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Study on Selection of *Bacillus* Sp. Strain Has Active Carbon Metabolism for Peat Treatment

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ABSTRACT

The objective of selecting strains of *Bacillus* sp. has high activity in carbon metabolism for peat treatment, firstly focusing on the ability to degrade lignin, cellulose, phosphorus and protein. From the gene pool, by surveying the microbial strains capable of degrading carbon-rich substrates including lignin, cellulose, insoluble phosphate, proteolytic, we have identified two *Bacillus* sp. strains contain *Bacillus polyfermenticus* and *Bacillus subtilis* strains have strong ability to metabolize carbon-rich substrates and decompose phosphorus in peat. The *Bacillus polyfermenticus* strain had cellulose, lignin, organophosphorus and starch-degrading ring diameters of 41, 40, 23 and 38 mm, respectively. The *Bacillus subtilis* strain had cellulose, lignin, organophosphorus and starch-degrading ring diameters of 40, 41, 24 and 29 mm, respectively. In addition, the content of humic acids formed after 48 hours of fermentation increased 2.5 times compared to the original without the addition of *Bacillus polyfermenticus* strain and increased 2.8 times compared to the original without the addition of *Bacillus subtilis* strain. Therefore, two strains of *Bacillus* sp. selected with high efficiency in producing humic acid products. At the same time, the selected microbial strains have a high biosafety level (level of 1, the safest level in the biosafety scale).

KEYWORDS: *Bacillus* sp, carbon metabolism, peat treatment

1. INTRODUCTION

The tropical conditions of our country are suitable for the formation of peatland. Vietnam's peat is distributed mostly in provinces and cities with a total peatland area of about 53 thousand hectares nationwide. However, the South of Vietnam has hot and humid conditions, with more rain, and more rivers than the North, so peat deposits with large reserves are concentrated mainly in the South; specifically the provinces in the Mekong Delta. In which, U Minh Thuong peat mine (in Kien Giang province) and U Minh Ha peat mine (in Ca Mau province) account for the largest reserves, about 60 percent of the total amount of peat in the country.

Our country's peat has very special characteristics: high carbon content, high humus content, high porosity, high water and micro-mineral retention capacity... Therefore, our country's peat can be used in many fields. However, the present inefficient exploitation and use of peatland not only does not bring many economic benefits, but also increases environmental pollution risk. Moreover, the widespread exploitation of peat makes peat mines damaged, affecting ecology, climate, flora, fauna...

Among microorganisms with high ability to produce extracellular enzymes, bacteria of the genus *Bacillus* are well known because they can use a variety of substrate sources to increase biomass and grow, so *Bacillus* has been being applied in many different fields of life in general and in peat treatment in particular in Vietnam. Due to their high extracellular enzyme activity, various common Bacillus species such as *B. subtilis, B. licheniformis, B. megaterium*, etc., when added to peat, have demonstrated higher organic matter decomposition efficiency. compared with naturally occurring strains in peat.

For that reason, we conducted the research project "***Study on selection of Bacillus sp strain. has active carbon metabolism for peat treatment***" with the aim of selecting strains of Bacillus sp. has high activity in carbon metabolism for peat treatment, firstly focusing on the ability to degrade lignin, cellulose, phosphorus and protein.

2. MATERIAL AND METHODS

2.1. Material

- *Bacillus* sp. strains were used in the selection study:

Bacillus polyfermenticus, Bacillus subtilis, Bacillus mycoides, Bacillus pumilus, Bacillus licheniformic.

- The carbon-rich raw material used in the study is a peat sample taken from a coal mine with the following composition: Organic content (OM) 55%, Hemicellulose 24.1%, Cellulose 17.1%, Lignin 18%, Compound containing nitrogen 0.8%, Humic acids 5%, pH 5.8.

- Chemicals and tools:

+ Chemicals used in the experiment: glucose, starch, CMC, agar, K_2HPO_4, peptone, KCl, NaCl, NaOH, malt extract, alkaline lignin, $MgSO_4.7H_2O$, $FeSO_4.7H_2O$, lugol, DNS reagent, distilled water …

+ Laboratory equipment: sterile autoclave, triangle flask, test tube, peptri dish, alcohol lamp, chang rod, inoculation stick, litmus paper, grease cotton, ...

2.2. Methods

2.2.1. The method of data collection

- Inheriting from documents, reports on testing results, reports on topics of institutes, research centers, universities, specialized management agencies, information on peat treatment, selection Bacillus strains metabolize carbon compounds.

- Collect information from books, newspapers, internet and other official information sources related to the research contents of the report, analyze and give evidence.

2.2.2. Survey method for microorganisms capable of metabolizing carbon-rich substrates and decomposing phosphorus in peat

The selection of microorganisms capable of converting carbon-rich substrates in peat is based on the determination of the ability to break down lignin substrates and cellulose substrates. Microorganism strains capable of strongly cleaving both substrates as above were selected.

a) Method to determine the ability to cleave lignin substrates of microorganisms

The method was adapted from Zuharlida Tuan harith et al., 2014. Method details include: each microbial strain was screened on a selective medium containing high malt (48 g/L) , alkaline lignin (4 g/L), $CaCO_3$ (2 g/L) and toluidene blue (0.025 g/L). Plates were incubated at 32°C for 48 h, monitored for growth, and development of cleavage rings (discoloration areas).

b) Method to determine the ability of microorganisms to break down cellulose substrates

Place the crude enzyme solution into the perforated holes in the petri dish containing Hans-CMC medium and place the agar plates at 37ºC. After 48 hours, put the Petri dishes in the refrigerator for 12 hours, then in the 40ºC incubator for 6 hours. Take out, put 5ml of lugol reagent in each peptide dish, spread evenly over the agar surface. Leave for 15 minutes and then decant all reagents coated with Lugol's solution. Observe the resolution ring that forms. The enzymatic activity of the selected bacterial strains was calculated by the resolution ring diameter (ΔD) (Nyi, Wikwiek, 2014): ΔD = D - d (mm). In there:

D: resolution ring diameter (mm);

d: diameter of agar hole (mm).

On the basis of determining the ability to produce extracellular enzymes, select strains with the largest resolution ring diameter and ≥20mm.

c) Method of determining phosphorus-degrading microorganisms

The sample was prepared by diluting 10 g soil into 90 ml sterile physiological saline, shake on a 150 rpm shaker, 10 minutes. Then, the sample solution was serially diluted in a 10 ml tube separately, like 10^{-2} to 10^{-5}. Take 0.1 ml of each concentration by pipet was cultured on petri plates added Pikovskaya media supplemented with lexitin and incubated at 30ºC for 48 to 72 hours & colonies were observed. Microorganisms that degrade insoluble phosphates are counted as the number of colonies in the petri dish forming a resolution ring (transparent ring) surrounding the colony.

d) Method of determining starch-degrading microorganisms

Determination of starch-degrading activity by diffusion method on agar plates. The research method was carried out as the method of determining the cellulose-degrading activity (Vietnamese Standard of 6168-2002) mentioned above, only replacing CMC with soluble starch. Starch hydrolysis activity is expressed as the difference between the resolution ring diameter and the bore diameter, measured in mm.

e) Method for extracting humic acid from peat and peat fermentation products

The extraction method of humic acid from peat and peat fermentation product was adapted from the method of B. Saito and M. M. Seckler, 2014. Details of the method included: The extraction was carried out in an apparatus with diameter 0.4L with a diameter of 0.09 m and a height of 0.097 m, with a stirrer and four 45° cutting blades with a diameter of 0.04 m. Rotation speed is 300 rpm.

The extraction experiment consisted of mixing 20g of dry peat with 100 mL of 0.5, 1.0, 1.5 or 2.0M KOH solution for 12 and 24 h. After extraction, the solution containing humic acid was separated from the insoluble fraction containing humin by centrifugation at 2500 rpm for 10 minutes. Experiments were performed in triplicate. All extraction experiments were conducted at room temperature (25 ± 2°C).

k) Method to determine the fermentative ability to convert carbon-rich substrates in peat of microorganisms to produce the end product of humic acid

Determination of fermentative ability to convert carbon-rich substrates in peat based on yield of humic acid products after fermentation. Selected microorganisms are cultured activated on selective culture medium with each strain, bacterial strains are cultured on king B medium. The culture fluid is added to the peat fermentation system (peat is

crushed to a size of about 74 µm, dissolved in water at a rate of 10 percent by weight/volume of peat, with a capacity of 5 liters (seed rate of 10% by weight/volume), fermentation is carried out. At a temperature of 30°C, stirring speed 150 rpm, gas supply level of 1 liter of air/1 liter of medium/min, culture was performed for 48 hours. Take samples and analyze the humic acid content, cellulose content, lignin content at the initial time of not seeding and at the time after fermentation 48 hours.

l) Methods of studying some morphological and physiological characteristics of selected microbial strains

Morphological characteristics of actinomycete strains were determined based on culture characteristics including: color and shape of colonies cultured on King B agar, color and characteristics of microbial fluid when shaking and static culture.

3. RESULTS AND DISCUSSION

3.1. Selection of microorganisms capable of metabolizing carbon and phosphorus-rich substrates that are difficult to dissolve in peat

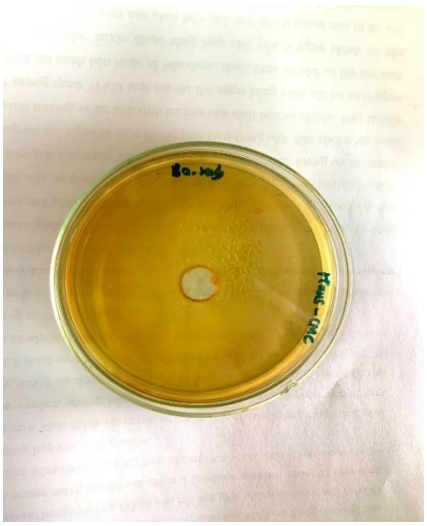
From the gene fund, we conducted a survey of microbial strains capable of degrading carbon-rich substrates including lignin, cellulose, phosphate, and proteolytic. The results are summarized in Table 3.1. showed that the strains with the most active degradation of cellulose, lignin, phosphorus and starch are the most active strains. These strains were selected for further research.

Table 3.1. The ability to degrade organic matter of the studied strains of microorganisms

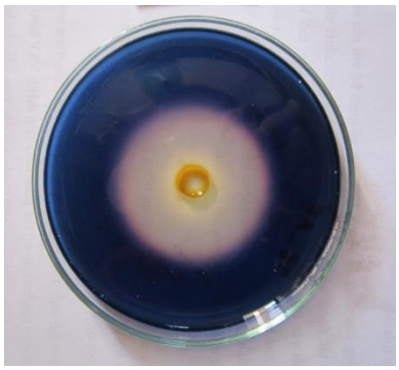
No.	Microorganisms strain	Cellulose degradation ability (D-d mm)	Lignin degradation ability (D-d mm)	Insoluble phosphorus degradation ability (D-d mm)	Starch degradation ability (D-d mm) (D-d mm)
1	*Bacillus polyfermenticus*	41	40	23	38
2	*Bacillus subtilis*	40	41	24	29
3	*Bacillus mycoides*	39	38	21	20
4	*Bacillus pumilus*	38	39	20	17
5	*Bacillus licheniformic*	30	35	22	26

From the chart and table 3.1, it is shown that the studied strains are capable of degrading cellulose, lignin, insoluble phosphorus and starch substrates (resolving rings are all larger than 20mm). Among them, two strains of *Bacillus polyfermenticus* and *Bacillus subtilis* are the strains with the strongest organic carbon and phosphorus metabolism activity. The *Bacillus polyfermenticus* strain had cellulose, lignin, organophosphorus and starch-degrading ring diameters of 41, 40, 23 and 38 mm, respectively. The *Bacillus subtilis* strain had cellulose, lignin, organophosphorus and starch-degrading ring diameters of 40, 41, 24 and 29 mm, respectively. These strains were selected for further research.

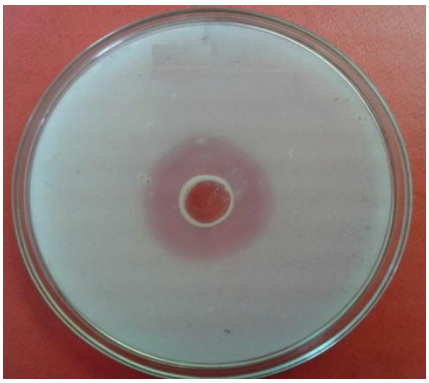
Cellulose degradation of Bacillus polyfermenticus strain



Cellulose degradation of Bacillus subtilisstrain



Cellulose degradation of Bacillus polyfermenticus strain

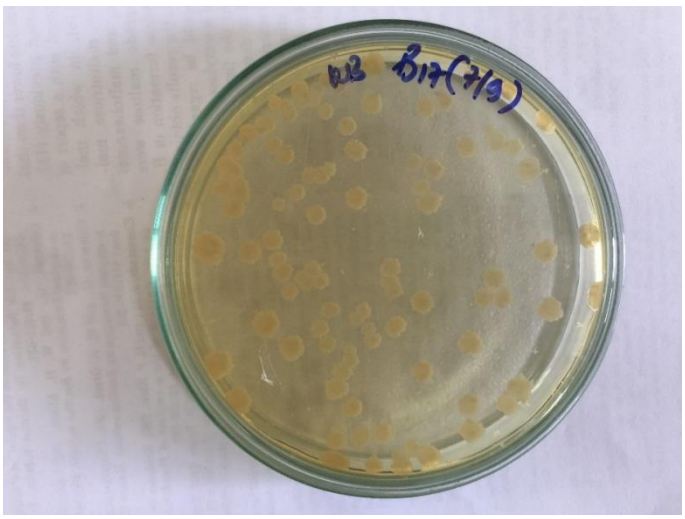


Cellulose degradation of Bacillus polyfermenticus strain

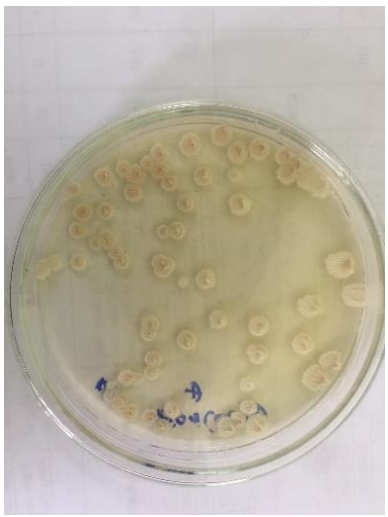
For further research, some growth and development characteristics of selected microbial strains were evaluated and investigated.

- The strain *Bacillus polyfermenticus* (SHB 17) is a bacterium (gram +) that was selected on King B media under the condition of culture temperature at 28±2°C. After 48 to 72 hours of culture, the colonies are irregular, with serrated edges 1.5 to 2 mm in diameter, clear yellow, odorless. When cultured by shaking on liquid medium (King B), after 48 hours the *Bacillus polyfermenticus* strain develops, causing the culture medium to turn pale yellow, on the wall of the flask, a thin white scum ring is formed and adheres tightly to the wall of the flask. Cell density test results after 48 hours of culture showed that SHB 17 reached a density of ≥ 5.10^9 CFU/ml. In static culture conditions, after 72-96 hours, the strain SHB 17 developed, making the liquid medium cloudy, the top was clearer, the surface of the medium was scum, the results of checking the cell density after 96 hours of culture showed found that SHB 17 reached a density of ≥ $5,10^6$ CFU/ml.

- The strain *Bacillus subtilis* is a bacterium (gram +) that was selected on King B medium under the conditions of culture temperature at 37±2°C. After 48 to 72 hours of culture, colonies are round, irregularly serrated, 2 to 4 mm in diameter, clear yellow, odorless. When cultured by shaking on liquid medium (King B), after 48 hours, the Bacillus subtilis strain developed, causing the culture medium to turn pale yellow, forming a thin white scum ring on the wall of the flask and adhered to the wall of the flask. Cell density test results after 48 hours of culture showed that *Ba.subtilis* reached a density of ≥ $4,6.10^9$ CFU/ml. Under static culture conditions, after 72 to 96 hours, the *Bacillus subtilis* strain developed, making the liquid medium cloudy, the top was clearer, the surface of the medium was scum, the results of checking the cell density after 96 hours of culture showed found that *Bacillus subtilis* reached a density of ≥ $8,3.10^6$ CFU/ml.



Colony shape of Bacillus polyfermenticus strain



Colony shape of Bacillus subtilis strain

3.2. Determination of microbial strains' ability to ferment carbon-rich metabolites in peat

The ability to ferment carbon-rich substrates in peat by selected microorganisms is shown in Tables 3.2. Research results show that, after 48 hours of fermentation with peat as a substrate, the acid content is large, 2 to 3 times higher than the initial time (0 hours when not added). microbial strains). At the same time, the content of cellulose, hemicellulose,

lignin, and organic content in the studied samples all had a certain decrease. This revealed that microbial strains (*Bacillus polyfermenticus, Bacillus subtilis*) efficiently cleaved and converted the carbon-rich substrates in the peat to the end product, humic acid.

Table 3.2. The fermentative capacity of peat-rich carbon-rich substrates of *Bacillus polyfermenticus*

Fermentation fluid analysis parameters	0 hours	After 48 hours of fermentation
Organic content (OM (%))	59	39
Hemicellulose (%)	25,5	18
Cellulose (%)	19,1	10,9
Lignin (%)	21	10
Humic acids (%)	5,4	13,5

From Table 3.2, it shows that *Bacillus polyfermenticus* strain has the ability to break down carbon-rich substrates: organic matter content decreased by 33.9% after 48 hours of fermentation; the content of hemicellulose, cellulose and lignin substrates decreased by 29.4%, 42.9% and 52.3%, respectively. In addition, the content of humic acids formed after 48 hours of fermentation increased 2.5 times compared to the original without the addition of Bacillus polyfermenticus strain.

Table 3.3. Fermentation ability to convert carbon-rich substrates in peat of *Bacillus subtilis* strain

Fermentation fluid analysis parameters	0 hours	After 48 hours of fermentation
Organic content (OM (%))	59	38,8
Hemicellulose (%)	25,5	18
Cellulose (%)	19,1	11,3
Lignin (%)	21	10
Humic acids (%)	5,4	15

From Table 3.3, it shows that the *Bacillus subtilis* strain is capable of cleaving carbon-rich substrates: the organic matter content, the hemicellulose, cellulose and lignin substrates decreased by 34.2%, 29.4%, 40, respectively. 8% and 52.3% after 48 hours of fermentation. Besides, the content of humic acids formed after 48 hours of fermentation increased 2.8 times compared to the original without the addition of *Bacillus subtilis* strain.

4. CONCLUSION

- From the agricultural microbial gene fund, the Bacillus sp. (including 2 strains, Bacillus polyfermenticus and Bacillus subtilis) have strong ability to metabolize carbon-rich substrates and decompose phosphorus in peat. The Bacillus polyfermenticus strain had cellulose, lignin, organophosphorus and starch-degrading ring diameters of 41, 40, 23 and 38 mm, respectively. The Bacillus subtilis strain had cellulose, lignin, organophosphorus and starch-degrading ring diameters of 40, 41, 24 and 29 mm, respectively.

In addition, the content of humic acids formed after 48 hours of fermentation increased 2.5 times compared to the original without the addition of Bacillus polyfermenticus strain and increased 2.8 times compared to the original without the addition of Bacillus subtilis strain. Therefore, 2 strains of Bacillus sp. Selected for high performance in producing humic acid products. At the same time, the selected microbial strains have a high biosafety level (level 1, the safest level in the biosafety scale).

- Thus, the selected combination of 2 strains of Bacillus polyfermenticus and Bacillus subtilis can be widely applied in practice to treat peat.

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