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Detection and Analysis of Metabolites from Facultative CO_2 and N_2 Fixing Bacteria

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Abstract: Facultative CO_2 and N_2 fixing bacteria are a special class of microorganisms that can simultaneously fix CO_2 and N_2 from the atmosphere and use it as a carbon source and nitrogen source, respectively. Discovery of useful metabolites from these microbes would be analogous to turning waste into wealth, as it would not only reduce production costs, but also provide an environmentally safe alternative to inorganic fertilizers. In this study, some common metabolites, including extracellular polysaccharides, extracellular proteins, lipids, alcohols, ketones, and esters were detected from thirty three facultative CO_2 and N_2 fixing bacteria using preliminary methods, such as the phenol - acid method, Coomassie brilliant blue method, method of loss of weight, and gas phase chromatography. The results showed that there are 4 types of facultative fixed CO_2, N_2 bacteria that can produce extracellular polysaccharides, 6 types that can produce extracellular protein, 4 types that can produce lipids, 2 types that can produce ethanol, 1 type that can produce acetone, and 2 types that can produce esters. This information will provide a theoretical basis for future research and applications.

Introduction

A very special type of microorganisms can fix CO_2 and N_2 from the atmosphere and utilize them as carbon source and nitrogen source, respectively. This group of microbes is called facultative CO_2 and N_2 fixing bacteria [1].

These kinds of microorganisms have been extensively discovery by our research group since 2012. Soil samples (3268 samples) from different regions were sampled and 33 kinds of facultative CO_2 and N_2 fixing microorganisms were screened using C and N free source culture medium.

Microbial metabolites have several beneficial applications for humans. Extraction of potential metabolites from facultative fixed CO_2 and N_2 microorganisms would greatly reduce the production cost by saving the additional carbon source and nitrogen source cost, recycle waste into wealth, and absorb CO_2 and N_2 from the atmosphere, and thus aid environmental protection [1].

In this study, metabolites from 33 different facultative fixed CO_2 and N_2 bacteria were tested. Our results provide a theoretical basis for follow-up application research in the future.

Among the many metabolites from microorganisms, the most valuable ones are extracellular proteins [2], extracellular polysaccharides [3], lipids, organic solvents, alcohols [4], ketones [5], and esters.

Materials and methods

Experimental materials

An air compressor, CO_2 tank, protease (Proteinase K (CWBIO) (DNase), nuclease I (CWBIO), porcine pancreatic lipase (Lipase, Sigma-Aldrich of America), and cellulase (Sigma-Aldrich of America) were used in the experiment.

The strains of facultative CO_2 and N_2 fixing microorganisms were as follows: BHJ, BSXJ, TMJ, MSJ, HSJ, 1HJ, 2HJ, 3 HJ, 4 HJ, 5HJ, 6HJ, 7HJ, 8HJ, 9HJ, 10HJ, 11HJ, 12HJ, 13HJ, 14HJ, 15HJ,

16HJ, 17HJ, 18HJ, 19HJ, 20HJ, 21HJ, 22HJ, 23HJ, 24HJ, 25HJ. 26HJ, 27HJ, and 28HJ. All strains were screened and named by the authors.

Solid and liquid microbial culture

The screening medium (i.e., carbon nitrogen free agar medium) was optimized based on the compositions of Azotobacter Medium and the carbon fixing medium. Its composition was as follows: KH_2PO_4 0.2 g, $MgSO_4 \cdot 7H_2O$ 0.3 g, $CaCl_2$ 0.2 g, NaCl 0.2 g, agar 18 g, trace element solution 2 mL, distilled water (added to 1000 mL). The medium was sterilized at 121 °C for 20 min. Preparation of the trace element solution was done as follows: $FeCl_3$ 0.3 g, $FeSO_4 \cdot 7H_2O$ 0.3 g, $MnSO_4 \cdot H_2O$ 0.15 g, $ZnSO_4$ 0.14 g, and $CoCl_2$ 0.2 g were added to a final volume of 1000 mL using distilled water, and the solution was filtered by the filter, sterilized by high pressure steam sterilization, and stored at 4 °C. No carbon and nitrogen liquid medium can be added without agar.

Liquid culture: facultative CO_2 and N_2 fixing bacteria were inoculated into a sterile, liquid bottle containing above mentioned media, and were incubated at 30 °C. The gas (N_2 78%) and CO_2 were supplied using the air compressor and the concentrations of the gases varied according with the type of bacteria.

Extraction of extracellular matrix of the facultative CO_2 and N_2 fixing bacteria

The bacterial concentration was monitored using a spectrophotometer. Once the optimum concentration was reached, a total volume of 10 ml was centrifuged, cleaned twice using deionized water, and stored. For extraction of the extracellular matrix, bacteria were placed in a centrifuge tube and Proteinase K or cellulase aqueous solution was added. After keeping on stirred ice bath for 3 h, the solution was centrifuged at 12000 R min^{-1} at 4 °C for 10 min, and the supernatant (extracellular matrix solution) was collected. Then, 0.9% NaCl and 0.1 $molL^{-1}$ NaOH solution were used for extraction once and twice, respectively, according to the above methods.

Detection of polysaccharide content in the target bacteria

The concentration of polysaccharide was determined using the phenol-sulfuric acid method. Briefly, 0.05 mL of 80% phenol was added to 2 mL of the bacterial sample (suitably diluted to a concentration below 0.1 g L^{-1}). Then, 5 mL of concentrated sulfuric acid was added quickly and set aside for 10 min. The solution was mixed well and incubated in a water bath at 25–30 °C for 10–20 min. OD_{490} values were measured at a wavelength of 490 nm. The concentration of polysaccharide of the target bacteria was calculated based on the standard curve [6].

Detection of exoprotein content in the target bacteria

The protein concentration was determined using the Coomassie brilliant blue method. Bacterial sample (0.1 mL, diluted the concentration of less than 1 $g.L^{-1}$) was added into 5 mL Coomassie blue solution. After mixing well, the solution was set aside for 5 min. OD_{595} values were measured at a wavelength of 595 nm. Protein concentration of the target bacteria was calculated according to the standard curve.

Detection of lipid content in the target bacteria

Lipid concentrations were measured using the weight-loss method. DNase I (10μL) and lipase solution (1 mL) was added to 2.5 mL of culture medium for enzymatic treatment at room temperature for 4 h. After washing twice with deionized water, the mixture was centrifuged at 8000 R. min^{-1}, and the precipitate was collected and weighed (A_1) after lyophilization. The culture medium without enzymatic treatment was used as a blank control group. The dry weight (A_0) of the target bacteria after lyophilization was calculated and the ratio of lipid to dry cell weight was given by $(A_0-A_1)/A_0$.

Detection of organic solvents, alcohols, ketones, and ester contents in the target bacteria

The organic solvent and alcohol **,** ketones, and ester contents in the target bacteria were detected using GC9720 gas chromatograph（Zhe Jiang FuLi Analtical Instruments Inc of China）. The conditions and procedures were as follows: concentrations of different bacteria were monitored using spectrophotometer. Ten milliliters of different bacterial samples (at optimum concentrations) were centrifuged, and the supernatant was analyzed using gas chromatography under following conditions: Column temperature, 100 °C to 250 °C, 5 °C /min; vaporization temperature, 250 °C; and detection temperature, 260 °C. The standard samples were alcohols, such as methanol, ethanol, propyl alcohol,

and n-butyl alcohol. Acetone butanone was the ketone representative, while ethyl hexanoate was the ester representative. The carrier gas was high purity deoxidized nitrogen, and the detector was flame ionization detector（FID）. The chromatographic column type was gas phase capillary column, and the chromatographic column was Chrom PLOT-Q. The column specifications were 30 m * 0.32 mm * 20 m.

Results and Analysis

Qualitative determination metabolites from facultative CO_2 and N_2 fixing bacteria

Metabolites from 33 different types of facultative CO_2 and N_2 fixing bacteria, including alcohols, ketones, and esters were detected qualitatively, as listed in Table 1. The results showed that only 2 types of the tested facultative bacteria (9HJ and 6HJ) could produce alcohols. In addition, only 1 strain of facultative CO_2 and N_2 fixing bacteria (28HJ) produced ketone. Other organic compounds were not detected.

Extracellular polysaccharides, extracellular proteins, and lipids from the 33 different strains of facultative bacteria were detected qualitatively, as listed in Table 2. The results showed that there 5 strains of these bacteria (2HJ, 21HJ, 19HJ, 9HJ, 6HJ) could produce extracellular polysaccharides, 6 strains of the facultative CO_2 and N_2 fixing bacteria (5HJ, 11HJ, 21HJ, 22HJ, 26HJ, 28HJ) produced extracellular proteins, 4 strains of bacteria (2HJ, 13HJ, 19HJ, 27HJ) produced lipids, and only 2 strains (15HJ，24HJ) produced esters.

Table 1 Qualitative detection of alcohols and ketones from 33 strains of bacteria cultured in fermentation liquid.

strain number	carbinol	alcohol	propanol	butanol	acetone	butanone
TMJ	-	-	-	-	+	-
BHJ	-	+	-	-	-	-
BSXJ	-	-	-	-	-	-
MSJ	-	+	-	-	-	-
HSJ	-	-	-	-	-	-
1HJ	-	-	-	-	-	-
2HJ	-	-	-	-	-	-
3HJ	-	-	-	-	-	-
4HJ	-	-	-	-	-	-
5HJ	-	-	-	-	-	-
6HJ	-	-	-	-	-	-
7HJ	-	-	-	-	-	-
8HJ	-	-	-	-	-	-
9HJ	-	-	-	-	-	-
10HJ	-	-	-	-	-	-
11HJ	-	-	-	-	-	-
12HJ	-	-	-	-	-	-
13HJ	-	-	-	-	-	-
14HJ	-	-	-	-	-	-
15HJ	-	-	-	-	-	-
16HJ	-	-	-	-	-	-
17HJ	-	-	-	-	-	-
18HJ	-	-	-	-	-	-
19HJ	-	-	-	-	-	-
20HJ	-	-	-	-	-	-
21HJ	-	-	-	-	-	-
22HJ	-	-	-	-	-	-
23HJ	-	-	-	-	-	-
24HJ	-	-	-	-	-	-
25HJ	-	-	-	-	-	-
26HJ	-	-	-	-	-	-
27HJ	-	-	-	-	-	-
28HJ	-	-	-	-	-	-

"-" is not detected, "+" is detected

Table 2 Qualitative determination of extracellular polysaccharides, proteins, lipids, and nucleic acids in 33 strains of bacteria cultured in fermentation liquid.

strain number	polysaccharid	protein	lipid	nuclein	esters
TMJ	-	-	-	-	-
BHJ	-	-	-	-	-
BSXJ	-	-	-	-	-
MSJ	-	-	-	-	-
HSJ	-	-	-	-	-
1HJ	-	-	-	-	-
2HJ	+	-	+	-	-
3HJ	-	-	-	-	-
4HJ	-	-	-	-	-
5HJ	-	+	-	-	-
6HJ	+	-	-	-	-
7HJ	-	-	-	-	-
8HJ	-	-	-	-	-
9HJ	+	-	-	-	-
10HJ	-	-	-	-	-
11HJ	-	+	-	-	-
12HJ	-	-	-	-	-
13HJ	-	-	+	-	-
14HJ	-	-	-	-	-
15HJ	-	-	-	-	+
16HJ	-	-	-	-	-
17HJ	-	-	-	-	-
18HJ	-	-	-	-	-
19HJ	+	-	+	-	-
20HJ	-	-	-	-	-
21HJ	+	+	-	-	-
22HJ	-	+	-	-	-
23HJ	-	-	-	-	-
24HJ	-	-	-	-	+
25HJ	-	-	-	-	-
26HJ	-	+	-	-	-
27HJ	-	-	+	-	-
28HJ	-	+	-	-	-

"-" is not detected, "+" is detected

Quantitative determination of extracellular polysaccharide in facultative CO_2 and N_2 fixing bacteria

Extracellular polysaccharides are widely used in the food industry. For instance, they can endow fermented dairy products with special texture and flavor, can be used as safe food additives, and may also be a good source of food grade polysaccharides that are widely used in various foods for thickening, stabilization, emulsification, gelling, and water retention purposes. Extracellular polysaccharides also play a role in biological activities, such as immune activity, anti-tumor, and antiulcer, and can therefore find applications in the medical field. At present, majority of the extracellular polysaccharides are produced from industrial fermentation. Quantitative analysis of liquid culture of facultative CO_2 and N_2 fixing bacteria in our experiments showed that 5 strains of facultative CO_2 and N_2 fixing bacteria, 2HJ, 21HJ, 19HJ, 9HJ, and 6HJ, produced extracellular polysaccharides at concentrations of 268 μg/mL, 150 μg/mL, 50 μg/mL, 489 μg/mL, and 480 μ/mL, respectively, as shown in Fig1. Among these, the stain 19HJ produced the highest concentration of extracellular polysaccharides. In subsequent studies, the culture medium composition could be optimized to further increase the yield.

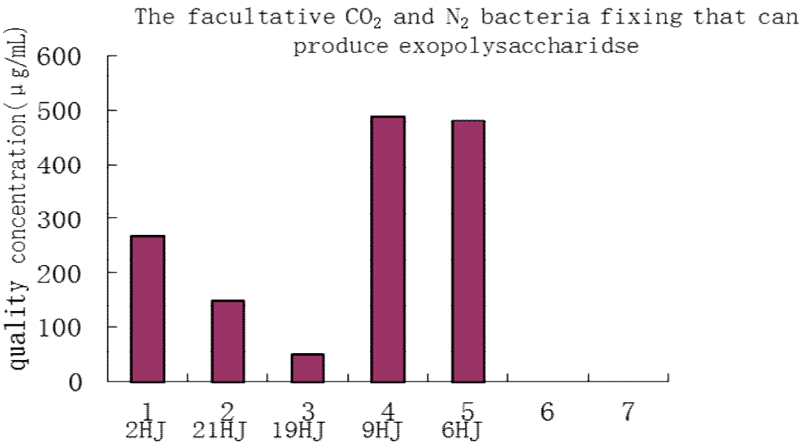

Fig.1 The facultative CO_2 and N_2 fixing bacteria can produce polysaccharides.

Quantitative determination of extracellular protein in facultative CO_2 and N_2 fixing bacteria

Extracellular proteins are proteins in the extracellular matrix, such as proteins, hormones, antibodies, digestive enzymes, and insulin, that are secreted by cells. At present， proteins or enzymes are generally extracted from the related tissues of animals or plants using biotechnology methods. However, these proteins are mostly used for industrial fermentation production. Our quantitative analysis showed that 6 strains of facultative CO_2 and N_2 fixing bacteria, 5HJ, 11HJ, 21HJ, 22HJ, 26HJ, and 28HJ, produced extracellular proteins at 7.8%, 2.5%, 16.8%, 12.6%, 23%, and 19%, respectively, as shown in Fig2. Among these, the strain 26HJ produced the highest percentage of extracellular proteins. In subsequent studies, the preparation of culture medium could be optimized, and the yield of extracellular proteins could be increased as required their application.

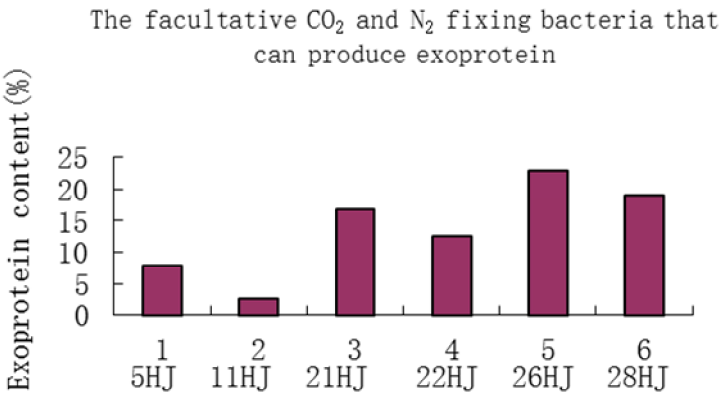

Fig.2 The facultative CO_2 and N_2 fixing bacteria can produce extracellular proteins.

Quantitative determination of lipids in facultative CO_2 and N_2 fixing bacteria

Lipids are widely used in medicine for supplying heat, participating in tissue formation, and supplying essential fatty acids. In industries, liposomes, as an important carrier of drugs and nutrients, can improve the stability of preparations, improve bioavailability and targeting, and have other broad application prospects. At present, lipids are extracted mainly from the related tissues of animals or plants using biotechnology methods. However, their production cost is quite high, which could be lowered by production from fermentation cultures of microorganisms. Our quantitative analysis showed that 4 strains of facultative CO_2 and N_2 fixing bacteria, 2HJ, 13HJ, 19HJ, and 27HJ, produced lipids at 18%, 6%, 12%, and 3%, respectively, as shown in Fig3. Among these, the strain 2HJ

produced the highest proportion of lipids. In subsequent studies, if the preparation of culture medium is optimized, the yield of lipids could be increased further.

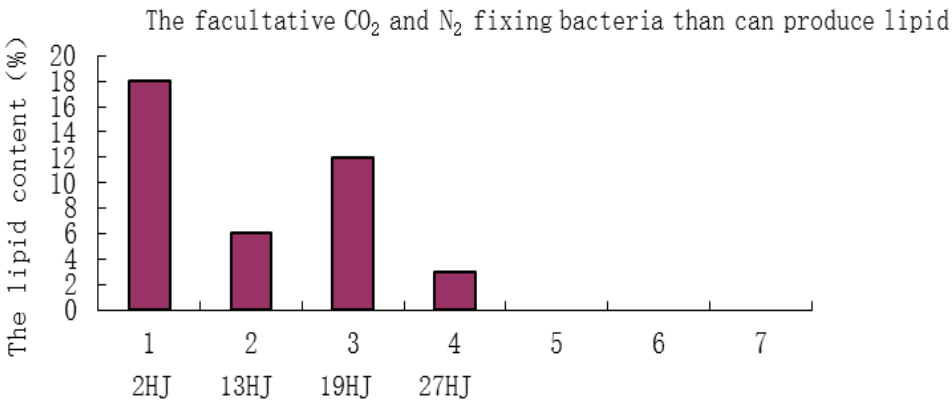

Fig.3 The facultative CO_2 and N_2 fixing bacteria can produce lipids

Quantitative determination of alcohols, ketones, and esters from facultative CO_2 and N_2 fixing bacteria

Alcohols, ketones, and esters are common organic solvents or energy sources for bacteria. The production of ethanol occurs via fermentation of sugars, while the production of acetone and ester occurs via chemical synthesis. However, the pollution levels and production costs of these processes are high. Biosynthesis has the potential to reduce production costs and environmental pollution. However, in this study, only 2 strains among the 33 facultative CO_2 and N_2 fixing bacterial strains that were studied produced alcohols with yields of 2.8% and 1.2%, respectively. Furthermore, there were only 2 strains that produced esters, and the yields were 0.4mg/L and 0.2mg/L, respectively. Finally, only one strain produced ketones, and the yield was relatively low (0.48mg/L). Thus, we acknowledge that there is a certain gap between current options and desired applications, but our results provide the basis of further applied research.

Discussion

Although carbon and nitrogen fixing bacteria have long been reported, their metabolites have rarely been reported. Currently used microbial fermentation procedures require addition of carbon and nitrogen, thus increasing production costs. However, the facultative CO_2 and N_2 fixing microorganisms analyzed in this study do not need additional carbon and nitrogen sources for their growth and reproduction. Instead, they use CO_2 and N_2 from the atmosphere as carbon and nitrogen sources. Their use in industry will not only reduce the resource costs, but more importantly, also channelize greenhouse gases CO_2 and N_2 to play a role in environmental protection. Although these facultative CO_2 and N_2 fixing bacteria that has been demonstrated in this study do not currently possess the metabolic capacity for industrial level of application, if the growth conditions of these bacteria are optimized or modified using microbial technology, these special microorganisms could contribute to industrial applications and environmental protection in the future.

Conclusion

There are 6 strains among the 33 strains of facultative CO_2 and N_2 bacteria fixing that produced extracellular proteins. Quantitative analysis showed that these 6 bacterial strains, 5HJ, 11HJ, 21HJ, 22HJ, 26HJ, and 28HJ, produced 7.8%, 2.5%, 16.8%, 12.6%, 23%and19%, respectively, of extracellular proteins. Five bacterial strains, 2HJ, 21HJ, 19HJ, 9HJ, and 6HJ produced extracellular polysaccharide at concentrations of 268 μg/mL, 150 μg/mL, 50 μg/mL, 489 μg/mL, and 480 μg/mL,

respectively. Four bacterial strains, 2HJ, 13HJ, 19HJ, and 27HJ, produced lipids at yields of 18%, 6%, 12%, and 3%, respectively. Two strains of BHJ and MSJ produced alcohols with outputs of 1.8% and 1.2%, respectively. Two strains of 15HJ and 24HJ produced esters at outputs of 0.4 mg/L and 0.2 mg/L, respectively. One strain was produced ketones, but the yield was quite low (0.48 mg/L). Currently, there is a gap between available options and desired applications, but our data provide value as the basis for further research. Although the yield is low, the facultative CO_2 and N_2 fixing bacteria are grown in the absence of carbon and nitrogen sources, reducing costs during production. At the same time, these microorganisms can effectively fix the CO_2 and N_2 in the atmosphere, and thus contribute to environmental protection.

Acknowledgements

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