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Isolation, characterization of *Bacillus* sp. producing heavy metal absorption γ-PGA

Phân lập, đánh giá tính chất chủng Bacillus sp. có khả năng sinh tổng hợp chất hấp thụ kim loại nặng γ-PGA

Research article

Nguyen Sy Le Thanh[1]*, Keitarou Kimura[2], Do Thi Tuyen[1], Le Thi Ngoc Anh[3]

[1]Institute of Biotechnology, Vietnam Academy of Science and Technology, 18 Hoang Quoc Viet, Cau Giay, Ha Noi, Vietnam; [2]Laboratory of Applied Microbiology, Food Research Institute, National Agriculture and Food Research Institute, Tsukuba, Ibaraki 305-8642, Japan; [3]Hanoi University of Agriculture, Vietnam

Poly-gamma-glutamic acid (γPGA), which is a biodegradable, non-immunogenic and unusual anionic amino-acid polymer consist of D- and L-glutamic acid units, was exploited for a wide array of useful applications. *Bacillus* are well known cellular system important for fermentation to synthesize γPGA, which is used as thickener, drugs carrier, cryoprotectant, humectant, biological adhesive, flocculants, or heavy metal absorbent. This study focused on the isolation of *Bacillus* spp. that is possible to produce γ-PGA from different soil samples from different places in Vietnam. Study the effect of precursors, temperature, carbon sources, times and pH on γ-PGA production. From 31 soil samples and 4 straws samples, strain 20.2 which produced the highest γ-PGA yields (riches 15.2 mg/ml), was identified as *Bacillus* sp. 20.2 by molecular biology method. The suitable conditions for growing of *Bacillus* sp. 20.2 strain to produce γ-PGA are at 37°C, pH 7 after 72 hours. Citric acid instead of glucose in a GSP medium is better for producing γ-PGA by strain *Bacillus* sp. 20.2.

Poly-gamma-glutamic acid (γ-PGA) là một polymer amino-acid gồm D và L-glutamic acid, có khả năng phân hủy sinh học, không gây miễn dịch, đã được ứng dụng rộng rãi trong công nghiệp, y học. Bacillus subtilis được biết đến là hệ thống tế bào ý nghĩa quan trọng trong quá trình lên men để tổng hợp γ-PGA. γ-PGA hòa tan trong nước, phân hủy sinh học và không độc đối với con người và môi trường. γ-PGA ổn định với nhiều protease vì các protease thường không nhận acid γ-glutamic (Obst et al., 2004). γ-PGA có cấu trúc đồng phân đơn giản, không gây miễn dịch. Do đó, γ-PGA đã được quan tâm ứng dụng trong các lĩnh vực như y học, công nghiệp thực phẩm, mỹ phẩm và đặc biệt là xử lý nước nhiễm kim loại nặng. Trong nghiên cứu này chúng tôi tập trung phân lập, tuyển chọn các chủng Bacillus có khả năng sinh tổng hợp PGA cao. Sau đó định danh và đánh giá khả năng sinh tổng hợp PGA từ chủng đã phân lập được. Kết quả cho thấy từ 34 mẫu rơm và đất, chúng tôi đã phân lập được chủng với mã số 20.2 có khả năng sinh PGA cao nhất đạt 15.2 mg/ml. Chủng này đã được định danh bằng phân tích trình tự gene 16S rRNA và thuộc loài Bacillus sp. *Môi trường thích hợp sinh tổng hợp PGA là GSP ở điều kiện 37°C pH7 sau 72 giờ nuôi cấy.*

Keywords: Poly-γ-glutamic acid, *Bacillus subtilis*, isolation, metal absorption

1. Introduction

Poly-gamma-glutamic acid (γPGA), which is a biodegradable, non-immunogenic and unusual anionic amino-acid polymer consist of D- and L-glutamic acid units, was exploited for a wide array of useful applications. *Bacillus subtilis* are well known cellular system important for fermentation to synthesize γPGA, which is used as a food additive and expected in medical application (Ogunleye, 2015, Kimura and Fujimoto, 2010). The synthesis of γPGA is governed by cell-density based signal transduction pathway (Kimura, 2009; Do, 2011) and directed by pgsBCA operon. The expression of the pgs operon is regulated by quorum-sensing components, ComPA, DegQ, DegS, DegU and cell motility related SwrA. Among them DegQ plays a pivotal role by stabilizing

* Corresponding author
E-mail: nslthanh@ibt.ac.vn

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DegS in a phosphorylated form that directly bounds to the promoter region of the pgsBCA operon (Do et al., 2011). γ-PGA is a naturally, negatively charged occurring polymer made up of L-glutamic acid or D-glutamic acid monomers or containing both monomers are joined by a linkage between the γ -carboxyl and the α-amino group (Ho et al., 2006; Shih and Van, 2001). The ability to participate in chemical reactions with the α-NH_2 group of α-COOH and γ-COOH groups in descending order of vascularization. In general, α-NH_2 binds to α-COOH to form an α-peptide linkage producing the α-PGA product by nucleophilic polymerization reactions. But when there is an appropriate catalyst system, the α-NH_2 group will bind to the γ-COOH group to form the γ-peptide linkage. γ- PGA has been produced extensively using bacteria, especially those of *Bacillus* sp. It is different from other proteins, because inside the cell, glutamate which is polymerized via the γ-amide linkages, is synthesized in a ribosome independent manner (Bodnár et al., 2008; Akagi et al., 2007). Thus, inhibitors of proteins, such as chloramphenicol, have no effect on the synthesis of γ-PGA (Adetoro et al., 2015). γ-PGA can be formed from more than 10,000 glutamic acid molecules via polymerization. Depending on the environment and on the microbial population, γ-PGA can be formed in three different types, one of which is made up of the whole D-γ-PGA, which is made up of all L -γ-PGA and one made of DL-γ-PGA polymerization. This distinction is made when bacteria are capable of directly or indirectly converting L-glutamic acid into D-glutamic acid and these two co-polymers produce DL-γ-PGA, which is called γ-PGA (Shih and Van, 2001).

γ-PGA is water soluble, biodegradable, edible, and non-toxic to humans, thus γ-PGA and its derivatives have been applied in many different fields. There are a lot of research in the world is carried out with the purpose of collecting volume of γ-PGA levels are highest from *Bacillus* (Akagi et al., 2005; Bajaj and Singhal, 2011; Gaborieau and Castignolles, 2011; Candela and Fouet, 2005). Due to its non-toxic and biodegradable properties, γ-PGA offers an eco-friendly alternative for wastewater treatment. γ-PGA with a molecular weight of ~5.8–6.2 × 106 Da appears to be superior to many conventional flocculants used in wastewater treatment plants operating downstream of food processing fermentation processes (Bajaj and Singhal, 2011). More interestingly, γ-PGA with a molecular weight of 9.9 × 105 Da could effectively remove 98 % of basic dyes from aqueous solution at pH 1 and could then be re-used. Study of poly γ-glutamic acid in Vietnam is still limited, mainly collaborative study of γ-PGA related parts. γ -PGA products are found only in Vietnam in the form of household cosmetics, biofilm ... and most of these γ-PGA products are of foreign origin: Japan, Korea, USA, Germany ... The researches of γ-PGA in Vietnam have been mainly obtained at the overview level, describing the process and description of practical application in some teaching materials.

2. Materials and methods

2.1. Samples soil, *Bacillus* strains

Total of 31 samples soil and 4 samples straws were obtained from several positions in many areas in Vietnam, and stored in plastic bags at 4^{o}C. *Bacillus subtilis* BSJ was used in this study from Lab of Enzyme Biotechnology.

2.2. Materials

Chemicals were used in this study are: Agarose, peptone, yeast extract, RNase, GeneRulerTM 1kb DNA ladder, agar, ethidium bromine, sodium glutamate, glucose, saccharose, lactose, NaCl, dNTP set. All the chemicals are in the purified form and provided by many famous and prestigious supplied such as Bio Basic, Fermentas, Qiagen, Sigma, etc.

2.3. Methods

Microorganism isolation

Placed 1 gram of soil, straws (cut into small pieces) into the falcon tube, add 10 ml of sterile water, shake for 10 minutes, transfer the solution into another falcon tube. Heated 85°C for 20 minutes (to kill non-spore forming microorganisms), then dilute to different concentrations: 10^{-1}, 10^{-2}, 10^{-3}. Pipetted 100 µl solutions onto a petri dish with solid GSP medium to spread, incubate at 37°C. The colonies appeared after one day. Colonies that presented morphology of *Bacillus* were selected to purify, streak to another GSP plate, incubated at 37°C overnight. Then colonies appeared separately. The *Bacillus* was distilled with 30% glycerol at -80°C. Select colonies characteristic of *Bacillus*. To purify *Bacillus* strains, streak colonies to another petri dish with solid GSP medium, incubate at 37°C overnight to single colonies appear. The *Bacillus* strains was stored at -80°C with 30% glycerol.

Selection of Bacillus strains capable of producing γ-PGA

The *Bacillus* strains, which were isolated on GSP medium, were cultured on 30 ml of liquid GSP medium in shake flasks, shaking 200 rpm for 24 hours at 37°C. Centrifuged (4500 rpm for 15 minutes) culture at 4°C to remove the cell, collected the supernatant. Then added 60 ml of alcohol 70% and 5 ml of 5M NaCl, placed at -20°C for 15 minutes. Centrifuged (12000 rpm for 10 minutes at 4°C), removed supernatant, dry and weigh the pellet and re-suspended with 3 ml sterile PBS 1X. Then pipetted 10 µl and mix with 2 µl loading dye 6X and checked by agarose gel electrophoresis at 100 V for 60 minutes on 1.0% agarose gels using TBE running buffer. The gels were stained with methylene blue for 10 min, followed by distaining with water.

Estimation of γ-PGA by measuring absorbance at OD_{216}

Measurement of γ-PGA was conducted based on optical absorption in the ultraviolet region, as γ-PGA absorbed the maximum at OD216 (Zeng et al., 2012). The technique for determining γ-PGA by the method of measurement was based on the following procedure: Centrifuged 0.5 ml of culture at 4500 rpm for 10 minutes, take the supernatant. Added 1ml of cold alcohol and 1 µl NaCl 5 M and let in -20°C, gently shake and centrifuged at

12.000 rpm for 10 minutes to obtain pellet. Then the pellet was dried at 40-50°C and dissolved by 0.5 ml sterilized water and then diluted 50 times. The solution is measured OD_{216}.

Identification of bacteria by DNA 16S ribosome analysis

Gene 16S ribosome was amplified from total genomic DNA by a pair of primers 9F: 5'-AGA GTT TGA TCC TGG CTC AG-3' and 926R: 5'-CCG TCA ATT CCT TTR AGT TT-3' (Weisberg et al., 1991). The sequences obtained after sequencing were analysed and handled with BLAST tool (The Basic Local Alignment Search Tool)-NCBI (National Center for Biotechnology Information) to determine the most closely related 16S rRNA species. A phylogenetic tree was also made based on the homology of known 16S rRNA sequences deposited in GenBank.

Effect of time, precursors on γ-PGA production

20.2 strain were cultured in a GSP medium pH 7 at 37°C, shaken 200 rpm. After periods every 24 hours, collecting the culture and measuring yield of the γ-PGA, then evaluating and selecting the time optimal to produce γ-PGA. The 20.2 strain was cultured in different medium, including GSP, GSP 1 (All sodium glutamate was replaced with soybean meal), LB medium.

Effect of carbon source on γ-PGA production

Bacillus uses carbon to grow and synthesize γ-PGA. Changing different carbon source in the GSP medium to select the optimum carbon source for fermentation such as saccharose and lactose. After every 24 hours, measuring yield of the γ-PGA and comparing, commenting and concluding.

Effect of temperature on γ-PGA production

The temperature affects very much on the synthesis γ-PGA of *Bacillus* strains. To find out the optimum temperature for γ-PGA production with high yields, we performed experiments with initial pH, medium, shaking speed conditions at three different temperatures: 30°C, 37°C and 40°C. Samples were collected after 72 hours to check for γ-PGA yields.

Effect of pH on γ-PGA production

The experiment was conducted with the initial pH of medium change as follows: 5, 6, 7, 8 and 9. Culture 20.2 strain in GSP medium at 37°C for 72 hours, shaken 200 rpm. Collecting the sample to measure for γ-PGA yields.

3. Results and discussion

3.1. Isolation *Bacillus* spp. synthesis γ-PGA

Soil samples and straws samples were diluted in sterilized water, spread on solid GSP plates. Because γ-PGA has a high viscosity and is produced by *Bacillus* spp. as a polymer outside of cells (Kubota et al., 1993), thus, we chose the mucoid colonies. Then streak colonies on solid GSP plates to purify colonies. Figure 1 shows the structure of colonies formed by bacteria strains in GSP plates. They have an irregular, large size, rounded-shape and raised with or without margin. Their colour is white and dull and they have a wet texture.

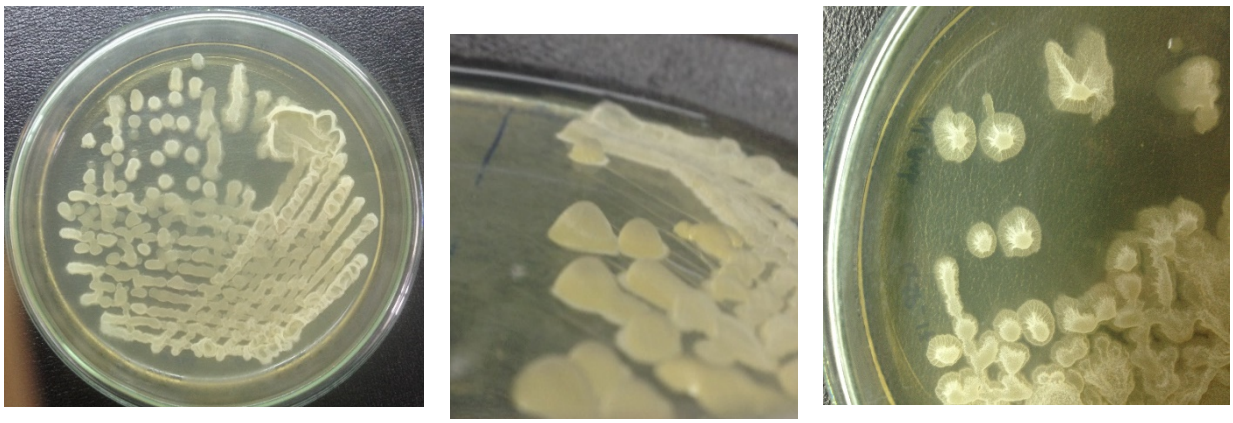

Figure 1. Single colonies of bacteria in GSP medium plates

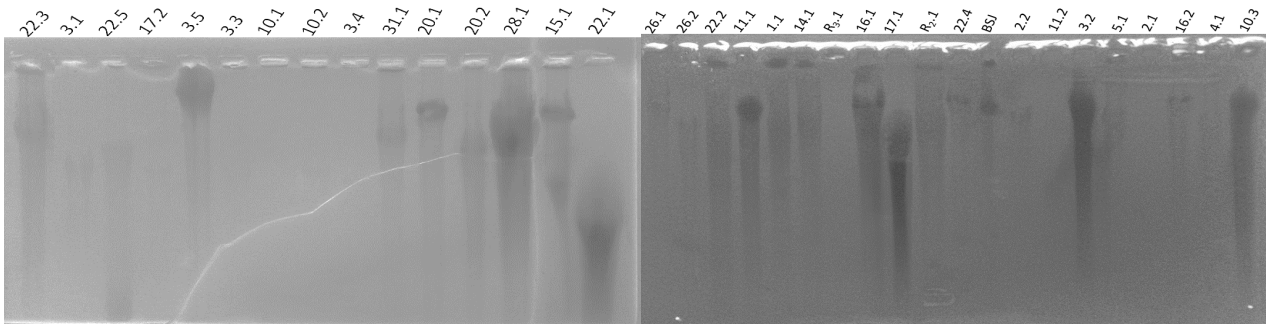

Figure 2. Gel electrophoresis γ-PGA on agarose 1%

After culturing bacteria strains in GSP medium, collected the culture and supernatant was precipitated with alcohol. The pellets, which was containing γ-PGA, was dried and weighted. The capacity of strain, which produce a high-yield of γ-PGA products was selected by. The results show that there are 26 strains (out of 35 strains was isolated) are capable of producing γ-PGA (Figure 2). Base on the total amount of γ-PGA was produced, we choose 1 strains namely 20.2 that producing the largest γ-PGA yields (15.2 mg/ml) for next experiments.

3.2. Identification of bacteria by 16S rRNA ribosome analysis

Total DNA of 20.2 strains above was extracted following the Sanger et al (2003) protocol. After gene 16S ribosome is amplified from total genomic DNA by PCR reaction with the heat cycle is mentioned in chapter materials and methods, we purify the PCR products for sequencing, use GeneJET PCR Purification Kit of Thermo according to the manufacturer's instructions. The total 919bp of 16S rRNA sequence was used for the identification strains 20.2.

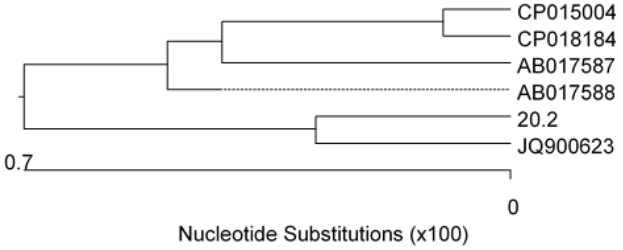

Figure 3. Sequence identically with *Bacillus subtilis* strain (A). The construction of phylogenetic tree based on 16S rRNA analysis of 20.2 strain (B).

Comparison of test sequences against non-redundant collection of GenBank database was performed with DNAstar software. The BLAST result in figure 3 showed that the 16S rRNA sequence of isolated 20.2 has 98% sequence identically with *B. subtilis* strain, which is identical with *B. subtilis* (CP018184), 98.1% identical sequence with *B.subtilis* (CP015004) and *B.subtilis* PM3 (AB017587), 98.1% sequence similarly with *B.subtilis* SSA3 (AB017587) and 98,8% with *B. subtilis* ME2 (JQ900623). A phylogenetic tree was also constructed based on the homology of known 16S rRNA sequences. Thus, we can conclusion that 20.2 is belong to *Bacillus* sp. strain family.

3.3. Effect of culture conditions on γ-PGA production

Effect of culture conditions on γ-PGA production
Effect of times on γ-PGA production

The experiments on the effect of time on γ-PGA production, 20.2 strain was cultured for 96 hours. Sample medium which were used for OD_{216} measurement, was collected every 24 hours. The results are shown in Figure 4.

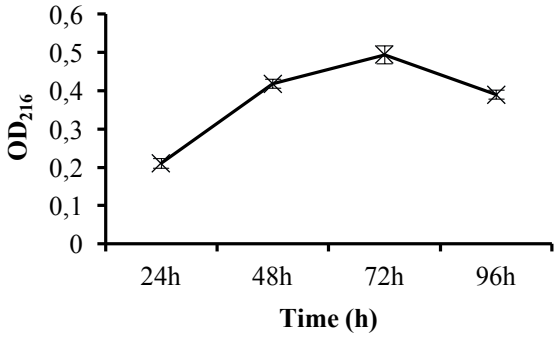

Figure 4. Effect of times on γ-PGA production

From the above chart, we can see that after 72 hours the maximum biomass of γ-PGA was obtained with OD_{216} = 0.494. After 24 hours of culture, γ-PGA yields were obtained is lowest, γ-PGA yields increased significantly for the next 24 hours. The amount γ-PGA decreased for 92 hours (OD_{216} = 0.3897). For 72 hours, because γ-PGA is produced in the culture medium that increases the viscosity of the medium, resulting in the decline of dissolved oxygen amounts for the bacteria. In addition, the formation of γ-PGA transforms some of the carbonaceous compounds that are a source of nutrients for the bacterium into γ-PGA, which result in depletion of nutrients, therefore, the growth of bacteria in the culture medium is affected. (Candela and Fouet, 2006). The period from 72h to 96h, γ-PGA output decreased. The exhaustion of the substrate and oxygen during the formation of the γ-PGA has forced the bacteria to adapt to this condition. This leads to the creation of enzymes that degrade the γ-PGA polymer into glutamic monomers, and glutamic monomers continue to be a source of nutrients to the bacteria.From the above results showed that the optimum time for producing γ-PGA by 20.2 strain is 72 hours.

Effect of precursors on γ-PGA production

The studies were submitted of strains capable of biosynthesis γ-PGA shown that the source of precursors for the formation of γ-PGA is varied such as: L-Glutamic, Sodium Glutamate, Soybean powder, etc (Bajaj, 2011; Bhat, 2013; Zeng, 2012; Zhu, 2013).

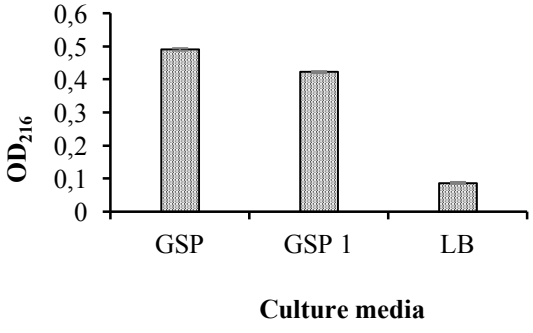

Figure 5. Effect of metabolic precursors on γ-PGA production

In order to assess the effect of the precursor source on γ-PGA biosynthesis, We cultured 20.2 strain in different media such as: GSP medium, GSP 1 medium with all glutamate sodium replaced by soybean meal and LB me-

dium. All media are in the same stable condition with temperatures of 37°C, pH 7 and shaking 200 rpm in 100 ml shake flasks. The chart in Figure 5 shows that the GSP medium is the medium for the highest production of γ-PGA with OD_{216} (after dilution 100 times) = 0.2625, GSP 1 medium containing soybean powder as a precursors sources producing lower γ-PGA yields. (OD = 0.2495). Whereas the LB medium does not contain γ-PGA precursors, γ-PGA yields is very low. Soybean power supplementation did not increase γ-PGA production because glutamic content in soybean was very low (10 g / kg).

Effect of Carbon sources on γ-PGA production

In the GSP medium, strain 20.2 uses glucose as a source of nutrients for growth. To be able to check glucose is really suitable nutrient source for strain 20.2. There is no need for appropriate carbon source research to help the 20.2 bacteria grow better, creating a premise for γ-PGA synthesis. Based on some other research, we chose lactose, lactose and saccharose instead of glucose for experiments (Cromwick et al., 1996). The experiment was conducted at the same temperature condition of 37°C, initial pH of 7 and shaken at 200rpm, cultured for 72 hours.

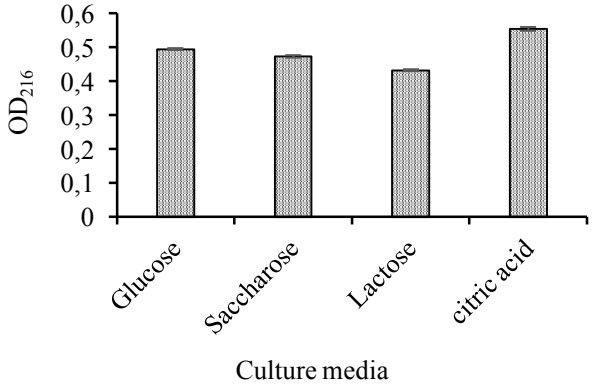

Figure 6. Effect of Carbon sources on γ-PGA production

Figure 6 shows the influence of carbon sources on the formation of γ-PGA. After 72 hours of culture, strain 20.2 using carbon source is citric acid produced the highest yield, the absorption at λ=216 nm is OD_{216} = 0.494. Cultured media with other carbon sources produced lower γ-PGA yields: glucose (OD_{216} = 0.494, saccharose (OD_{216} = 0.4723), lactose (OD_{216} = 0.4310). When citric acid acts as the main source of carbon for growth and development of bacteria, citric acid can be directly used without intermediate conversion. For glucose, lactose, and bacterial saccharose, the bacteria absorb these substances more slowly than citric acid because bacterial cells needs conversion time to be converted these substrates to intermediate compounds before citric acid (Shih et al., 2010). Thus, the use of citric acid for γ-PGA biosynthesis is most suitable for bacteria *Bacillus* sp. 20.2.

Effect of temperature on γ-PGA production

In order to find out the temperatures suitable for the biosynthesis of γ-PGA, we cultured 20.2 strain in 30ml GSP medium, pH 7, shaken 200rpm in shake flasks at different temperatures of 30°C, 37°C and 40°C. Finish culture after 72 hours, collect the cultures, γ-PGA was extract and measured OD_{216}. The results are shown in Figure 7.

The results of this experiment (Figure 7) show that 20.2 strain produced the highest γ-PGA at 37°C, OD216 = 0.4940. At 30°C, the γ-PGA yields were very low (OD216 = 0.2977). When strain was culture at 40°C, γ-PGA yields was obtained lower than γ-PGA yields at 37 (OD216 = 0.4687). Temperatures directly affect the growth of bacteria, so the temperature also directly affects the production of γ-PGA. In conclusion, the optimal temperature of incubation for the 20.2 strain was 37°C.

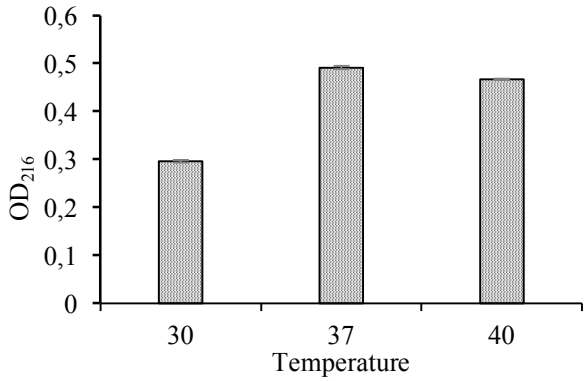

Figure 7. Effect of temperature sources on γ-PGA production

Effect of pH on γ-PGA production

The initial pH value significantly influences the growth of microbial cells. Each microorganism has a pH condition that is suitable for growth. To study the effect of pH on medium to strain 20.2, investigate at pH from 5 to 9, cultured 20.2 strain on 30ml GSP medium in 100ml shake flasks, shaken 200 rpm at 37°C for 72 hours. Then culture was taken, extracted and measured OD_{216}. The results are shown in Figure 8.

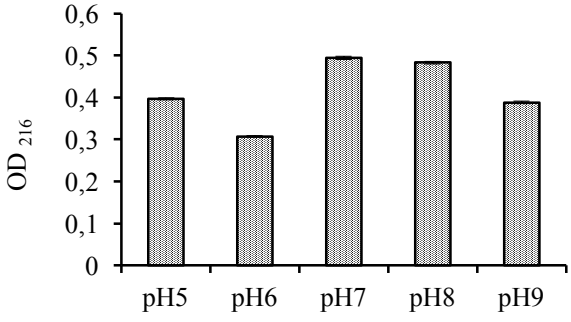

Figure 8. Effect of pH on γ-PGA production

The graph in Figure 8 shows the effect of pH on the GPA biosynthesis of the strain. 20.2. In the weak acidic medium (pH 5) and alkaline medium (pH 9), the γ-PGA production ability of 20.2 strain is very weak. The formation of γ-PGA increased sharply at pH 6-8. In the medium pH 7, the 20.2 strains produced the largest PGA yields (OD_{216}= 0.4940).At pH 6 and pH 8, the production of γ-PGA is lower and the OD_{216} measurement is 0.3967 and 0.4800 respectively. Thence, we can conclude that pH 7 is the optimal pH for γ-PGA production of 20.2 bacteria.

4. Conclusion

In conclusion, from 31 soil samples and 4 straws samples, we isolated 35 strains, of which 26 strains are capable of producing γ-PGA and 1 strains soil samples namely 20.2 were selected which produced the highest γ-PGA yields. By molecular biology method, we identified these two strains, strain 20.2 named *Bacillus* sp. 20.2. The suitable conditions for growing of *Bacillus* sp. 20.2 strain to produce γ-PGA are at 37°C, pH 7 after 72 hours. Replacing citric acid instead of glucose in a GSP medium is better for producing γ-PGA by strain *Bacillus* sp. 20.2.

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