ISOLATION AND CHARACTERIZATION OF CELLULOSE-DEGRADING BACTERIA FROM THE SPENT MUSHROOM COMPOST

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ABSTRACT

The edible and medicinal mushroom industry in Vietnam is undergoing significant development. Mushrooms are not only a source of clean food but also contain pharmaceutical substances for disease prevention and treatment. Statistics have revealed that the production of 1 kg of mushrooms generates 5 - 6 kg of post-harvest substrates. These substrates contain various polysaccharides, including hemicellulose, cellulose, and residual mycelia. If left untreated, they could contribute to environmental pollution. However, with appropriate treatment, these post-harvest substrates can be transformed into bioproducts, enhancing soil quality and providing nutrients for crops. This study aims to isolate and characterize bacterial strains capable of synthesizing cellulase, an enzyme that breaks down cellulose, from post-harvest substrate compost. The mushroom samples were collected from the mushroom farms in Ha Noi city and Thai Binh province and a total of 44 bacteria strains were isolated. By the agar well diffusion method, 27 strains of bacteria exhibiting cellulase activity were obtained. Notably, the strains FG16 and FG18 had the highest cellulase activity (0.253 U/ml and 1.137 U/ml), respectively. Therefore, these two strains were selected for further experiments. The optimal conditions for cellulase synthesis by strains FG16 and FG18 were found to be as follows: 1.0% and 0.5% CMC added to the culture medium, pH: 5.0 and 7.0, temperatures: 40°C and 45°C, with the raw enzyme collected after 24 hours. Both strains FG16 and FG18 are bacilli, gram positive, positive for MR (Methyl Red), VP (Voges Proskauer) reactions and capable of biosynthesizing some extracellular enzymes such as amylase, chitinase and protease. Based on compairing nucleotide 16S rRNA, strain FG18 closed to strain *Bacillus subtilis* NR112629.1, thus it was called *Bacillus subtilis* FG18

Keywords: *Mushroom compost, cellulose-degrading bacteria, cellulase, Bacillus subtilis.*

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1. INTRODUCTION

Vietnam is an agricultural country, every year the quantity of agricultural by-products reaches millions of tonnes. These by-products are good materials for mushroom cultivation and include rice straws, corn leaves, stems, grasses, etc. The total mushroom production of Vietnam is ranked 9th in the region, equal to 0.3% of China's mushroom production and 0.23% of the world's total mushroom production. The materials for mushroom cultivation are very rich in lignocellulose. Cellulose is the most abundant renewable organic molecule in the biosphere with an estimated annual yield of 4.0 x 10^9 tons. Cellulose is a biological polymer made up of thousands of D-glucose units linked together by β-1,4 glycoside bridges [1]. A large amount of lignocellulose waste is generated through agricultural, forestry and testing processes, especially from agricultural industries such as paper production, textiles, mining and wood processing industries.

However, plant lignocellulose biomass is considered to be biodegradable "waste" and it could be converted into valuable products such as biofuels, chemicals and cheap sources of energy for fermentation, animal food production and human nutrients. The conversion of lignocellulose compounds into fermentable sugars through organism-derived cellulase has been considered as a feasible process and provides potential products to

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minimize the use of fuel, thus, reducing environmental pollution [2]. Raw materials used in mushroom production are often by-products from agro-forestry production such as straw, corn stalks, cotton and sawdust. The production of 1 kg of mushroom would release 5 - 6 kg of post - harvest substrate.

This substrate contains many lignocellulose compounds, nutrients produced when mycelium grows, especially mycelia remaining in the substrate. This is a very good nutrition source for microorganisms to use. Without treatment, this amount of waste would be degraded causing environmental pollution, and negatively affecting the lives of people and farms producing mushrooms because the organic matter would degrade causing water pollution, also spreading a foul smell during decomposition causing air pollution. If it is treated properly, the post-harvest substrate would be converted into bioproducts for not only improving soil quality but also supplying proper nutrition to plants. To solve this problem, microbial strains with strong cellulose-degrading ability are required. Therefore, the objective of this study was to isolate and screen strains of microorganisms that possess cellulose degradation activity to create an initial microbial source for future applied research.

2. MATERIALS AND METHODS

2.1. Material

The bacterial strains using in this article was isolated from posy-harvested mushroom substrate.

Post-harvest substrate samples were collected from Fargreen mushroom farm in Hung Dung commune, Hung Ha district, Thai Binh province and Van Giang mushroom center, located in Lien Nghia commune, Van Giang district, Hung Yen province. Samples were kept in sealed plastic bags and brought immediately to the laboratory for isolation.

2.2. Media

Isolation and maintenance medium for cellulolytic bacteria (g/l) (cellulose agar media): 0.5 g of KH_2PO_4, 0.25 of $MgSO_4$, 2 g of cellulose, 15 g of agar and 2 g of gelatin dissolved in 1000 ml of distilled water at pH 6.8 - 7.2.

LB medium (Luria Bertani): 5.0 g of yeast extract, 10.0 g of peptone and 10.0 g of NaCl, dissolved in 1000 ml of distilled water at pH 7.0 - 7.2.

2.3. Tools and equipment

Tools and equipment used in this study belong to the laboratory Microbial Department – Faculty of Biotechnology – Vietnam National University of Agriculture: Autoclave, technical scale, analytical scale, optical microscope, shaking machine, incubator, oven, pipettes, refrigerator, microwave and pH meter.

2.4. Methods

2.4.1. Methods of isolation, purification

The collected samples were processed and isolation of microorganism strains was conducted following the previously described by Pratima *et al.* (2011) [3]. Ten (10 g) of each sample was mixed thoroughly with 90 ml of sterile distilled water, the solution obtained had a dilution concentration of 10^{-1}. 1 ml of solution at 10^{-1} dilution concentration was taken and diluted with 9 ml of sterile distilled water to make the solution at 10^{-2} dilution concentration. The process of dilution was continued until the solution at 10^{-8} dilution concentration was obtained. Bacterial isolation was carried out on selective media. Pipette was used to take 100 µl of diluted solution at different concentrations (10^{-5}, 10^{-6} and 10^{-7}) and spread on the surface of the agar dish. Petri dishes containing inoculated samples were placed in an incubator at 30^0C, after 2 days colonies were collected. Single colonies were streaked on Petri dishes containing agar and kept in an incubator at 30^oC.

2.4.2. Cellulose hydrolysis capacity of isolated bacterial strains

The bacterial strains were checked by agar well diffusion method on agar plates described by Vo Van Phuoc Que and Cao Ngoc Diep (2011) [4]. Experiments were performed on LB medium-added carboxymethyl cellulose (CMC). Isolated bacterial strains were spotted on plates, and incubated for 2 days at 30˚C. The agar plates were then dyed with Lugol solution. CMC hydrolyzing bacteria would create a colorless area around their colony (halo). Formula to calculate hydrolysis ability (D):

D = [(Halo diameter - Colony diameter)/Halo diameter] x 100

Determination of cellulase activity by quantification of reducing sugar with DNS reagent

Cellulase activity was determined accurately based on the amount of reducing sugar formed after reaction by the spectrophotometric method [5]. Cellulase enzyme acts on the CMC molecule and

releases reducing sugar molecules, glucose, into the culture medium. Reagent 3,5-Dinitrosalicylic acid (DNS or DNSA) is yellow, when reacting with reducing sugar, DNS is reduced to 3-amino-5-nitrosalicylic acid (reddish brown) which is capable of absorbing strong light at wavelength 540 nm. The color intensity of the reaction mixture is directly proportional to glucose concentration in the medium. Based on the standard curve of pure glucose with the DNS reagent, the sugar concentration of samples could be calculated.

Effect of cultural conditions on the cellulase-producing ability of selected bacterial strains

Effect of incubation time

Selected bacterial strains were cultures in a liquid medium, shaken for 120 rounds/minute, at 30°C. After 24, 48, 72 and 96 hours, culture media were centrifuged and the raw enzyme was collected. The experiment was repeated 3 times.

Effect of induced substrate concentration

Liquid media-supplied induced substrate (CMC) with different concentrations: 0, 0.5, 1, 1.5 and 2% were prepared. Selected bacterial strains were cultures in liquid medium, shaken for 120 rounds/minute, at 30°C for the optimal culture time surveyed from the previous experiments. After that, culture media were centrifuged and the raw enzyme was collected to determine cellulose activity. Since then, optimal substrate concentration was determined. The experiment was repeated 3 times.

Effect of culture temperature

Culture medium with the optimal substrate concentration as examined above was prepared. Selected strains were grown in the liquid medium with appropriate substrate concentration, and shaken for 120 rounds/minute for optimal culture time, at different temperatures: 25, 30, 35, 40 and 45°C. Culture media were centrifuged and raw enzyme was collected to determine cellulase activity. Since then, the optimal temperature was determined. The experiment was repeated 3 times.

Effect of pH

Selected strains were grown in the liquid medium added substrate with optimal concentration, at optimal temperature as investigated, shaken for 120 rounds/minute. NaOH 1N and HCl 1N were used to adjust initial pH of the culture medium to reach different pH concentrations: 3, 4, 5, 6, 7, 8, 9, 10 and 11, respectively.

Selected strains were grown in the liquid medium, and shaken for 120 rounds/minute at the optimal temperature of each strain during the optimal culture time as studied above. Culture media were centrifuged and raw enzyme was collected to determine cellulase activity. Since then, optimal pH was determined. The experiment was repeated 3 times.

Effect of carbon source

Various induced substances including grind husk, dextrin, starch, chitin, xylose, lactose, fructose, D-glucose, mannitol, maltose, sorbitol and CMC were used. pH was adjusted to the optimal value after adding substrates. Selected strains were grown in the liquid medium, and shaken for 120 rounds/minute at optimal temperature and time as investigated in the above experiments. Culture media were centrifuged and raw enzyme was collected to determine cellulase activity. Since then, the optimal carbon source was determined. The experiment was repeated 3 times.

Investigating the ability to degrade print paper of bacterial strains

Strains of bacteria with the strongest CMC hydrolysis ability were selected, and cultured in 50 ml of liquid medium containing 0.1 g of finely ground print paper. After 7 days of culture, paper degrading ability was determined based on the loss of dry print paper. Paper degrading ability was calculated following the formula as below [4].

[(Initial weight – Post weight)/Initial weight] x 100

Similar experiments were conducted to evaluate the ability to degrade straw of bacterial strains which were able to degrade print paper the best. 1.0 g of dry straw was soaked in 100 ml of liquid medium. After 10 days of culture, degrading ability was assessed based on the loss of dry straw. Degrading ability was calculated following the formula as below:

[(Initial weight – Post weight)/Initial weight] x 100

All experimental results were analysed by Excel software vesion 2016.

2.4.3. Molecular identification of the strain FG18

The colony of the strain FG18 was sent to the Phu Sa company to identify based on comparing the nucleotide sequence of the 16S rRNA from the strain FG18 with nucleotide sequences of 16S rRNA of other bacterial strains, deposited on GeneBank (https://blast. ncbi. nlm. nih.gov/Blast. cgi). The phylogenetic tree was conducted by using MEGA 7 software.

3. RESULTS AND DISCUSSION

3.1. Isolation of cellulose-degrading bacteria

Post-harvest mushroom substrate samples were spread on isolation medium supplied with carbon source as CMC. After two days, a preliminary assessment of CMC degrading ability was based on the ability to create a bright ring around the colony. From different post-harvest substrate samples, 44 bacterial strains were isolated, of which 26 strains possessed cellulose-degrading ability with hydrolysis ability from 23.08 to 88.24% (Figure 1 and table 1).

From the above results, we selected two strains of bacteria with the strongest hydrolysis ability FG16 (88.24%) and FG18 (71.43%) to continue further research.

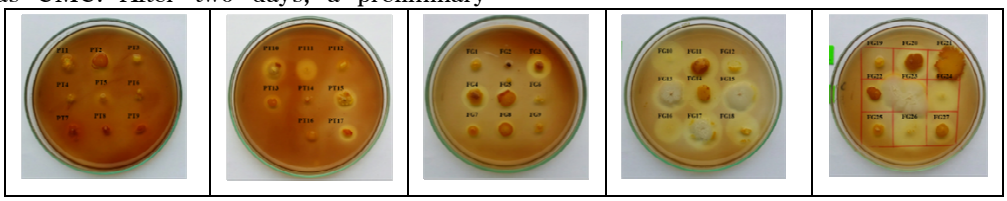

Figure 1. CMC hydrolysis ring created by bacterial strains

Table 1. CMC hydrolysis ability of bacterial strains

Bacterial strain	D	d	Hydrolysis ability (%)	Bacterial strain	D	d	Hydrolysis ability (%)
FG16	1,7	0,2	88.24	FG11	2.2	1.2	45.45
FG18	2.1	0.6	71.43	FG4	1.8	1	44.44
FG3	1.7	0.6	64.71	FG17	2.3	1.3	43.48
FG14	2.2	0.8	63.64	FG21	2.5	1.5	40.00
PT11	1.3	0.5	61.54	FG26	2.7	1.7	37.04
FG10	2	0.8	60.00	FG25	1.1	0.7	36.36
FG24	2.2	0.9	59.09	FG13	2.5	1.6	36.00
FG27	1.8	0.8	55.56	PT15	1.4	0.9	35.71
PT13	1.1	0.5	54.55	FG22	1.2	0.8	33.33
PT17	1.1	0.5	54.55	FG7	1	0.7	30.00
PT10	1.5	0.7	53.33	FG15	2.4	1.8	25.00
FG12	1.7	0.8	52.94	FG5	1.3	1	23.08
FG20	2.4	1.3	45.83	FG8	1.3	1	23.08

D - hydrolysis ring diameter (mm); d - colony diameter

3.2. Effect of culture conditions on cellulase synthesis ability

Effect of incubation time on cellulase activity

Two selected strains were grown in a liquid medium, and shaken for 120 rpm at 30°C in LB broth to investigate the ability to produce cellulase after 24, 48, 72 and 96 hours of culture. OD_{540} value was measured to determine the optimal culture time period for selected bacteria to show the highest activity. The result is shown in figure 2.

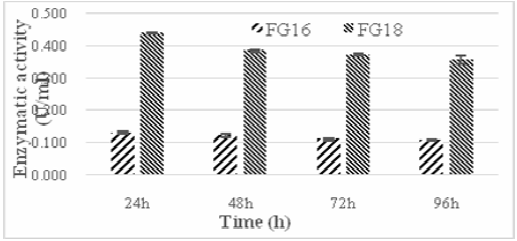

Figure 2. Effect of culture time on cellulase activity

Both studied bacterial strains showed the highest cellulase activity after 24 hours of culture.

Cellulase activity after 48 hours of culture decreased and continued to decrease after 72 and 96 hours. The reason was due to prolonged culture time, nutrient source in the medium was exhausted, and the accumulation of metabolic products would change the initial favorable factors of the environment such as temperature and pH, sometimes accumulating toxins harmful to cells. This would affect the growth of the studied bacterial strain as well as the enzyme production and activity. Farjana Islam and Narayan Roy (2018) when investigating the effect of culture time on the cellulase activity of *Paenibacillus* sp. C1 showed the highest cellulase activity after 24 hours of culture [6]. Our results were similar to that research.

Based on the experimental results, we selected the optimal culture time for the production of cellulase in selected bacterial strains as 24 hours for subsequent experiments.

Effect of concentration of induced substrate on cellulase activity (induced substrate was CMC)

CMC is both a source of carbon nutrition and an inducer for bacteria to produce cellulase. The presence of CMC in the culture medium is useful for cellulase production. Therefore, we studied the effect of CMC concentration in the culture medium on the cellulase activity of selected strains.

Two selected bacterial strains were cultured in LB broth supplemented with CMC substrate at different concentrations: 0, 0.5, 1, 1.5 and 2%, shaken for 120 rounds/minute at 30°C. After 24 hours, the raw enzyme was collected and cellulase activity was determined. The results are shown in figure 3.

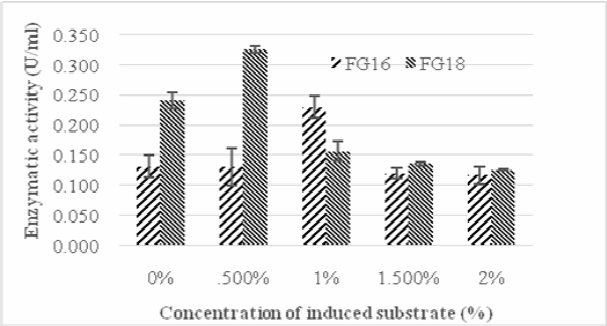

Figure 3. Effect of induced substrate concentration on cellulase activity

The results showed that the highest cellulase activity of strain FG16 was at 1.0% of CMC and strain FG18 was at 0.5% of CMC. When the concentration of induced CMC was increased (1.5%, 2%), cellulase activity decreased. Thus, if the amount of induced substrate (namely CMC) in the culture medium was too high, the growth phase would last longer, the final and secondary products would accumulate more, thus inhibiting the synthesis of cellulose. Our experimental result about strain FG16 were quite similar to the study of Farjana Islam and Narayan Roy (2018) when they investigated the effect of induced substrate concentration (CMC) on the cellulase activity of strain *Paenibacillus* sp. C1, the cellulase activity of this strain was highest at 1.0% of CMC substrate [6]. The research about strain FG18 was similar to the study of Faiz Rasul *et al.* (2015) [7] on strain SM-3M when 0.5% of the induced substrate was applied, cellulase activity was the highest. Therefore, we chose 0.5% of CMC substrate as the optimal substrate concentration for strain FG16 and 1% of CMC substrate for strain FG18 to conduct subsequent experiments.

Effect of temperature on cellulase activity

The culture temperature is a characteristic of the organism and it has a profound effect on the yield and duration of the enzyme synthesis phase [8]. In the range of temperature when the enzyme may exist, when the temperature is increased 10°C, the reaction rate increases twice. When the temperature is too high or too low, it affects the growth of bacteria and the process of cellulase biosynthesis. Therefore, we experimented to determine the effect of temperature on cellulase activity.

Two selected bacterial strains were cultured in LB broth containing 1.0% of CMC substrate for strain FG16 and 0.5% of CMC substrate for strain FG18, shaken for 120 rounds/minute at different temperatures 25, 30, 35, 40, 45 and 50°C. After 24 hours, the raw enzyme was collected and cellulase activity was determined. The results are shown in figure 4.

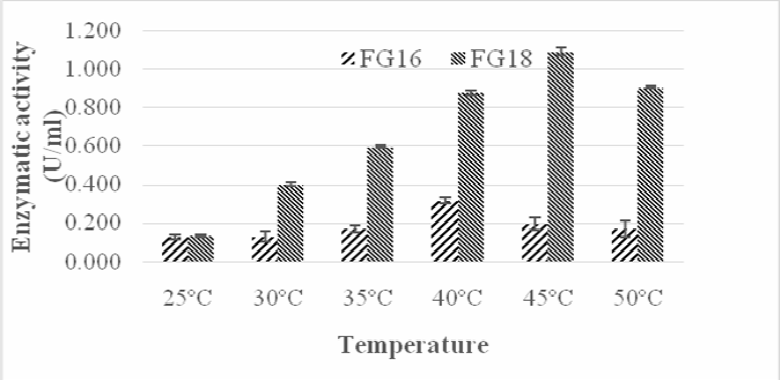

Figure 4. Effect of temperature on cellulase activity

Enzyme's nature is protein, when the temperature is too high or too low, the ability of enzyme biosynthesis and activity of enzyme would be influenced. From the diagram above, the cellulase activity of strain FG16 and FG18 reached the highest values at 40°C and 45°C, respectively.

The result of strain FG16 was similar to the study of Shilpa Lokhande and Pethe AS (2017) [9] about studying the effect of temperature on cellulase activity of bacterial strain *Bacillus thuringiensis*, showing that at 40°C, cellulase activity was the strongest. In the study of Vipul Verma *et al.* (2012), *Bacillus* sp. showed strong cellulase activity when cultured at 45°C. Therefore our result of strain FG18 was quite similar to this study [10].

Effect of pH on cellulase activity

Enzymatic activity strongly depends on pH. pH affects the level of ionization of substrate, the level of ionization of enzymes and the center of enzyme activity, enzyme-substrate complexes and the stability of enzyme proteins [11]. Thus, we chose the pH range from 3 to 11 to do this experiment.

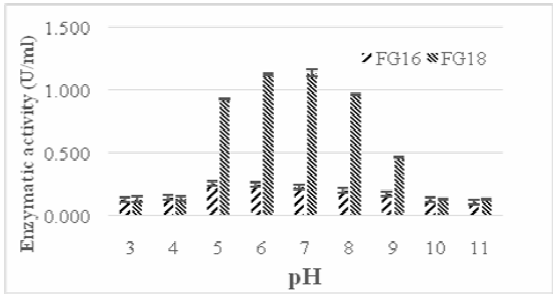

Figure 5. Effect of pH on cellulase activity

Two bacterial strains were cultured in LB broth added 1.0% of CMC as substrate at 40°C for strain FG16, 0.5% of CMC for strain FG18 at 45°C, shaken for 120 rounds/minute at different pH 3, 4, 5, 6, 7, 8, 9, 10 and 11. After 24 hours of culture, raw enzyme was collected and cellulase activity was determined. The results are shown in figure 5.

Cellulase enzymatic activity of two bacterial strains FG16 and FG18 expressed at different pH values, but it was highest at pH 5 and 7, respectively. Therefore, pH of the culture medium greatly affects cellulase biosynthesis ability. Vipul Verma *et al.* (2012), when studying strain *Bacillus* sp., also reported that cellulase activity was the highest at pH 7 [10]. Meanwhile, Bajaj *et al.* (2009) concluded that the cellulase activity of strain M-9 reached the highest value at pH 5 [12]. Our results were similar to previous studies. Therefore, we chose pH 5 and 7 for our two strains FG16 and FG18, respectively, for further experiments.

Effect of carbon source on cellulase activity

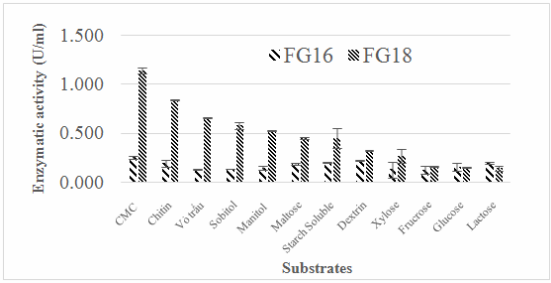

Figure 6. Effect of induction source on cellulase activity

Substrates are substances that have the ability to incorporate into the active center of the enzyme. Induced substrates are the substances that enzymes participating in the reaction catalyze degradation or metabolism of those substrates. These substrates are capable of stimulating the ability to synthesize corresponding induced enzymes. Cellulase is an induced or structural enzyme. In addition, in culture media of enzyme-synthesizing microorganisms, the

addition of induced substrates would stimulate microbial enzyme biosynthesis. Therefore, different substrate sources influence enzymatic activity and finding an appropriate source of induced substrate is of great importance.

To find the best-induced substrate for cellulase synthesis, bacterial strain FG16 was cultured at 40°C in LB broth added 1% of different substrate sources and strain FG18 was grown at 45°C in the medium supplied with 0.5% of different substrates. Both strains were shaken for 120 rounds/minute. Different substrates included CMC, grind husk, dextrin, starch, chitin, glucose, fructose, lactose, sorbitol, xylose, maltose and mannitol. After 24 hours of culturing, the raw enzyme was collected and cellulase activity was determined. The results are shown in figure 6.

Various induced substrates of different origins such as CMC, grind husk, dextrin, starch, chitin, glucose, fructose, lactose, sorbitol, xylose, maltose and mannitol showed different effects on the cellulase activity of two bacterial strains. CMC was the best source of induced substrate for the production of cellulase e in both studied strains.

Among different substrates, CMC had the highest ability to stimulate cellulase production [6]. Therefore, our result was similar to previous research. Based on this, we chose CMC as the induced substrate.

Investigating the ability to degrade print paper of bacterial strains

Table 2. Ability to degrade straw and print paper of two bacterial strains FG16 and FG18

Bacterial strain	Print paper degrading ability after 7 days (%)	Straw degrading ability after 10 days (%)
FG16	32.43	18.42
FG18	39.83	19.85

For straw, two strains of bacteria also showed good degrading ability. After 10 days, the degradation was very clear, the ability to degrade straw was 18.42% and 19.85% for FG16 and FG18 (Table 2).

Some results of biochemical tests of FG16 and FG18 are presented in table 3.

Table 3. Results of biochemical tests

Bacterial strain	FG16	FG18
MR reaction	-	-
VP reaction	+	+
Siderophore production	+	+
Chitinase production	+	+
Protease production	+	+
Citrate reaction	+	+

3.3. Molecular identification of the strain FG18

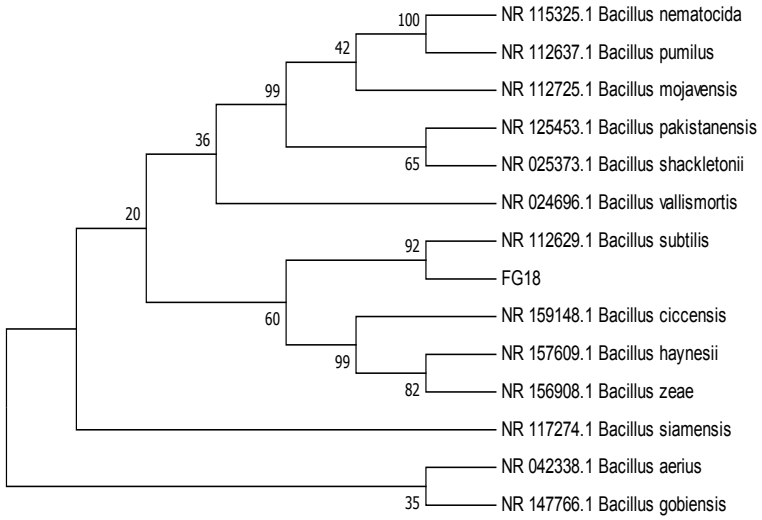

Figure 7. Phylogenetic tree of strain FG18

16s rRNA gene of strain FG18 was sequenced at Phu Sa company and compared with the 16s rRNA sequence of bacteria on the gene bank by BLAST software. The result showed that the 16s rRNA sequence of strain FG18 was 99% similar to strain *Bacillus subtilis*. Therefore this strain was named as *Bacillus subtilis* FG18.

4. CONCLUSION

After growing mushrooms on substrate samples, we isolated 26 strains that possessed cellulose - degrading ability with hydrolysis ability ranging from 23.08 to 88.24% and selected strain FG16 and FG18 with the highest cellulase activity, 0.253 U/ml and 1.137 U/ml, respectively. These two strains were able to degrade straw and paper.

Strain FG16 synthesized cellulase enzyme most strongly when it was cultured in LB broth containing 1.0% of CMC substrate, at pH 5, 40°C, shaken for 120 rounds/min for 24 hours. FG16 was capable of degrading print paper and straw.

Strain FG18 was able to degrade straw and paper. FG18 synthesized cellulase enzyme most strongly when cultured in LB broth containing 0.5% of chitin as substrate, at pH 7 and 45°C for 24 hours. This strain was 99% homologous to the strain *Bacillus subtilis* NR112629.1, thus it was called *Bacillus subtilis* FG18.

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