure 4).

Pectate lyase (PGL): The effect of organic solvents on PGL activity was investigated at pH6 and pH10. It was observed that the activity decreased at pH6, at increasing concentrations of methanol. DMSO increased the activity at 1% and 5% concentrations and started to decrease at 10% concentration. Methanol also decreased the activity at increased concentrations. Ethanol and acetone reduced the activity at the concentrations of 1% and 5%, but led to a higher activity at 10% concentration. (Fig.4). Organic solvents did not significantly affect the activity at 1% concentration at pH10. Ethanol and DMSO reduced activity at 5%, but not at 10% concentration (Figure 4).

Pectin methylesterase (PME): The effect of organic solvents on PME activity at different concentrations was investigated under optimum conditions. While 1% methanol, ethanol, and acetone increased the activity, the activity appeared to decrease at higher concentrations. Surprisingly, however, DMSO inhibited the enzyme at 1% concentration, while increasing at higher concentrations (Figure 4).

3.3.3 Effects chemical reactifs

Activities were measured in reaction media containing different chemical reagents and under optimum conditions (Figure 5).

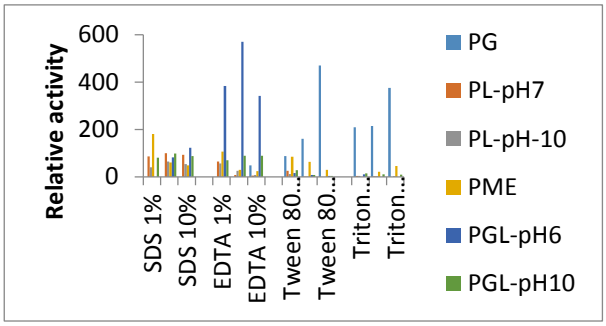

Figure 5. Effects of chemical reactifs.

Polygalacturonase (PG): Activities were measured at optimum conditions by adding SDS, EDTA, Tween-80 and Triton-X100 at 1%, 5% and 10% concentrations to the reaction medium. No activity was observed in samples containing SDS (1%) and EDTA (5%); however, increased activity was determined in reactions containing tween-80 and triton-X100. In samples containing ten percent chemical reagents, tween-80 and triton-X100 increased the activity. While EDTA inhibited the enzyme at 1% and 5% concentrations, a significant activity was observed at 10% concentration (Figure 5).

Pectin lyase (PL): As there were two different optimum pHs for PL, the effect of chemical reagents on both pHs was investigated. In the reactions containing 1% and 10% SDS at pH7, 14% and 7% activity loss was observed, and no activity loss was observed at 5% SDS. Other chemicals decreased the activity at increasing concentrations. While Triton X100 almost completely inhibited the activity at pH10, 5% SDS caused only 35% loss of activity (Figure 5).

Pectate lyase (PGL): PGL activity was measured at pH6 and pH10. EDTA and 10% SDS increased the activity at pH6 (Fig. 5) and caused only a 10% decrease at pH10 (Fig. 5). Other chemical reagents were found to almost inhibit PGL at both pH points.

Pectin methylesterase (PME): The effect of chemical reagents on PME activity at different concentrations was investigated under optimum conditions. It was determined that the activity increased at 1% concentrations of both SDS and EDTA, but decreased at increasing concentrations. At higher concentrations Tween-80 inhibited the enzyme, but the activity increased at increasing concentrations of triton-X100 and there remained a 45% activity at 10% concentration (Figure 5).

Some of the examples of research involving Bacillus members can be summarised as follows:

Al-Rajabi and Mahasneh (1999) have studied pectin and pectate lyase activities isolated from Bacillus polymyxa strain [16]. It has been reported that 1mM EDTA in pectate lyase activity causes 48% reduction in nitrilotriacetate and 35% and 53% in pectin and pectate lyase activity, respectively.

Kashyap et al. (2000) have purified pectinase from the DT7 strain of Bacillus sp., using gel filtration and ion exchange chromatography. It has been stated that pectinase activity increased in the presence of 100mM CaCl2 and mercaptoethanol and that EDTA decreased the activity by 17% [17].

Joo et al. (2003) have shown that ta protease from the Bacillus clausii I-52 strain was stable in the presence of non-ionic surfactants such as Triton-X-100 and Tween-20, but also in strong anionic surfactant such as SDS [18]. A serine protease of Bacillus clausii has been used for the production of food additives within the regulations of FAO / WHO common food standards program (2010).

Mei et al. (2013) have isolated a pectinase from Bacillus halodurans M29 strain and cloned in E. coli JM109. It was determined that EDTA decreased the activity of this pectinase [19].

A pectinase of Bacillus circulans has been purified (Raju and Divakar, 2013). This enzyme appeared to have a maximum activity in Tween-80, and Tween-20 and SDS also increased its activity [20].

Sassi et al. (2016) have purified a polygalacturonase from a mutant strain Penicillium occitanis CT1. This enzyme was found to be highly active in the presence of Triton X-100, DTT, β-mercaptoethanol, urea, iodoacetamide, and iodoacetic acid, whereas EDTA and SDS cause a 20% and 10% decrease in its relative activity [21].

Madu et al. (2016) have purified a 38 kDa pectinase from Bacillus licheniformis and they have measured its relative activity at 1mM, 5mM and 10mM concentrations of NaCl. They have reported 127%, 117% and 98% relative activities respectively. Here again the enzyme activity decreased in 1mM and 5mM EDTA to 77% and 59%, respectively [22].

In a previous study, it has been shown that the pectinolytic enzymes of *B. clausii* reside in the alkaline side of the pH spectra. Thus these enzymes could be used in textile processing, in the degumming of plant fibres, in the treatment of pectic wastewaters, and in paper making.

4. Conclusions

Although studies with other species of *Bacillus* genus are frequently encountered in the literature, there is little information on the pectinases of *Bacillus clausii*, especially on pectin- and pectate lyases. In addition, *Bacillus clausii* is a probiotic species [23-26]. This makes it more interesting to understand its extracellular enzyme content. This study aimed at the discovery of pectinolytic enzymes of this probiotic species.

The study showed that specific activities of the enzymes of interest could be identified and assessed in an extracellular protein mixture. Its results could have strong biotechnological implications in industrial processes where the use of purified enzymes are unnecessary. As the producing organism was a probiotic bacterium, its crude extracellular products could directly be used. The work shed some light on some of the industrial properties of four pectinolytic enzymes produced by one isolate of *Bacillus clausii*.

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Author's Contributions

Sevinç Berber: Performed the experiment and result analysis and helped in manuscript preparation.

Serap Çetinkaya: Conceived and designed research, conducted experiments, contributed new reagents or analytical tools, analyzed data, wrote the manuscript.

Ethics

There are no ethical issues after the publication of this manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

References

1. McNeil, M., Darvill, A.G., Fry, S.C. and Albersheim, P. 1984. Structure and Function of The Primary Cell Walls of Plants. *Annual Review of Biochemistry;* 53: 625-663.

2. Abbott, D.W. and Boraston, A.B. 2008. Structural Biology of Pectin Degradation by Enretobacteriaceae. *Microbiology and Molecular Biology Reviews;* 72: 301-316.

3. Vries, R.P. and Visser, J. 2001. Aspergillus Enzymes Involved in Degradation of Plant Cell Wall Polysaccharides. *Microbiology and Molecular Biology Reviews;* 65: 497-522.

4. Abbott, D.W. and Boraston, A.B**.** 2008. Structural Biology of Pectin Degradation by *Enretobacteriaceae*. *Microbiology and Molecular Biology Reviews*; 72: 301-316.

5. Khairnar, Y., Krishna, V.K., Boraste, A., Gupta, N., Trivedi, S., Patli, P., Gupta, G., Gupta, M., Jhadav, A., Mujapara, A., Joshi, B. and Mishra, D. 2009. Study of Pectinase Production in Submerged Fermentation Using Different Strains of *Aspergillus niger*. *International Journal of Microbiology Research*; 1: 13-17.

6. Hoondal, G.S., Tiwari, R.P., Tewari, R., Dahiya, N. and Beg, Q.K. 2002. Microbial Alkaline Pectinases and Their Industrial Applications: A Review. *Applied Microbiology Biotechnology*; 59: 409-418.

7. Kashyap, D.R., Vohra, P.K. Chopra, S. and Tewari, R. 2001. Applications of Pectinases in the Commercial Sector: A Review. *Bioresource Technology*; 77: 215-227.

8. Mojsov, K., 2012. Biotechnological applications of pectinases in textile processing and bioscouring of cotton fibers. *II International Conference Industrial Engineering and Environmental Protection Zrenjanin, Serbia*, 314-322.

9. Sharma, N., Rathore, M. and Sharma, M. 2012. Microbial Pectinase: Sources, Characterization and Applications. *Reviews in Environmental Science and Bio/Technology*; 12: 45-60.

10. Berber, S. 2018. Productıon, Isolatıon and Characterızatıon of Extracellular Pectinase from Soil Bacteria. Master's Thesis, *Cumhuriyet University Institute of Science;* Sivas, 21.

11. Berber, S. and Çetinkaya, S. 2020. An Isolate of *Bacillus clausii* Appears to Possess Four Distinct Pectinolytic Activities. *Iğdır University Journal of the Institute of Science;* 10, in press.

12. Kocabay, S. 2015. Producdution, Isolation and Characterization of Extracellular Amylase Enzyme from Gaıta Bacreria. Master's Thesis, *Cumhuriyet Üniversitesi Fen Bilimleri Enstitüsü*; Sivas, 96.

13. Whitaker, J.R. 1990. Microbial Pectolytic Enzymes. Microbial Enzymes and Biotechnology, Fogarty, W. M. ve Kelly, C. T. (Ed.), *Department of Industrial Microbiology, University College, Dublin*; 133-177,

14. Pickersgill, R.W. and Jenkins, J.A. 2003. The Structures and Active Sites of Pectinases. Advances in Pectin and Pectinase Research, Voragen, F., Schols, H. ve Visse, R. (Ed.), *Wageningen University, Netherlands*; 267-277.

15. Gummadi, S.N., Monaj, N. and Kumar, S. 2007. Structural and Biochemical Properties of Pectinases. Industrial Enzymes Structure, Function and Application, Polaina, J., MacCabe, A. P. (Ed.), *Instituto de Agroquimica y Technologia de Alimentos, Valencia*; 99-117.

16. Al-Rajabi, I.I. and Mahasneh, A.M. 1999. Partial Characterization of an Alkalophilic Extracellular Crude Pectinases From a *Bacillus polymyxa* Strain. *Qatar University Science Journal*; 18: 67-80.

17. Kashyap, D.R., Chandra, S., Kaul, A. and Tewari, R. 2000. Production, Purification and Characterization of Pectinase from a *Bacillus sp. DT7*. *World Journal of Microbiology and Biotechnology*; 16: 277-282.

18. Joo, H.S., Kumar, C.G., Park, G.C., Paik, S.R. and Chang, C.S. 2003. Oxidant and SDS-stable alkaline protease from *Bacillus clausii* I-52: Production and Some Properties. *Journal of Applied Microbiology*; 95: 267-272.

19. Mei, Y., Chen, Y., Zhai, R. and Liu, Y. 2013. Cloning, Purification and Biıchemical Properties of a thermostable Pectinase from *Bacillus halodurans M29*. *Journal of Molecular Catalysis B: Enzymatic*; 94: 77-81.

20. Raju, E.V.N. and Divakar, G. 2013. Production of Pectinase by Using *Bacillus circulans* Isolated From Dump Yards of Vegetable Wastes. *IJPSR*; 4: 2615-2622.

21. Sassi, A.H., Tounsi, H., Trigui-Lahiani, H., Bouzouita, R., Romdhane, Z.B. and Gargouri, A. 2016. A Low-Temperature Polygalacturonase from *P.occitanis*: Characterization and Application in Juice Clarification. *International Journal of Biological Macromolecules*; 91: 158-164.

22. Madu, O.J., Okonji, R.E., Torimiro, N. and Agboola, F.K. 2016. Purification and Characterization of Pectinase from *Bacillus licheniformis* obtained from a Cassava Waste Dump. *Journal of Advances in Biology*; 8: 1685-1695.

23. Ciffo, F. 1984. Determination of the spectrum of antibiotic resistance of "*Bacillus subtilis*" strains of enterogermina. *Chemioterapia;* 3: 45–52.

24. Cutting, S.M. 2011. *Bacillus* probiotics. *Food Microbiol.;* 28: 214–220.

25. Lippolis, R., Siciliano, R.A., Mazzeo, M.F., Abbrescia, A., Gnoni, A., Sardanelli, A.M. and Papa, S. 2013. Comparative secretome analysis of four isogenic Bacillus clausii probiotic strains. *Proteome Sci.;* 11: 28.

26. Urdaci, M., Bressollier, P. and Pinchuk, I. 2004. *Bacillus clausii* Probiotic Strains Antimicrobial and Immunodulatory Activities. *Journal of Clinical Gastroenterology*; 38: 86-90.