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Isolation of *Bacillus licheniformis* TT01 to apply it in Compost Production from Quail Manure

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A *Bacillus licheniformis* TT01 strain has been isolated from raw quail manure compost on the 25th day of the incubation. Studying on physiological characteristics showed that this isolate could grow best in the LB medium, pH from 7.0 to 7.5, at 40 °C for 15 to 25 hrs. *Bacillus licheniformis* TT01 showed a significant ability of producing extracellular enzymes including amylase, protease, cellulase and having strong antibacterial effect on *Salmonella typhi* ATCC 14028. This strain can be applied to improve compost producing from quail manure, enhance the role of microorganisms in composting as well as control pathogens in the manure.

Key words: amylase, *Bacillus licheniformis* TT01, cellulase, protease, quail manure, *Salmonella typhi* ATCC 14028, Vietnam.

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Raising quails has been receiving much attention from farmers working in the livestock industry in the Central Vietnam because of high economic benefits. However, a great of manures discharged from breeding farms brings negative effects on environment. Commonly, quail manure is treated by drying and used directly as a fertilizer. However, when fertilizing into anaerobic soils, non-degraded organic compounds in the manure undergo further degradation by microorganisms to release organic acids and toxic gas that contribute to soil acidification and consequently affect the crops. In addition, harmful microorganisms remaining in untreated manure can cause diseases to plants. Therefore, effective management of quail manure is urgent. One of the best methods for the agricultural sustainable development and ensuring human health and safety is the compost production from quail manure. To shorten the composting time, accelerate the composting process and improve the quality of compost, it is necessary to supplement efficient microorganisms.

Bacillus licheniformis is most commonly found in soil and on feathers of ground-dwelling birds [1]. This organism has been reported to produce some of the most important extracellular enzymes including amylase, protease, cellulase and lipase. It has been proved that *Bacillus licheniformis* is a producer of commercially feasible quantities of various industrial enzymes [2] Therefore, isolating these indigenous strains (including those from raw quail manure) and studying on their biological characteristics is essential for the application in many fields.

In this study, the main purpose was to isolate from quail manure the useful microorganism of *Bacillus licheniformis* which is highly adaptable to the climate conditions and geographic characteristics of the Central Vietnam to improve the quality of compost producing from quail manure and maximize the role of microorganisms in decomposing of organic substances, as well as to control pathogens in soil aimed at contributing to the creation of clean agricultural products for the human consumption. The morphological

Abbreviations: CMC, carboxymethyl cellulose; LB medium, Luria–Berthani medium; OD_{600}, optical density at wavelength of 600 nm.

characteristics of the isolated strain were studied, the taxonomical identification using the 16S rRNA gene analysis was performed, the phylogenic tree was built, the optimum conditions of growth were investigated, the antibacterial properties and the activities of some extracellular enzymes were studied.

EXPERIMENTAL

Isolation of bacteria

10 g manure samples from quail farming were collected, washed in 90 mL sterile water and serially diluted by 10^9 times. Three last dilution samples (0.1 mL) were cultured on nutrient LB agar plates at 40 °C for 24 h. After the growth started, a single loop full of bacteria colonies was transferred each 24 h onto another nutrient agar plates to isolate. The identification of the genus was based on the morphological and biochemical characteristics of the *Bacillus* species.

16S rRNA gene analysis and sequencing

The DNA extraction and sequencing of 16S rRNA gene was performed by the Sanger's method, after which the BLAST NCBI analysis was used and the phylogenic tree was built by the maximum likelihood method.

Study on effects of medium conditions on the isolate's growth

To activate the isolated strain, the cells were inoculated into a 250 mL Erlenmeyer flask containing 100 mL LB medium (adjusted to pH 6.0 and sterilized by autoclaving at 121 °C, 1 AT for 15 min), placed in a rotary shaker (180 rpm) and cultured for 12 h.

For fermentation, the culture was introduced up to the concentration of 1% (v/v) in the growth LB medium with different pH, 6.0, 6.5, 7.0, 7.5 or 8.0, and grown at 40 °C for 30 h. Then the selected pH values were combined with various temperatures including 25 °C, 30, 35, 40, 45, 50 °C to investigate the temperature effect. The cell growth was monitored by measuring OD_{600} on a spectrophotometer.

Screening of isolate for antibacterial activity

The antibacterial effect of the isolated strain was studied using *Salmonella typhi* ATCC 14028 as a test microorganism. The *B. licheniformis* and *S. typhi* strains were seeded in the same Petri dish symmetrically or perpendicularly and then incubated at 37 °C for 15 h. The antibacterial activity of *B. licheniformis* TT01 was seen as growth-inhibiting zones on the *S. typhi* ATCC 14028 strain lawn.

Screening of isolate for extracellular enzymes production

The screening of the alpha-amylase activity was based on the color development resulting from the iodine binding to starch [3–5]. The isolated strain was grown on 0.5% starch agar plate at 37 °C for 24 h. After the incubation, the strain was flooded with a iodine – potassium iodide solution for the detection of the alpha-amylase activity.

The protease production by the bacterial strain was screened on plates of agar supplemented with 5% NaCl and 1% casein (MNA). The plates were incubated overnight at 37 °C. The protease-producing strains were selected on the basis of based the zone of clearance [6].

Screening for extracellular cellulase production of the strain was carried out on agar plates containing CMC as a substrate [7, 8]. The cellulolytic activity was detected by staining of undigested CMC in the plate regions without the enzymatic activity, while the areas exposed to cellulase gave clear halos surrounding the sites of the enzyme application.

The procedure of composting was performed in cooperation with Dr. Bao An (Danang city, Vietnam) under the following conditions: height of composted mass, 1.4 m; width, 2.5 m; temperature 37 °C; humidity 35–40% and pH 7.5.

Statistical analysis

All experiments were conducted three times. The Excel software (2010) was used to analyze the obtained data.

RESULTS AND DISCUSSION

Bacteria isolation

Nine bacteria strains marked as TT01, TT02, TT03, TT04, TT05, TT06, TT07, TT08 and TT09 were obtained from pretreated quail manure. All of them could actively grow in the LB media at 40 °C. However, only the TT01 strain was obtained from the raw compost on the 25th to 30th days. Therefore, TT01 was selected for the identification and study on its biological characteristics in order to apply in composting.

TT01 was cultured in plates with LB agar at 40 °C for 24 h. It was established that the TT01 colonies are white, smooth and have a turbid film on the surface. The Gram-staining revealed that the strain under study is Gram-positive and rod-shaped (Fig. 1).

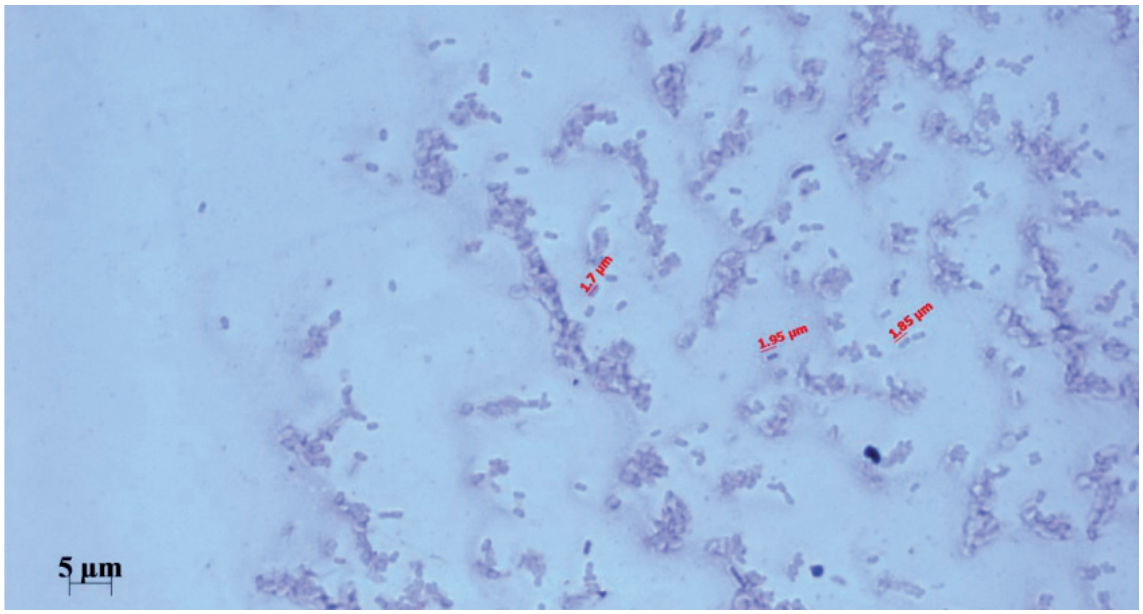


Fig. 1 Morphology of *Bacillus licheniformis* TT01 strain cells

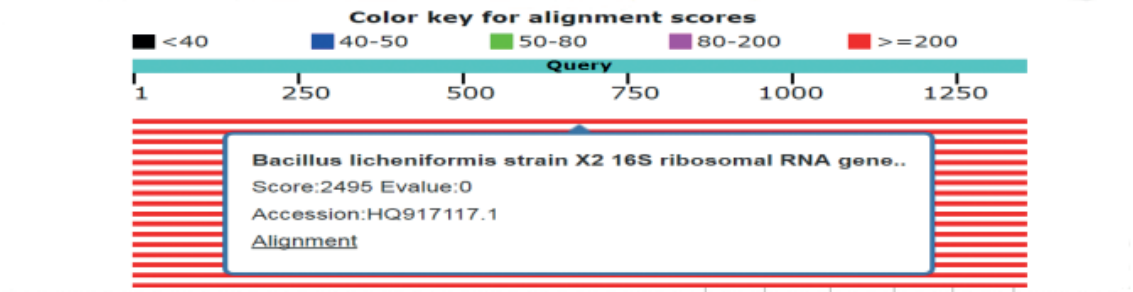
Gene analysis and sequencing

The extraction of DNA and sequencing of 16S rRNA gene of TT01 strain were performed by the Sanger method. The results of searching using BLAST NCBI are shown in Fig. 2.

The results shown in Fig. 2 and Fig. 3, in particular the alignment with the sequence of the *B. licheniformis* HQ917117.1 strain proved that the strain under study belongs to the *Bacillus* genus. The isolated strain was named *Bacillus licheniformis* TT01. To confirm the TT01 strain was aboriginal for the quail manure microbiota, a quail manure sample was autoclaved at 121 °C for 1 h, and then inoculated with the isolated TT01 strain. After 15 days, white spots appeared and were spread after 20 days. The same phenomenon was observed 25 days after the inoculation of the raw quail manure compost with the TT01 strain, which confirms that the *B. licheniformis* TT01 strain was a single one to be obtained from the raw compost on the 25th to 30th days.

Study on pH and temperature effect on TT01 strain growth

The data in Fig. 4 indicate that the TT01 strain can grow well on the LB medium within the pH range 7.0–7.5 at 40 °C. By the contrast, pH 6.0, 6.5, and 8.0, and temperature of 25 °C, 30, 35, 45 and 50 °C are not suitable for this strain growth. As compared to the result of Ngo et al. [9], *B. licheniformis* TT01 has less heat resistance ability. However, this strain is sufficiently tolerant to the composting



Description	Max score	Total score	Query cover	E value	Ident	Accession
Bacillus licheniformis strain X2 16S ribosomal RNA gene, partial sequence	2495	2495	100%	0.0	100%	HQ917117.1
Bacillus licheniformis strain CICC 10336 16S ribosomal RNA gene, partial sequence	2495	2495	100%	0.0	100%	GQ375245.1

Fig. 2. Results of BLAST NCBI searching of *Bacillus licheniformis* TT01 strain

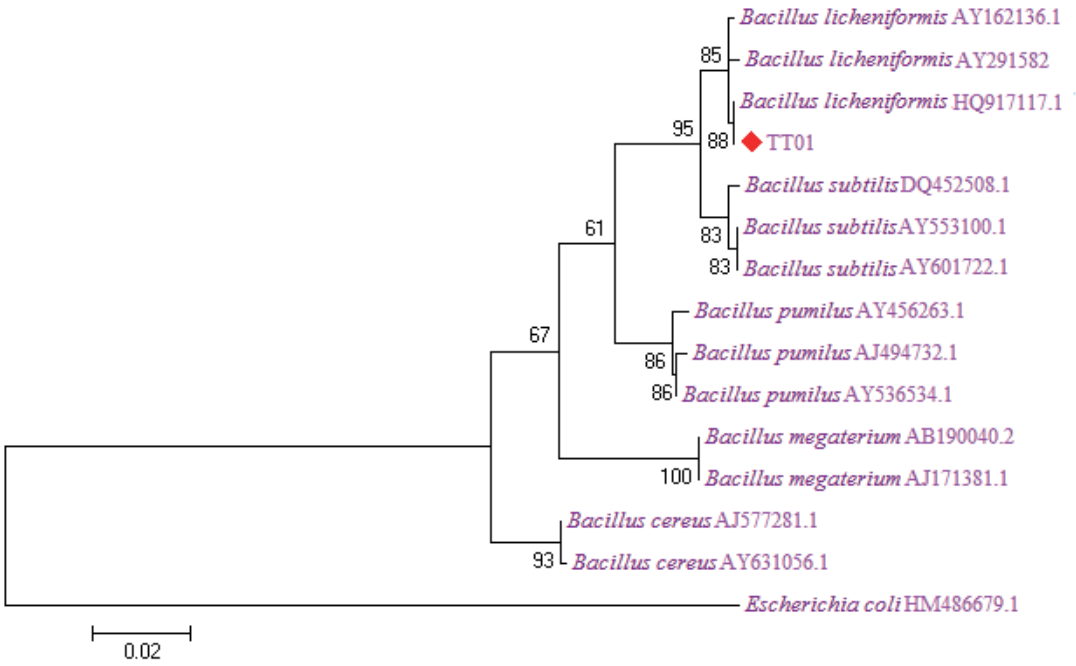


Fig. 3. Phylogenic tree for *Bacillus* genus strains (including *B. licheniformis* TT01 strain)

conditions and is capable of being involved in the process of producing a microbial organic fertilizer from quail manure. Within the first couple of days when heat is rapidly generated due to the metabolism of various microorganisms, *B. licheniformis* TT01 forms spores [9]. When the active composting stage passes, and the temperature decreases gradually, this strain seems to grow fast and produce useful enzymes to decompose organic materials in the quail manure. By the time composting is completed (from the 25th to 30th day), an organic fertilizer product also contains the beneficial microorganism of *B. licheniformis* TT01.

To analyze the dynamics of the cell growth under the optimum conditions (Fig. 4), the TT01 strain culture was sampled every day and its OD_{600} was measured. The results showed that the cell growth increased exponentially after 10h incubation, reached maximum after 20 h and then entered the stationary phase (Fig. 5).

Analysis of antibacterial activity of TT01 strain against *Salmonella typhi* ATCC 14028

After 25–30 days of incubation, the raw quail manure compost changed the color from golden yellow to dark yellow and was covered by a white layer.

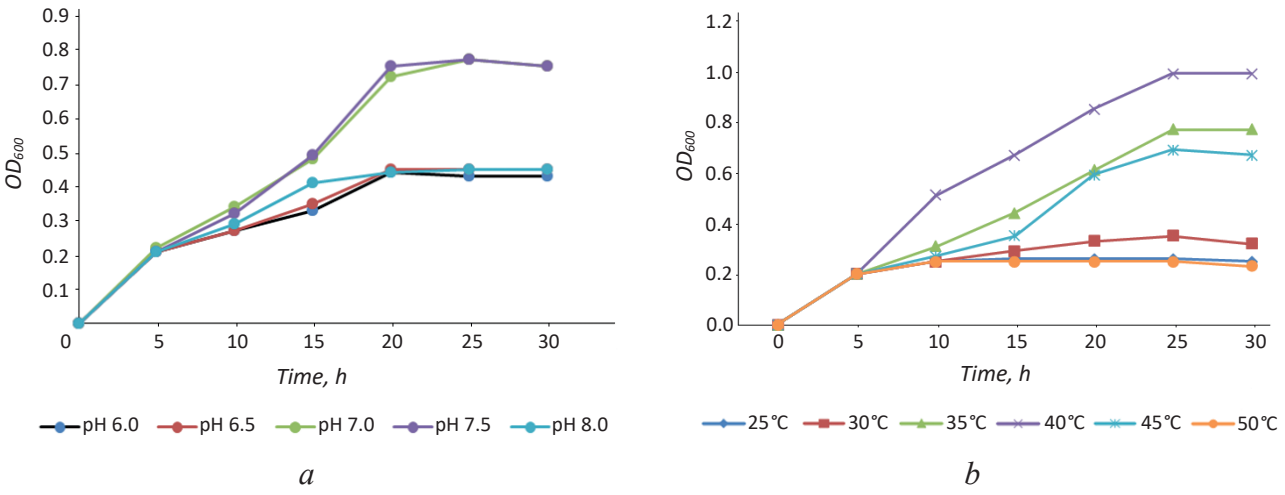


Fig. 4. Effect of pH (*a*) and temperature (*b*) on *B. licheniformis* TT01 strain growth

Only the *B. licheniformis* TT01 bacterial strain was isolated from this sample which indicates that the strain TT01 has a promising biological characteristic of inhibiting the growth of other microorganisms. To determine the rate of antagonism of the strain TT01 to *Salmonella typhi* ATCC 14028, the methods of half-agar plate and perpendicular culturing were used. Both methods showed that after the 15h incubation, *B. licheniformis* TT01 inhibited the growth and development of *S. typhi ATCC* 14028 strain (Fig. 6).

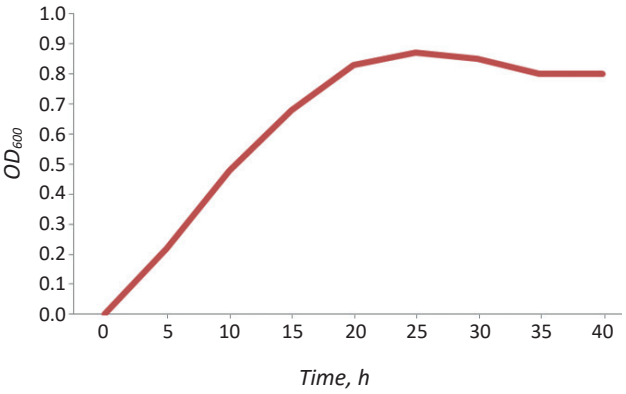


Fig. 5. Dynamics of *B. licheniformis* TT01 strain growth at optimum pH (7–7.5) and temperature (40 °C)

Analysis of capacity of TT01 strain to produce amylase, protease and cellulase

The strain TT01 was cultured in a shaken LB broth at 40 °C for 24 h. The supernatant was collected by centrifuging at 10000 g for 10 min to test the enzyme activity. The obtained results (Fig. 7) indicated that the strain TT01 has promising ability of producing all the three analyzed enzyme activities.

Fig. 7 shows that the TT01 strain isolated in this work from the raw quail compost has a useful capacity of producing enzymes including amylase, protease and cellulase.

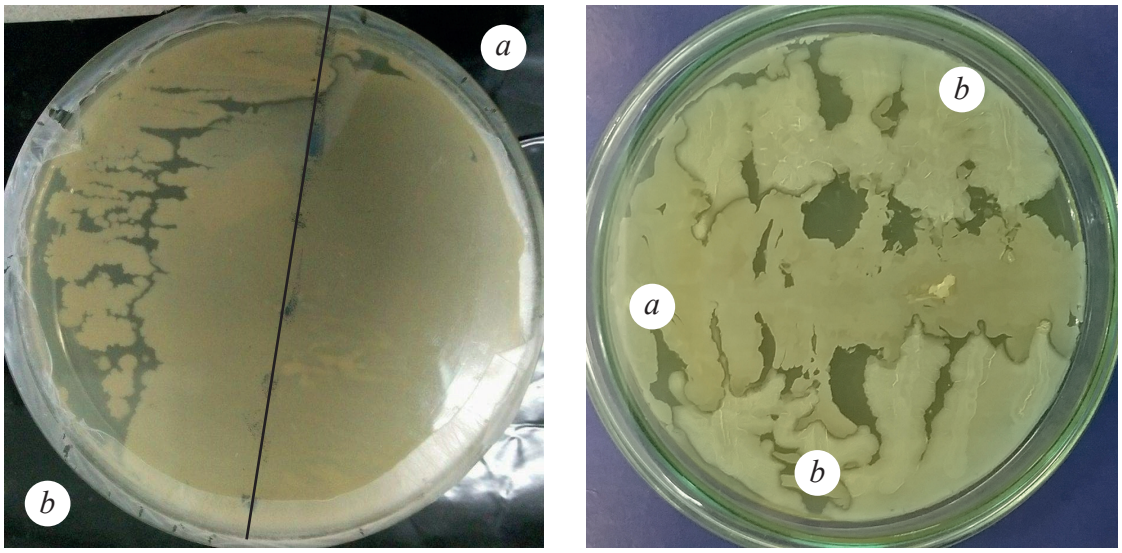


Fig. 6. Antibacterial activity of TT01 strain against *Salmonella typhi* ATCC 14028 measured by methods of half-agar plate (left) and perpendicular culturing (right): (*a*), *Bacillus licheniformis* TT01, (*b*) *Salmonella typhi* ATCC 14028

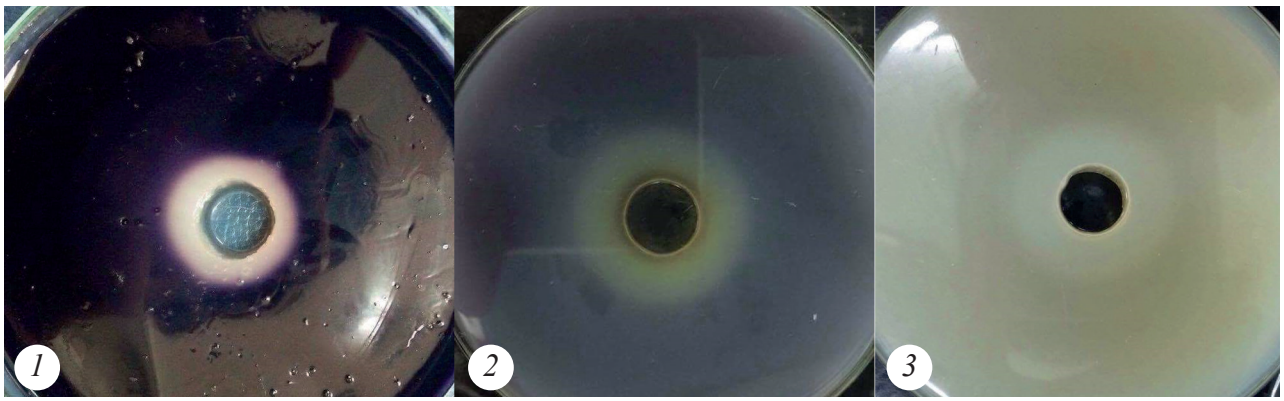


Fig. 7 The ability of TT01 strain enzymes to degrade specific substrates on agar plates: *1*, amylase; *2,* protease; and *3*, cellulase

Thus, a *Bacillus licheniformis* TT01 was isolated from raw quail manure compost and identified on the basis of 16S rRNA gene analysis. The pH and tempertature optimum for the strain growing was proved to be 7.0–7.5 and 40 °C, respectively. *Bacillus licheniformis* TT01 showed a significant ability of producing some extracellular enzymes including amylase, protease, cellulase and having a strong antibacterial effect on *Salmonella typhi* ATCC 14028.

Therefore, our data prove that *Bacillus licheniformis* TT01 has remarkable biological activities, and a further research is needed to investigate the introducing of this strain in the process of quail manure composting. It can result in a bioorganic fertilizer which reduces the risk of the environmental pollution and nutrient wasting in Quang Nam province, Da Nang city, in particular, as well as in the Central Vietnam and Highlands, in general.

REFERENCES

1. Burtt, E. H., Ichida J. M. Occurrence of feather-degrading bacilli in the plumage of birds. *Auk,* 1999, 116, 364–372.
2. Robert Rastall. Novel enzyme technology for food applications. Cambridge. Woodhead Publishing, 2014.
3. Fuwa H. A new method of microdetermination of amylase activity by the use of amylase as the substrate. *J. Biochem.,* 1954, 41, 583–603.
4. Violet M., Meunier J.C. Kinetic study of the irrversible thermal denaturation of *Bacillus licheniformis* α-amylase. *Biochem. J.* 1989, 263, 665–670.
5. Xiao Zhiz-Huang, Storms R., Tsang A. A quantitative starch–iodine method for measuring alpha-amylase and glucoamylase activities. *Analytical Biochem*., 2006, 35, 146–148.
6. Suganthi C., Mageswari A., Karthikeyan S., Anbalagan M., Sivakumar A., Gothandam K.M. Screening and optimization of protease production from a halotolerant *Bacillus licheniformis* isolated from saltern sediments. *J. Genetic Enginee. Biotechnol.,* 2013, 11(1), 47–52.
7. Dashtban M., Schraft H., Qin W. Fungal bioconversion of lignocellulosic residues; opportunities and perspectives. *Int. J. Biol., Sci.,* 2009, 5, 578–595.
8. Dashtban M., Maki M., Leung K.T., Mao C., Qin W. Cellulase activities in biomass conversion: measurement methods and comparison. *Crit. Rev. Biotechnol*., 2010, 3, 302–309. doi: 10.3109/07388551.2010.490938
9. Ngo Xuan Manh, Nguyen Thi Lam Doan, Vo Nhan Hau, Ngo Xuan Dung. selection of optimal culture conditions for Bacillus licheniformis (strain BCRP) to synthesize thermostable α-amylase. *Sci. Dev.* Part VI, 2008, 5, 460–466.