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Characterisation of Calcium Carbonate Formed by *Bacillus Sphaericus* Via Fermentation of Urea

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Abstract: Microbiologically induced calcium carbonate precipitation (MICCP) has emerged as rather promising green technology compares to conventional calcium carbonate process. *Bacillus sphaericus* LMG22557 is a superior MICCP-capable microorganism that can potentially utilise urea from diverse sources. This study focuses on maximising and characterising the calcium carbonate produced from fermentation of synthetic urea and waste urea from chicken manure. Morphologies of calcium carbonate attributed from fermentation of urea and precipitation by different calcium source were studied by microscopic observation, Fourier-Transform Infrared (FTIR) and X-Ray Diffraction (XRD). Precipitation from calcium chloride produced more vaterite while precipitation from calcium nitrate tetrahydrate produced pure calcite. Higher specific yield productivity (0.2502 mol/g cell/h) was achieved with 200 rpm and 40 g/L urea concentration. The growth of *B. sphaericus* in chicken manure solution was achievable but with very low yield of bicarbonate ions (less than 0.0008 mol/g cells/h). In brief, *B. sphaericus* is able to produce calcium carbonate from synthetic urea while further study is required for chicken manure to be used as substitute.

Keywords: Calcium carbonate, *Bacillus sphaericus*, MICCP, urea, chicken manure

1. Introduction

Calcium carbonate is a chemical compound that is extensively used as supplement, fillers and coating materials in a wide range of industries [1]. The two major classes of calcium carbonate are ground calcium carbonate (GCC) and precipitated calcium carbonate (PCC). PCC as the higher quality and purified material is often selected for more advance and demanding industries such as food and pharmaceutical industries [2]. However, both materials rely on limestone mining industry that is likely to cause severe environmental pollution . An alternative approach is to produce calcium carbonate via microbiologically induced calcium carbonate precipitation (MICCP). This promising technology offers an environmental friendly solution to the conventional mining activity.

Bacillus species are the common group of bacteria capable of performing MICCP through urea hydrolysis [3]. The precipitation process started with the hydrolysis of one mole urea to one mole ammonia and one mole carbamic acid. Spontaneous hydrolysis results in formation of one mole ammonia and one mole carbonic acid. Further equilibration of carbonic acid in water produces bicarbonate ion. The increase in pH causes the shifting of bicarbonate equilibrium that leads to the formation of carbonate ions. The carbonate ions are responsible in precipitation reaction when sufficient calcium ions are presence [4]. *Bacillus sphaericus* promotes high ureolytic activity, suggesting that high productivity of calcium carbonate [5]. Crystal shape and anhydrous polymorphs of the precipitate formed by the bacteria depends on calcium sources used in the precipitation reaction [6], [7].

Based on Chastain, et al. [1] [8] chicken manure contains elements such as nitrogen (N), phosphorous (P), potassium (K), and other metal elements. Most of nitrogenous waste in chicken manure is in the form of uric acid and urea [9], [10]. The former might be converted into the latter during the first week excretion through microbial activity. Hence, waste urea from chicken manure has the potential to substitute synthetic urea used in conventional MICCP. The success of this process will lower the cost and improve the sustainability of MICCP.

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To date, most published MICCP study using *B. sphaericus* have been focused on self-healing concrete application albeit rather high calcite production [11], [12]. This research attempts to maximise production of bicarbonate ions, compare the productivity of MICCP using different sources of urea i.e. waste urea from chicken manure and synthetic urea, and characterise the precipitates from addition of different calcium source. In brief, this research serves to improvise conventional MICCP using abundant and low cost substrate.

2. Methodology

Two different substrates namely synthetic urea and waste urea from chicken manure were used in this study. Additional parameter, effect of urea concentration was studied only for the former as according to Arunachalam et al. [22].

2.1 Inoculum preparation

Bacillus sphaericus LMG 22557 (Belgian Coordinated Collection of Microorganisms, Ghent) was used in this research. The UYE medium formulation was prepared according to that suggested by Shirakawa et al. [14], which consisted of 20 g/L yeast extract (HiMEDIA, India) and 20 g/L synthetic urea (Bendosen Laboratory Chemicals, Norway). The pH of medium components was adjusted to 8. Both solutions were autoclaved for 15 minutes at 121°C and mixed afterward.

The inoculum was prepared by subculturing the pure bacterial colony that grown on UYE agar plate into 8 ml UYE broth then into 72 ml UYE broth (10% serial transfer). The subculture was incubated at 30°C and 100 rpm using the incubation shaker (Ecotron, Infors HT, Switzerland). The bacterial growth was monitored by measuring the absorbance of the culture using UV-*vis* spectrophotometer (U-1900, HITACHI, Japan). Incubation period was decided based on exponential growth phase of the bacteria.

2.2 Fermentation of synthetic urea

The fermentation was performed in 2 L shaker flask with working volume of 0.8 L. The effect of urea concentration on the growth rate of *B. sphaericus* and the production of bicarbonate acids was carried out by using 20 g/L yeast extract and three levels of synthetic urea; 20, 30 and 40 g/L of synthetic urea. As predetermined in preliminary experiment (not shown), 200 rpm of agitation speed and incubation temperature at 37°C were used as the fermentation condition. During the fermentation, the absorbance reading and the concentration of bicarbonate acids of the culture were determined in intervals of 2 hours until stationary phase was reached. Next, the cells were harvested and removed by centrifugation (Kubota, Japan) at 6,000 rpm for 10 minutes.

Each level of parameters was studied using two replicates. The specific growth rate of *B. sphaericus* and specific yield productivity of bicarbonate acids were calculated. The statistical significance of data was analysed by one-way analysis of variance (ANOVA) and Tukey HSD's post hoc test which were conducted using SPSS version 20.0 (IBM Corp., New York).

2.3 Fermentation of chicken manure

The chicken manure was collected from hen, *Gallus gallus domesticus* of 6 month-old reared at Animal Research and Service Centre (ARASC) of Universiti Sains Malaysia, Penang, Malaysia.

A 250g of hen manure was weighed and mixed in 1 L of distilled water. The mixture was stirred and mixed well. Then, the solution was centrifuged at 9,000 rpm for 10 minutes. The supernatant was filtered using medium-flow filter. After the second centrifugation and filtration, the supernatant was autoclaved at 121°C for 15 minutes.

The growth of *B. sphaericus* and bicarbonate acids production were studied without yeast extract (0 g/L) and with yeast extract (20 g/L). The absorbance reading of the culture and bicarbonate acids concentration was determined as described in section 2.2. This study was performed in triplicates. The specific growth rate of *B. sphaericus* and specific yield productivity of bicarbonate acids were calculated.

2.4 Alkalinity test

Total alkalinity was determined according to Snoeyink and Jenkins [2] [15]. A 50 mL sample (supernatant) was collected and titrated with 0.02 N sulfuric acid, H_2SO_4. The initial pH and pH changes were measured using pH meter (pH 510, EUTECH Instruments, USA). The volume of sulfuric acid titrated to reach the end points of the reactions (from pH 8.3 to pH 4.5) was calculated and recorded.

Total alkalinity was calculated based on the folowing formula:

$$Total\ alkalinity, mg\ HCO_3^-/L = \frac{A\ x\ N\ x50000}{50mL\ of\ sample}$$

Where A = Volume of standard acid used (mL) and N = Normality of standard acid used

2.5 Precipitation

Precipitation of calcium carbonate was initiated by addition of calcium salt, calcium chloride, $CaCl_2$ (Bendosen Laboratory Chemicals, Norway) or calcium nitrate, $Ca(NO_3)_2.4H_2O$ (Bendosen Laboratory Chemicals, Norway). After the concentration of bicarbonate acids were determined from the alkalinity test, equal molar of calcium salt was added into the sample. Then, the solution was mixed well.The precipitates were allowed to settle down and it was filtered by medium-flow filter paper (Nice Chemicals, India). The precipitates were washed thrice using distilled water and were oven-dried at 70°C for 24 hours prior to characterisation analysis.

2.6 Identification and Characterisation of Precipitates

a. Compound Microscopy

The precipitates were suspended in distilled water and were observed under light microscope (OLYMPUS, Japan) with appropriate magnification. The microscopic images of the precipitates were captured using image analyser software (OLYMPUS, Japan).

b. Fourier-Transform Infrared (FTIR) analysis

A 2.0 mg of dried sample was weighed and potassium bromide, KBr (Sigma-Aldrich, USA) was added until total weight of 100 mg was reached. The sample mixture was grinded using an agate mortar and pestle. Then, the sample mixture was pressure-pressed using a press (Carver, USA) for few minutes until a transparent thin plate was formed. The plate containing the sample was put onto the sample holder and was analysed using FTIR at wavelength ranges of 4000-400 cm^{-1}. The FTIR spectra were analysed using OMNIC software (Thermo Fisher Scientific, USA).

c. X-Ray Diffraction (XRD) analysis

A 2-3 g of precipitate was weighed and heated in the oven at 105°C for 1 hour. Then, the dried precipitate was allowed to cool down inside a desiccator. The samples were then carefully put into the sample space to fill the sample space compactly. The filled sample space was attached to the sample holder before they were transferred into the X-ray diffraction machine. The precipitate samples were analysed using X-ray diffractometer (Bruker D8 Advance, BRUKER, USA) with the help of semi-quantitive Topas software (BRUKER, USA).

3.0 Results and Discussion

3.1Compound microscopy

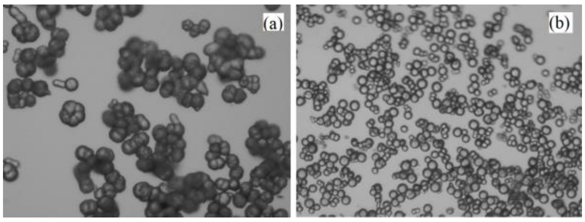

Fig. 1 Calcium carbonate produced from addition of calcium nitrate tetrahydrate (a) and calcium chloride (b) under microscope (100X)

The precipitates formed from addition of calcium nitrate tetrahydrate and calcium chloride are morphologically different (Fig. 1). However, both the former and the latter are relatively homogenous spherical in shape while the former is exceptionally larger in particle size.

3.2 Fourier-Transform Infrared (FTIR) analysis

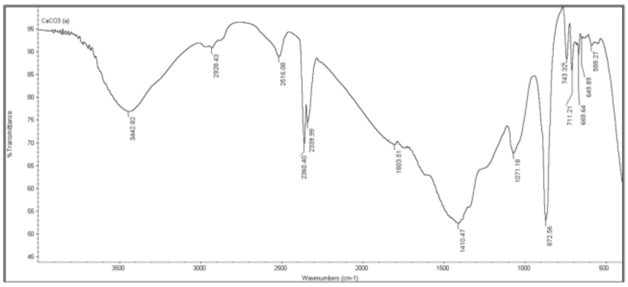

Fig. 2 FTIR spectra of precipitates formed from addition of calcium chloride into fermentation supernatant.

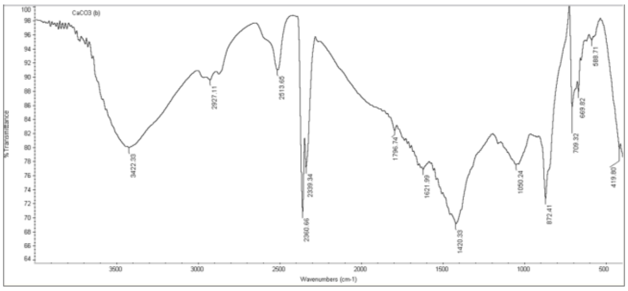

Fig. 3 FTIR spectra of precipitates formed from addition of calcium nitrate tetrahydrate into fermentation supernatant

Fig. 2 shows FTIR spectra of the precipitates indicating that strong absorption around the wavelengths of 2928, 2516, 1804, 1410, 872 and 711 cm^{-1} while Fig. 3 shows strong absorption around the wavelengths of 2927, 2514, 1797, 1420, 872 and 709 cm^{-1}. This indicates the presence of calcium carbonates among the precipitates. According to Ji, et al. [8][16], carbonate of calcite would incur significant absorption in various range of wavelengths (cm^{-1}) such as 3050-2850, 2650-2500, 1820-1790, 1500-1400, 877, 730-710. The different absorption spectra were caused by different vibration modes of carbonate ions of calcium carbonates which consisted of symmetric stretching, out-of-plane bending, double degenerate planar asymmetric stretching and double degenerate planar bending [17], [18]

3.3 X-Ray Diffraction (XRD) analysis

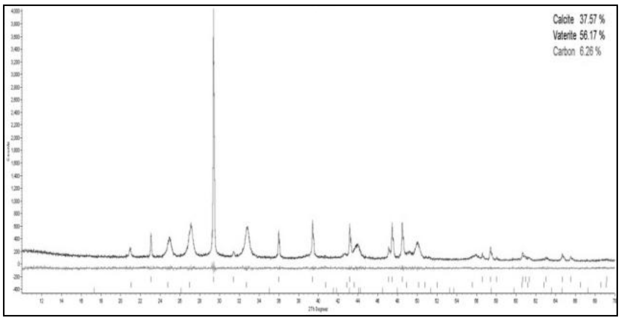

Fig. 4 Diffractogram of precipitates formed from addition of calcium chloride (Calcite 37.57%, Veterite 56.17% and 6.26%)

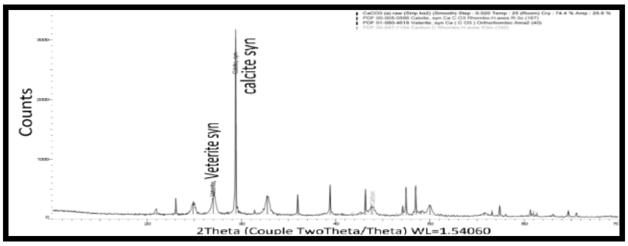

Fig. 5 Topas semi-quantitative analysis of precipitates formed from addition of calcium chloride (Crystal 74.4% and 25.6%).

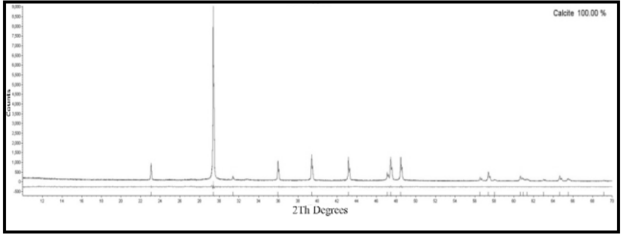

Fig. 6 Diffractogram of precipitates formed from addition of calcium nitrate tetrahydrate (Calcite 100%)

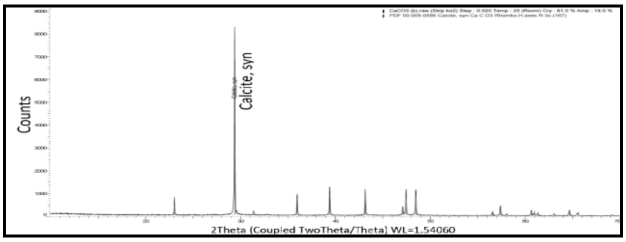

Fig. 7 Topas semi-quantitative analysis of precipitates formed from addition of calcium nitrate tetrahydrate (Crystal 81% and amp 19%)

Fig. 4 shows that precipitate formed from addition of calcium chloride consisted of calcite and vaterite polymorphs. The sizes of the particles were estimated on average 115.4 nm for calcite and 32.0 nm for vaterite. From Topas semi-quantitative analysis (Fig. 5), more vaterite (56.17%) was formed than calcite (37.57%) when calcium chloride was used for precipitation.

Fig. 6 and Fig. 7 present 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.

3.4 Effect of urea concentration on growth rate of *B. sphaericus* and production of bicarbonate acids

A one-way analysis of variance was conducted to evaluate the null hypothesis that there was no difference in growth rate or specific yield productivity when different urea concentration was used during the fermentation. The growth rate of *B. sphaericus* according to three levels of urea concentration were 20 g/L (M = .5868, SD = .002899), 30 g/L (M = .4876, SD = .01520) and 40 g/L (M = .4132, SD = .01167). While, for specific yield productivity were 20 g/L (M = .5868, SD = .002899), 30 g/L (M = .4876, SD = .01520) and 40 g/L (M = .4132, SD = .01167). The Welch test was significant for grow rate (p = .019) and specific yield productivity (p = .008). Therefore, it could be concluded that there was statistical significant difference on the specific growth rate and specific yield productivity when different levels of urea concentration were used in the fermentation. All pairs of means of specific growth rate and specific yield productivity for different agitation speed were significantly different at confidence level of 95% since p<.05.

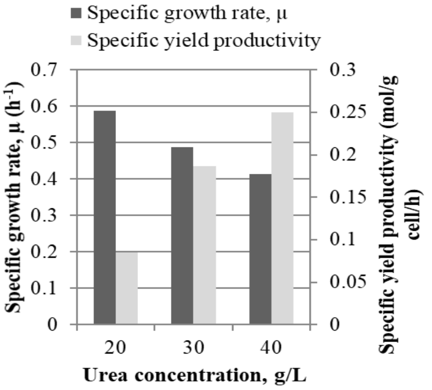

Fig. 8 Specific growth rate of *B. sphaericus* and specific yield productivity of bicarbonate acids when different urea concentrations were used during fermentation

Specific growth rate of *B. sphaericus* showed decrement trend while the specific yield productivity of bicarbonate acids showed increment trend with increasing urea concentration used during fermentation (Fig. 8). The increase in urea concentration contributed more substrate for urease produced by the bacteria, thus, increasing ureolytic activity. Eventually, more bicarbonate acids were produced. According to Krajewska [9][19], urease normally obeyed Michaelis-Menten rules where the enzyme activity increased with concentration of substrate. Ureolytic reactions produced ammonia during the process [20].

The concentration of ammonia produced in the culture increased as the ureolytic activity increased. Leejeerajumnean, et al. [10][21] discovered that up to certain concentration of ammonia might inhibit the growth of some bacteria. Therefore, the specific growth rate of *B. sphaericus* decreased may due to the increasing ammonia production.

3.5 Potential of chicken manure for production of bicarbonate acids

The growth rate of *B. sphaericus* according to presence of yeast extract were 0 g/L (M = .132, SD = .00611) and 20 g/L yeast extract (M = .142, SD = .00171). While specific yield productivity of the bacteria were 0 g/L (*M* = .000484, *SD* = .0000292) and 20 g/L (*M* = .000748, *SD* = .000177). There were no significant difference for grow rate (p=.055) and specific yield productivity (p= .120) when yeast extract was absence or presence at 20 g/L during the fermentation.

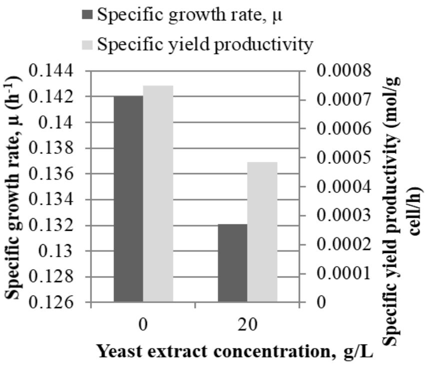

Fig. 9 Specific growth rate of *B. sphaericus* and specific yield productivity when fermentation was conducted using chicken manure with and without addition of yeast extract

The specific yield productivity of bicarbonate acids produced by *B. sphaericus* in chicken manure were very low which were less than 0.0008 mol/g cell/h (Fig. 9). However, growths of *B. sphaericus* were observed in chicken manure solutions, both with ($\mu = 0.142\ h^{-1}$) and without yeast extraction ($\mu = 0.1321\ h^{-1}$). Pretreatment of chicken manure prior to usage and supplementary synthetic urea during fermentation might be needed to produce higher bicarbonate acids.

4.0 Conclusion

Precipitation of carbonates ion using calcium nitrate tetrahydrate produced mostly calcite while vaterite was mainly produced when calcium chloride was used for precipitation of calcium carbonate in fermentation supernatant without cells. All calcite despite the precipitation methods, had sizes around 110-130 nm. Higher specific yield productivity (0.2502 mol/g cell/h) was gained with 40 g/L urea concentration and 200 rpm. Although very low specific yield productivity (less than 0.0008 mol/g cell/L) was obtained in fermentation using chicken manure, chicken manure could be potential additional nutrient for bacteria growth as growth of *B. sphaericus.*

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