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A new potential of calcium carbonate production induced by *Bacillus sphaericus* in batch fermentation

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Abstract: Bacteria is believed to induce calcium carbonate precipitation as it meets the concept of bioconcrete. *Bacillus sphaericus* was found to produce enzyme urease capable to hydrolyse urea, thus generate carbonate ions. The excess presence of calcium ions in the system will precipitate calcium carbonate. Therefore, the used of bioreactor to produce calcium carbonate can meet the demand for calcium carbonate as it can be produced in large-scale production thus maximize the production of calcium carbonate. The aim of this paper conducted by using 2.5 L bioreactor was to investigate the precipitation of $CaCO_3$ and to optimize the production of H_2CO_3 by using different parameters such as agitation and aeration. As this is a preliminary study, the artificial urea is used as the carbon source of the *Bacillus sphaericus*. The determination of the kinetics of $CaCO_3$ precipitation in the 2.5 L batch bioreactor such as specific growth rate (μ) and doubling time (Td) were studied after fermentations were stopped. The composition of calcium carbonate characteristic from the fermentation of *Bacillus Sphaericus* in 2.5 L bioreactor of pH 8.0 and 30 °C were proved by X-Ray Diffraction. The highest specific growth rate (0.141 h^{-1}) was obtained. The optimum parameters condition to produce bicarbonate ions and calcium carbonate was at 50 rpm with 2.0 vvm aeration rate.

Keywords: Calcium carbonate precipitation, *Bacillus sphaericus*, urea hydrolysis, submerged fermentation, kinetic study

1. Introduction

Microbiologically induced calcium carbonate precipitation (MICP) is a bio-geochemical process that create calcium carbonate precipitation (CCP) within the soil matrix [1]. During MICP, microorganisms can produce one or more metabolic products (CO_3^{2-}) that react with ions (Ca^{2+}) in the environment, thus create subsequent precipitation of minerals. Formerly, the formation of calcium carbonate precipitation was planned to occur via various mechanisms such as photosynthesis, urea hydrolysis, sulfate reduction, anaerobic sulfide oxidation, biofilm and extracellular polymeric substances [2]. However, the precipitation of calcium carbonate via urea hydrolysis is the most widely used method by using microorganism. [3,4,5].

The ammonium and carbonate were catalyzed by urease from the hydrolysis of urea. Urea hydrolysis begin when one mole of urea is hydrolysed to one mole of ammonia and one mole of carbonic acid, then it spontaneously hydrolysed to another one mole of ammonia and carbonic acid [6,7,8]. The ammonia (NH3) and carbonic acid (H_2CO_3) are then equilibrated in water to form bicarbonate and two moles of ammonium and two moles of hydroxide ions. The formation of hydroxide ions in the system will increase the pH level, which can shift the bicarbonate equilibrium, result in the formation of carbonate ions [9]. With the presence of calcium ions, the precipitation of $CaCO_3$ will be initiated if there are sufficient calcium ion and carbonate ion in the solution [5].

The role of CCP-capable bacteria to atmospheric CO_2 fixation and removal is substantial. Thus, various study on CCP-capable bacteria has been a steadily growing field [10,11,12,13] as it is able to solve environmental and industrial problems. The use of calcium carbonate precipitation that being microbially induced encompass the disposal of heavy metals and radionuclides, to enhance the quality of construction substances and the seclusion of CO_2 in atmosphere [2]. Calcium carbonate is being used to seal fractures and treat cracks in concrete and improve the strength and

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durability of cementious materials [14,15]. However, the increase of concentrations of CO_2 in the world atmosphere cause a major environmental global issue [16,]. An attempt was made to remove CO_2 from the environment by utilizing biotechnological application. Thus, the microbially induced calcium carbonate precipitation is an effective method by converting CO_2 into dissimilar carbonate minerals' crystals such as calcite, aragonite, dolomite and magnesite [2].

Different species of bacteria were recognized to be used to produce different amounts of urease and calcium carbonate precipitation. Bacillus group is a known species of bacteria used to produce urease and calcite precipitation [2]. Bacillus also has been reported as one of the bacteria that can induce carbonate minerals [18]. Their capability to develop well, can take up heavy metals and able to facilitate precipitation of calcium carbonate to form calcite has made the bacteria the auspicious microbes for biomineralization purpose in construction industry [19]. Thus, this study was focused on the potential of *Bacillus sphaericus* bacteria cell to produce calcium carbonate. The higher the concentration of bacterial cells of *B.sphaericus* can increase the precipitation of calcium carbonate from urea hydrolysis. For the growth of the bacteria, suitable pH and temperature has been decided to be at pH 8 and temperature of 30 °C because the pH 8 is the optimum pH for urease [6] and the enzyme will active only at particular pH for urea hydrolysis [2].

However, there has no study has been done on the production of calcium carbonate using bioreactor. From the previous, only a study has been conducted on the fermentation of *B.sphaericus* using shake flask to produce calcium carbonate and it is not reliable to be used in the industry [19]. Hence, fermentation in bioreactor is more suitable to be used for the calcium carbonate production in a large scale. The present study attempts to investigate the production of bicarbonate ions and calcium carbonate by *B.sphaericus* using urea medium in 2.5 L bioreactor. The aim of this study was to optimize the production of bicarbonate ion, HCO_3^- using different parameters include agitation and aeration. This study also was determined the kinetics of urea hydrolysis and $CaCO_3$ precipitation in the 2.5 L batch bioreactor such as specific growth rate (μ) and doubling time (Td).

2. Materials

The bacterial strain used in this work was *Bacillus sphaericus* (LMG 22257), bought from Belgian Co-Ordinated Collections of Micro-Organisms (BCCM). The strain was grown on urea agar containing 3 g/L nutrient broth (Bendosen), 2.12 g/L $NaHCO_3$(Merck) and 10 g/L urea(Merck) and 15 g/L agar powder(Merck). The strain was grown on urea agar for 24 hours at 30ºC in laboratory incubator (INFORS HT, Switzerland). The culture required two times sub-culturing before they can optimally be used in experiment (Limited, 2008).

3. Methods

3.1 Submerged fermentation

The starter culture was prepared by inoculating single colony of *B.sphaericus* into 200 mL of urea growth medium (20 g/L yeast extract and 20 g/L urea solution) in 500 ml flask. The flask was agitated on an orbital shaker at 100 rpm under 30 ºC. The 10% (v/v) starter culture was transferred into the sterile bioreactor.

The batch fermentation was carried out in 2.5 L bioreactor (Minifors, Infors HT, Switzerland) with working volume up to 1.5 L. The experiments were carried out at different parameters such as agitation (50, 100, 150 and 200 rpm) and aeration (1.0, 1.5, 2.0 vvm) to optimize the upstream parameters. The cultivation was continued for 24 h and the temperature was kept at 30 ºC. The pH of 8.0 was regulated by using 2 molar H_2SO_4 and 2 molar NaOH.

3.2 Determination of growth and kinetics fermentation

Two types of sampling were carried out, which were sampling for absorbance reading and sampling for the alkalinity test to determine the bicarbonate ions produced by *B.sphaericus*. The growth of *B.sphaericus* was measured for the optical density (O.D) by using UV-vis spectrophotometer (U-1900, HITACHI, Japan) until the stationary phase for 2 hours interval measured. The concentration bicarbonate ions were monitored by using a pH meter and the 50 mL sample was taken for every 4-hour interval to determine the HCO_3^- ion productivity.

3.3 Precipitation study

The cells were harvested and removed by centrifugation (Kubota, Japan) at 6,000 rpm for 10 minutes after fermentation. The alkalinity of the supernatant was determined by alkalinity test. To determine the alkalinity, 50 ml volume of sample was titrated with a standard solution 0.02 N H_2SO_4 to a pH value in the approximate endpoint of pH 4.5 (Snoeyink and Jenkins, 1980). The formula to calculate the concentrations of HCO_3^- is:

$$Alkalinity, mg\ HCO3^- / L = \frac{A \times N \times 50000}{50\ ml\ of\ sample}$$

Precipitation of calcium carbonate was establishing by adding calcium source which is calcium nitrate tetrahydrate, $Ca(NO_3)_2.4H_2O$ (Bendosen). After the concentration of bicarbonate ions were determined from the alkalinity test, equal molar of calcium source was added into the sample. The solution was mixed well and centrifuged at 4000 rpm for 10 minutes. The precipitates were rinsed three times with distilled water. The pure precipitates were filtered by medium-flow filter paper (Nice Chemicals, India) and were dried in the oven at 70°C for 24 hours prior to characterization analysis.

3.4 X-Ray Diffraction Analysis

Two grams of precipitate samples were analysed using X-ray diffractometer (Bruker D8 Advance, BRUKER, USA) with the help of semi-quantitive Topas software (BRUKER, USA).

4. Results and Discussion

4.1 Batch fermentation analysis

The results that has been studied at different agitation speed and aeration fermentation were summarized at Fig. 1. The fermentation process under agitation speed of 50 rpm with aeration rate 2.0 vvm producing the higher amount of bicarbonate ion. From the results on different aeration speed, the high oxygen supply to the strain enhances the growth of the bacteria to produce the bicarbonate ion. This is due to characteristic of *B.sphaericus*, which classified as a strict aerobe and oxygen has a strong regulatory effect [21]. However, the effect of agitation speed of bioreactor does not give significant effect on bicarbonate ion production. The research reveals slow agitation enough to mix oxygen, heat and nutrients well and be transferred efficiently in the bioreactor. Furthermore, slow agitation could reduce power consumption and prevent the shear forces which could ruptured the strain and thus effect the product formation [22].

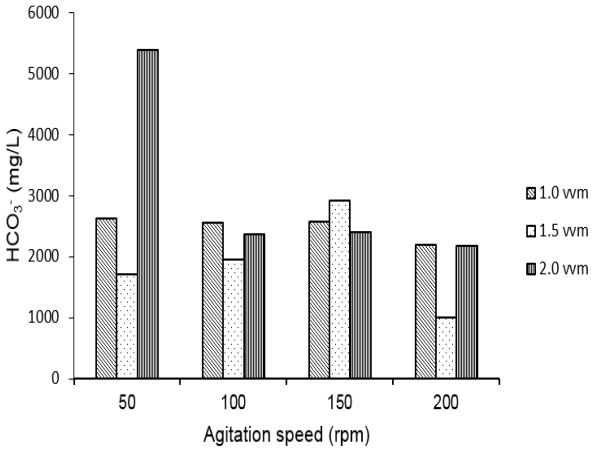

Fig. 1 Bicarbonate ion production

The amount of calcium carbonate produced has the correlation with the production of bicarbonate ion. After determining the concentration of bicarbonate ion from the alkalinity test, precipitation of calcium carbonate was initiated by adding the calcium source which was calcium nitrate ($Ca(NO_3)_2.4H_2O$). The results show that agitation speed of 50 rpm with aeration rate 2.0 vvm has the highest production of calcium carbonate (3.4 g/L).

Fig. 2 depicts the growth profile of *Bacillus Sphaericus* grown in 2.5 L bioreactor. The growth curve showed a lag phase from 0 to 2 h. The biomass increased rapidly form 2 h to 10 h, which marked the log phase. At 12 h, the growth reached stationary phase, and the death phase thereafter. The lag phase is very short as the bacteria already adapted to the favourable environment and had sufficient source to proliferate [22]. The maximum cell concentration (1.58 g/L) was obtained during the fermentation under agitation speed 50 rpm with aeration rate 2.0 vvm.

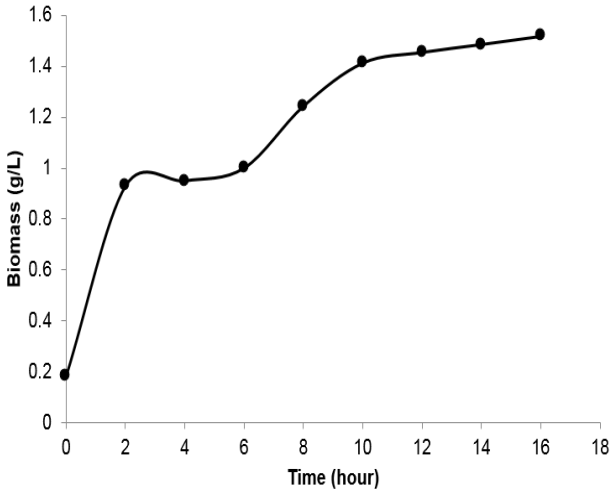

Fig. 2 The growth curve of *Bacillus Sphaericus* during fermentation at 50 rpm with 2.0 vvm.

Kinetic growth of bacteria results (Table 1) for different agitation and aeration showed the specific growth rate and the doubling time. It is to examine the kinetics of bicarbonate ion that being produced from *Bacillus Sphaericus*. From the experimental result, the highest specific growth rate achieved was 0.141 (h^{-1}) with 4.916 h to double itself. It showed that the agitation speed of 50 rpm with aeration rate 2.0 vvm was the most optimize condition to produce HCO_3^- ion. A higher specific growth rate is often desirable to grow cells faster to a desirable cell density as it can reduce fermentation time and thus improved overall productivity.

Table 1: The kinetic growth of bacteria at different agitation and aeration

Agitation speed (rpm)	Aeration (vvm)	Specific growth rate, μ (h^{-1})	Doubling time, Td (h)
50	1	0.053	13.078
	1.5	0.127	5.458
	2	0.141	4.916
100	1	0.035	19.804
	1.5	0.059	11.748
	2	0.106	6.539
150	1	0.086	8.060
	1.5	0.099	7.001
	2	0.123	5.635
200	1	0.072	9.627
	1.5	0.076	9.120
	2	0.071	9.763

4.2 XRD analysis

The calcium carbonate produced were subjected to XRD analysis to distinguish the stable polymorphs of calcium carbonate, calcite. By referring to diffractogram

of precipitates in Fig. 3 and Topas semi-quantitative analysis in Fig. 4, calcite has the highest-intensity peaks at relatively small °2θ. There was 100% rhombohedral calcite produced from addition of calcium nitrate tetrahydrate. It was analysed that 81% of the precipitates were in crystalline form while the rest was in amorphous form (19%). The average crystal size of calcite was calculated by the Topas semi-quantitative software as 133.7 nm. Therefore, this proved that the calcium carbonate produced from *B.sphaericus* has 100 % purity of calcite compared to other type of calcium carbonate such as from the limestone, chalk and marble.

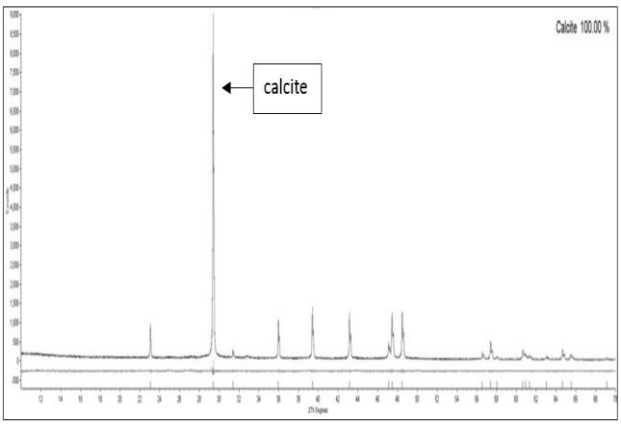

Fig. 3 Diffractogram of precipitates formed from addition of calcium nitrate tetrahydrate

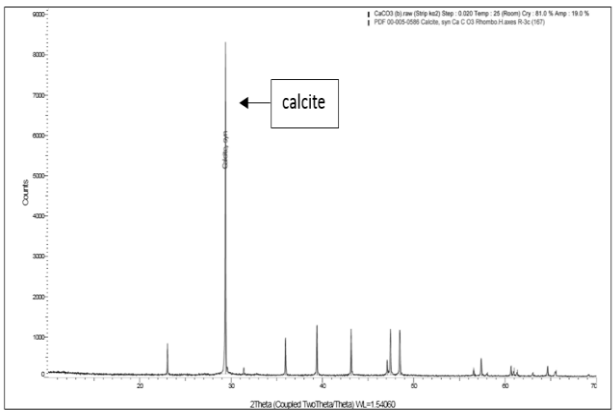

Fig. 4 Topas semi-quantitative analysis of precipitates formed from addition of calcium nitrate tetrahydrate

5. Summary

The growth rate and characteristic of *Bacillus Sphaericus* (LMG 22257) by using 2.5 L bioreactor was conducted. It showed agitation speed at 50 rpm and aeration rate of 2.0 vvm has the highest production rate of bicarbonate ion. At the same agitation speed and aeration also, it has the highest specific growth rate (0.141 h^{-1}). In overall, the precipitation of calcium carbonate by using calcium nitrate produced 100% purity of calcite compared to other type of calcium carbonate including limestone, chalk and marble. This study proved that the use of bioreactor has a potential for the large-scale production of calcium carbonate. Thus, this calcifying bacterium may be utilized for sealing the minor cracks in bioconcrete technology.

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