

Article

Bioleaching of Gold from Silicate Ore by *Macrocooccus caseolyticus* and *Acinetobacter calcoaceticus*: Effect of Medium, Amino Acids and Growth Supernatant

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Abstract: The aims of this work were to study the gold leaching by the isolated bacteria from silicate ore. Three strains were isolated and identified as *Macrocooccus caseolyticus*, *Acinetobacter calcoaceticus*, and *Bacillus sp.* MBEA40. However, only *M. caseolyticus* and *A. calcoaceticus* were capable of gold bioleaching. In order to examine only the effect of microorganisms involved in the gold bioleaching process, minimal medium and ethanol mineral salt medium without amino acids were used for culturing *M. caseolyticus* and *A. calcoaceticus*, respectively. The result showed that the growth supernatant (in the absence of microorganisms) of both strains might be more suitable to leaching gold from ore than leaching by microorganisms (in the presence of microorganisms) directly. This might be due to the fact that there is no interference of gold absorption and metal toxicity in microorganisms in the long-term operation. The result also confirmed that amino acids/peptides/proteins produced by microorganisms might be involved in gold bioleaching, as shown in the high-performance liquid chromatography (HPLC) results. The Fourier transform infrared spectroscopy (FTIR) study also found that amine groups and carboxylic groups played important roles in gold bioleaching by *M. caseolyticus* and *A. calcoaceticus*. In addition, the bioleaching process had significantly higher gold leaching than mixed pure amino acids due to the growth supernatant containing mixed amino acids/peptides/proteins and other compounds. Therefore, the growth supernatant of *M. caseolyticus* and *A. calcoaceticus* can be applied in gold bioleaching under neutral pH conditions, which is considered to be a safe, not corrosive, and environmentally friendly leaching process. This study is also needed further study in order to increase the percentage of gold bioleaching and decrease times.



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Keywords: amino acids/peptides/proteins; gold; bioleaching; *Macrocooccus caseolyticus*; *Acinetobacter calcoaceticus*

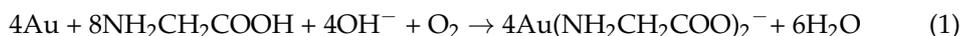
1. Introduction

Generally, the gold extraction process from ores is the cyanidation method [1]. This method is the greatest reagent for the gold extraction process and is widely used in various mining areas worldwide. Concurrently, cyanide has high chemical toxicity for human health and causes environmental problems such as the leakage of cyanide into groundwater, which has a strong effect on humans [1]. Moreover, this method is difficult to manage and incurs high operating costs [2]. Alternative methods for gold extraction processes that are less toxic, effective, and inexpensive need to be implemented [3,4].

Various methods have been suggested as the alternative way to extract gold, including using thiocyanate, thiourea, thiosulphate, bromine, and chloride [5–7]. However, these methods also have negative effects on gold extraction; for instance, the thiosulphate system has high reagent consumption and leads to gold extraction being performed in a less

efficient manner [3,4]. Therefore, the cyanidation process is still used in various mining industries. Another method of interest with a lesser effect on human health and the environment is bioleaching. This method represents the ability of microorganisms to extract gold from various sources, such as electronic waste and mineral ores. Previously, researchers have proposed various strains that were capable of extracting gold, such as *Acidithiobacillus*, *A. ferrooxidans*, and *A. thiooxidans* [8–13]. Nevertheless, *Acidithiobacillus*, *A. ferrooxidans*, and *A. thiooxidans* are grown in extreme environments, for example, high temperatures and acidic conditions, which can increase the cost of treatment and need further management of the harmful end product. Thus, the ability of other strains needs to be investigated under mild conditions such as at neutral pH (pH 7) and moderate temperature (30–35 °C).

Another alternative way also interests, which is using amino acids; it has been widely applied to the gold extraction process. Moreover, the mechanism of gold–amino acid bonds has previously been reported by Komnitsas [14] and Reith [15]. The complexes formed between gold and amino acids are mainly through binding with nitrogen atoms in the amine group to form a covalent bond, while the oxygen atom in the carboxylic group could form ionic bonds. On the other hand, Eksteen and Oraby [16,17] and Altinkay [18] also proposed that gold can be bound with glycine, as shown in Equation (1). Oraby's [19,20] study also confirmed amino acids as a reagent, which can be used to leach gold, replacing traditional methods. In addition, the study of Maneesuwannarat [21,22] found that gallium can be bound with amino acids as produced by *Cellulosimicrobium funkei*, which was isolated by contaminated soil. However, the real process of gold extraction depends on other factors, particularly pH, oxidizing agents, and oxidation-reduction potential (Eh) of the system. A study by Brown [23] explained that the extraction efficiency might be increased when using hydrogen peroxide as an oxidizing agent in copper extraction.



Then, the aim of this study was to investigate the isolated strains that had the capability to extract gold from ore, as well as to examine the way in which these strains can leach gold under mild conditions. Firstly, three isolated microorganisms from silicate ore were selected to study an effect on gold bioleaching. Meanwhile, the percentage of gold bioleaching by isolated strains was investigated. High-performance liquid chromatography (HPLC) and Fourier transform infrared spectroscopy (FTIR) were used to study the compounds that these strains produced and the function groups of the compounds that interact with gold, respectively. In addition, gold leaching by mixed synthetic amino acid concentrations found in growth supernatant and growth supernatant was also compared.

2. Materials and Methods

2.1. Ore Sample Preparation

The ore sample was collected from a gold mining site area in Pichit Province, Thailand. The composition of ore was determined by X-ray fluorescence (XRF), as shown in Table 1. Gold content cannot be detected, because it is too low a concentration, which the XRF can detect. Then, one gram of ore was digested by nitric acid (HNO₃) and hydrochloric acid (HCl) and analyzed by inductively coupled plasma spectroscopy (ICP-OES JY 2000). The initial gold concentration was approximately 16.54 mg/Kg. The ore was grounded and sieved through a standard 200 mesh screen (particle size smaller than 75 µm). Silicate ore was sterilized by autoclaving before being used in the bioleaching experiment (121 °C for 20 min).

2.2. Screening and Isolate of Microorganisms for Gold Bioleaching

In order to study the gold bioleaching by isolated strains from silicate ore, one gram of ore was inoculated in a Luria Bertani medium (LB: 1% tryptone, 0.5% yeast extract, and 1% sodium chloride) and incubated for 24 h at 150 rpm and 30 °C. A total of 1 × 10⁸ CFU/mL of initial stock was used for the serial dilution method. A total of 1 mL of initial stock was

mixed with 9 mL of LB medium including 10^{-1} to 10^8 times, respectively. Afterward, 20 μ L of 10^{-3} to 10^6 was pipetted into the LB agar medium using the spread plate technique and incubated at 30 °C for 3 days. Three different colonies were found and also used to investigate gold bioleaching efficiency in this experiment.

Table 1. Chemical component of ores (wt%).

SiO ₂	CaO	Al ₂ O ₃	MgO	Fe ₂ O ₃	K ₂ O	SO ₃	MnO	TiO ₂	ZnO
65	9.61	7.6	6.67	4.2	3.35	1.69	0.42	0.238	0.191
Na ₂ O	P ₂ O ₅	BaO	PbO	CuO	V ₂ O ₅	Ag	SrO	Cr ₂ O ₃	Rb ₂ O
0.143	0.0942	0.0811	0.0437	0.0348	0.031	0.0269	0.0079	0.00722	0.00575

2.3. Microbial Identification

Three microorganisms isolated from silicate ore were identified, and characterization followed the method of [21]. Then, the phylogenetic tree was used for checking the percentage of similarity with the NCBI database, performed by the MEGA7 software (Pennsylvania State University, Centre County, PA, USA).

2.4. Gold Concentration Analysis

After bioleaching experiments, the sample was left for 30 min to precipitate the suspended particles of the ore. Then, a 4 mL liquid sample was collected and filtered by filter paper for measuring by ICP-OES JY 2000. Gold standard for ICP-OES JY 2000 was used (SIGMA-ALDRICH Switzerland). The efficiency of gold bioleaching from ores was calculated according to the following Equation (2):

$$\% \text{ Au leaching} = C_L \times 100 / C_I \quad (2)$$

where C_I is the initial gold concentration = 16.54 mg/Kg, and C_L is the gold concentration after the bioleaching process.

2.5. Bioleaching of Gold by Isolated Microorganisms

2.5.1. Bioleaching by Microorganisms (in the Presence of the Microorganism)

Pure culture of three isolated microorganisms (*Macrocooccus caseolyticus*, *Acinetobacter calcoaceticus*, and *Bacillus sp.* MBEA40) was prepared in various media, including the LB medium, growth medium, minimal medium, and ethanol mineral salt medium. Composition of the LB medium contained 0.5% yeast extract, 1% tryptone, and 1% sodium chloride, while growth medium contained 0.5% yeast extract, 0.5% glucose, 1% casein peptone, and 0.5% sodium chloride. The composition of the minimal medium was 0.5% glucose, 4 g NH₄SO₄, 0.3% KH₂PO₄, 0.6% of Na₂HPO₄, and 0.05% NaCl [23]. In addition, the ethanol mineral salt medium (500 mL) also contained 10.9 g of K₂HPO₄·3H₂O, 3.57 g of KH₂PO₄, 2 g of NH₄SO₄, 0.1 g of MgSO₄·7H₂O, and 2% of Ethanol. Then, 10% (*v/v*) of 1×10^8 CFU/mL was added in a 250 mL Erlenmeyer flask containing each medium and one-gram autoclaved ore. The experiments were set in triplicate and shaken at 150 rpm, 30 °C, at various times (1 day, 7 days, 15 days, and 28 days).

2.5.2. Bioleaching by Growth Supernatant (in the Absence of the Microorganism)

The growth supernatant was prepared by adding 10% (*v/v*) of 1×10^8 CFU/mL as a pure stock isolated microorganism in 250 Erlenmeyer flasks containing each medium including the LB medium, growth medium, minimal medium, and ethanol mineral salt medium. Then, the growth supernatant was taken after 3 days of cultivation and centrifuged at 4000 rpm for 10 min. The growth supernatant was separated by filtering through a membrane filter (CA 0.2 μ Sartorius stedim biotec). Then, 25 mL of growth supernatant was added to the 250 mL Erlenmeyer flask containing 1 g of autoclaved ore. The experiments were set in triplicate and shaken at 150 rpm, 30 °C, at various times (1 day, 7 days, 15 days, and 28 days).

2.6. Amino Acid Analysis

2.6.1. Derivatization of Standards and Samples

Amino acid analysis was performed according to the protocol from Maneesuwannarat [21,22].

2.6.2. Chromatographic Conditions

The samples were run by HPLC. The HPLC system was an Alliance Water Model E2695 Pump with an autosampler (Milford, MA, USA). The condition for analysis of samples was following Maneesuwannarat [21,22]. The mobile phase including phase B was 100% of acetonitrile, phase C was pure water, and phase D was acetate buffer. The wavelength of fluorescence for excitation and emission were set at 250 and 395 nm. The data were analyzed using Millennium software (Millennium Systems International, Parsippany, NJ, USA)

2.7. Gold Leaching by Mixed Amino Acids

In order to confirm that growth supernatant of isolate strain can leach gold from silicate ore, the efficiency of gold bioleaching by growth supernatant containing amino acids/peptides/proteins was compared to mixed synthetic amino acid under the same concentration found in the growth supernatant. Twenty-five milliliters of mixed amino acids (including aspartic acid, serine, glutamic acid, glycine, histidine, arginine, threonine, alanine, proline, cysteine, tyrosine, valine, methionine, lysine, isoleucine, leucine, and phenylalanine) at the same concentration of growth supernatant of both strains was added in the 250 mL Erlenmeyer flask containing 1 g of autoclaved ore. The experiments were set in triplicate and shaken at 150 rpm, 30 °C, for 15 days.

2.8. Fourier Transform Infrared Spectroscopy (FTIR) Analysis

FTIR was used to investigate functional groups involved in gold bioleaching. The sample solution was taken from the growth supernatant of *M. caseolyticus* and *A. calcoaceticus* before and after gold bioleaching experiments. These samples were centrifuged at 4000 rpm for 5 min. Then, the samples were filtered using a membrane before use. The clear solutions were filtered again using a 0.2 µm membrane filter. The sample was measured by FTIR (Bruker TENSOR 27 instrument). Analysis was performed using OPUS software version 6.0 and IR mentor PRO. The FTIR spectra were recorded from 4000–500 cm⁻¹.

2.9. Statistical Analysis

Data were analyzed by SPSS statistics version 17 to perform a one-way analysis of variance (ANOVA); significantly different means were assessed by Duncan's multiple range tests at a 95% confidence level.

3. Results and Discussion

3.1. Screening, Isolation, and Ability of Gold Bioleaching Bacteria

Microorganisms used in this study were screened and isolated from silicate ore. Three strains were isolated including Y1, W1, and E1; all of these were identified by 16S rRNA sequencing analysis. The results indicated that the Y1 strain belonged to *Macrococcus caseolyticus*, W1 was identified as *Acinetobacter calcoaceticus*, and E1 was *Bacillus* sp. MBEA40, respectively, as shown in Figure 1. Afterward, isolated strains were investigated for abilities of gold bioleaching from silicate ore. The results of gold leaching were represented in LB medium individual (as a control), in the presence of the microorganism and growth supernatant (in the absence of the microorganism) as shown in Figure 2. The strain *M. caseolyticus* (Y1) had the highest efficiency to leach gold from ore approximately 46% after 15 days of cultivation under growth supernatant conditions (Figure 2A), while *A. calcoaceticus* (W1) was able to extract gold approximately 42% after 7 days of cultivation in the presence of microorganism conditions (Figure 2B). However, after 15 days, gold bioleaching was decreased from 42% to 23% in the presence of microorganism conditions

(Figure 2B). It was due to the fact that this organism itself can absorb gold into its cell that gold leaching efficiency was decreased. Interestingly, gold can be leached by the LB medium itself (control), at a level of approximately 14–24% after 15 days (Figure 2). This is consistent with the previous studies by Maneesuwannarat [21,22] showing that amino acids in the LB medium can leach gallium from gallium arsenide. This was possible because amino acid contained in the medium can be affected by gold bioleaching, while the efficiency of gold bioleaching by *Bacillus* sp MBEA40 had a similar level as the control condition (20%). It might be possible that this strain could not grow well and led to lower ability in gold bioleaching (Figure 2C). Therefore, in this study, the LB medium was changed to other mediums that contained no amino acids in subsequent studies. Then, *M. caseolyticus* and *A. calcoaceticus* were chosen for study in the next experiments.

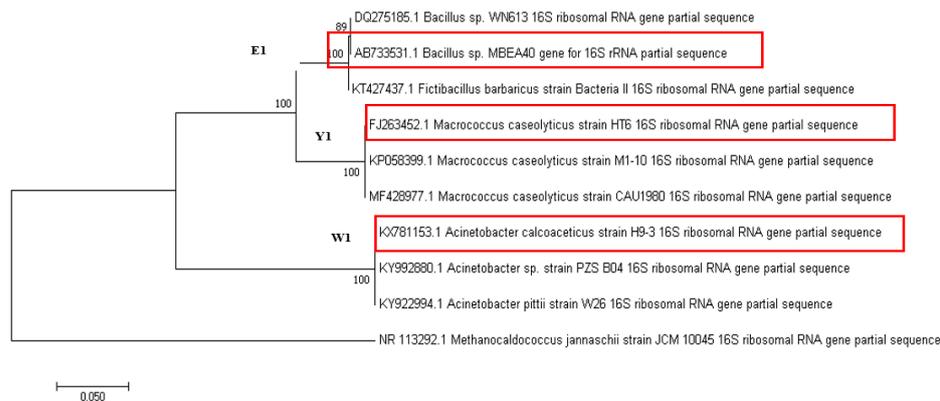


Figure 1. Phylogenetic tree of isolated strains from silicate ores, based on 16 rRNA sequencing analysis and using the MEGA7 software for generating the phylogenetic tree.

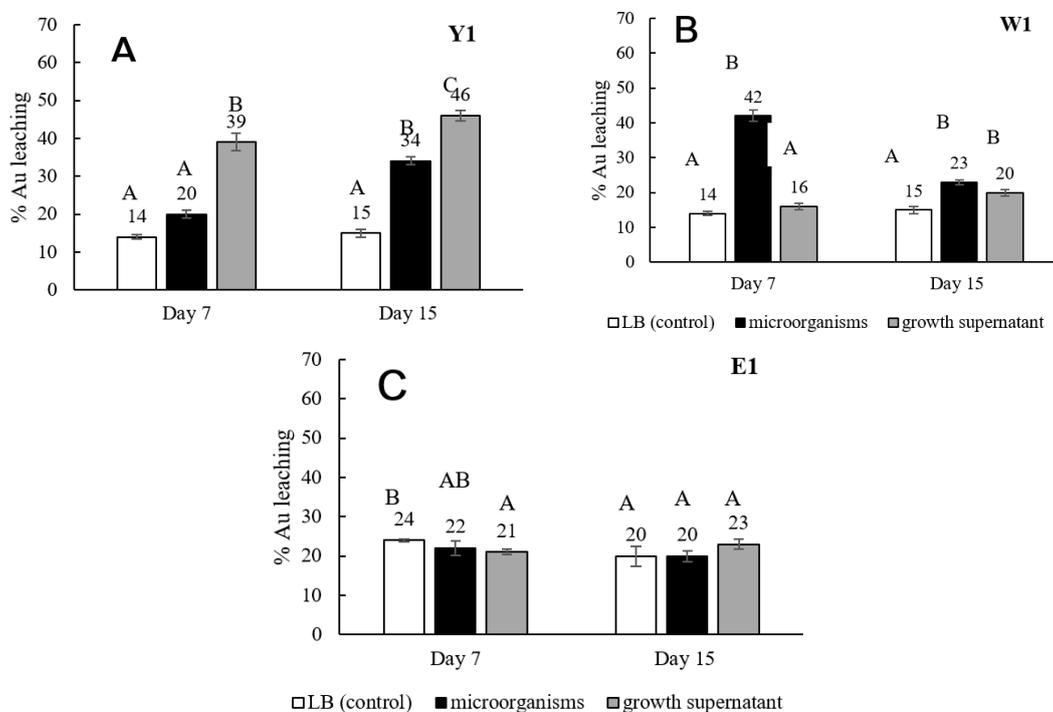


Figure 2. Bioleaching of gold from silicate ores by (A) *Macrocooccus caseolyticus* (Y1), (B) *Acinetobacter calcoaceticus* (W1), and (C) *Bacillus* sp. MBEA40 (E1) under 7 days and 15 days. Various strains were cultured in Luria Bertani medium (LB) and shaken at 150 rpm, 30 °C (a particle size of ores approximately $\leq 75 \mu\text{m}$). Data were expressed as the mean \pm SD ($n = 3$). Data were analyzed by Duncan’s test ($p < 0.005$). The capital letters compared the percentage of gold bioleaching by three isolated strains (A—Y1, B—W1, and C—E1) under control, in the present of microorganism and growth supernatant

3.2. Effect of Medium Component on Gold Bioleaching from Ores

Based on the result in Figure 2, LB medium solely can leach approximately 14–24% of gold from ore after 7 days. To avoid the effect of the medium, isolated strains (including *M. caseolyticus* and *A. calcoaceticus*) under the minimal medium (MMg) were also investigated for the efficiency of gold bioleaching. The result showed that the efficiency of gold bioleaching in the presence of the microorganism and growth supernatant by *M. caseolyticus* under the minimal medium was approximately 30% and 29%, respectively (Figure 3A), which was not significantly different. This was consistent with gold bioleaching by *A. calcoaceticus* under the minimal medium (MMg), which was also not significantly different in the presence of cells and growth supernatant condition (Figure 3B).

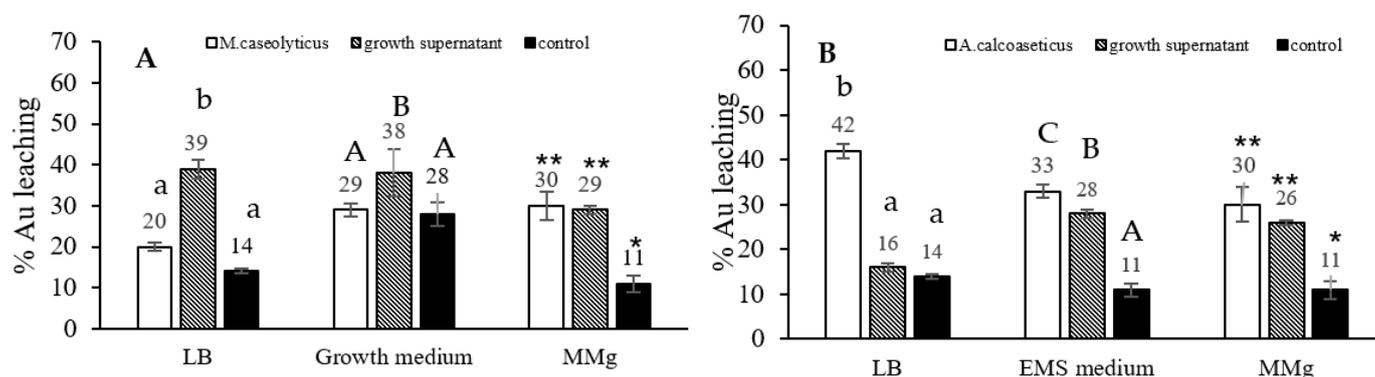


Figure 3. Bioleaching of gold from silicate ores under various media by (A) *M. caseolyticus* and (B) *A. calcoaceticus* under microorganisms and growth supernatant conditions for 7 days. Luria Bertani (LB), minimal medium with glucose (MMg), ethanol mineral salt (EMS), and growth medium. Data were expressed as the mean \pm SD ($n = 3$). The lower case letters, capital letters, and asterisks (*) were compared percentage of gold (Au) leaching by medium (control), in the presence of micro-organism and growth supernatant condition at various time (control: LB/growth medium/minimal medium/ethanol mineral salt medium). Data were analyzed by Duncan's test ($p < 0.005$).

This was because *M. caseolyticus* and *A. calcoaceticus* are able to grow under the minimal medium condition, and it can leach gold in the presence of cells, while the growth supernatant of both strains also leached gold well, probably because *M. caseolyticus* and *A. calcoaceticus* can produce some compounds that affect to gold leaching efficiency. However, both strains under the minimal medium had lower capability as compared to other medium (LB medium) conditions (Figure 3A,B). This evidence confirmed that the ingredient of the medium (LB medium) had a direct effect on gold leaching, which was similar to Maneesuwannarat [21,22], and that the medium constituent will be affected by gallium bioleaching.

Thus, the selective medium cultured two isolated strains and evaluated the abilities of gold bioleaching. Our hypothesis was that isolated strains cultured in the medium can grow well and have more ability to produce compounds to increase the efficiency of gold bioleaching. The growth medium was proper for *M. caseolyticus* and the ethanol mineral salt medium (EMS) was for *A. calcoaceticus*. The result showed that *M. caseolyticus* under the growth medium was able to leach gold from silicate ores in the presence of the microorganism (29%) and growth supernatant (38%) conditions (Figure 3A). Surprisingly, growth medium solely (28%) (as a control) showed a similar level to in the presence of the microorganism condition. It implied that microorganisms cannot grow well in the ore under growth medium conditions. The growth medium also contained 0.5% yeast extract, 1% casein peptone, 0.5% glucose, and 0.5% sodium chloride, resulting in containing more amino acids in this medium. This evidence also confirmed that the medium constituent can be affected in gold bioleaching directly. This was also consistent with other previous studies that found that yeast extract in LB medium could affect leach metals such as copper and gallium [21–24]. Therefore, it was probably because, under growth medium conditions,

the efficiency of gold bioleaching was affected by amino acids in the medium, while *A. calcoaceticus* with ethanol mineral salt medium can leach gold for both conditions, including in the presence of the microorganism (33%) and growth supernatant (28%), as shown in Figure 3B. Interestingly, the growth supernatant of *A. calcoaceticus* from the EMS medium (28%) showed the efficiency of gold bioleaching higher than from MMg (26%) and LB medium (16%), respectively. As mentioned previously, the EMS medium was more suitable for *A. calcoaceticus* and did not contain amino acids [25,26]. This result also confirmed that, under the EMS medium, *A. calcoaceticus* can produce compounds and lead to an effect on gold bioleaching directly. Therefore, *M. caseolyticus* cultured in the MMg medium and *A. calcoaceticus* culture in the EMS medium were investigated regarding their capabilities in gold bioleaching.

3.3. Influence of Amino Acids on Gold Bioleaching

The efficiency of gold bioleaching by *M. caseolyticus* under the minimal medium and *A. calcoaceticus* with the ethanol mineral salt medium were investigated for a longer time to understand how isolated strains can leach gold. The result showed that gold leaching in the presence of the microorganism (48%) and growth supernatant (49%) by *M. caseolyticus* was increased after 28 days (Figure 4A,B). Afterward, the amino acid content in the growth supernatant of this strain was analyzed before and after leaching. It was found that *M. caseolyticus* produced various amino acids, and after bioleaching for 28 days, amino acids were not found in the system (Table 2). It might be possible that amino acids/peptides/proteins or other compounds that this strain produced were bound with gold directly. This result was similar to Maneesuwannarat [27], in which amino acids found in the supernatant were involved in gallium leaching. Moreover, this result also showed that no amino acids interfered in the initial minimal medium (as control) (Table 2). It demonstrated that the efficiency of gold bioleaching by the growth supernatant of *M. caseolyticus* cannot be affected by amino acid from the medium. A similar result was also found in the growth supernatant of *A. calcoaceticus*; gold was leached approximately 43% after 28 days of cultivation (Figure 4D), while in the presence of the microorganism, results showed a decrease after 28 days (23%) (Figure 4C). It might be possible that, on day 28, the organism started to absorb gold into its cell and caused a decrease in gold leaching [28]. Moreover, amino acid content by *A. calcoaceticus* was also investigated and similar contents to the previous strain (*M. caseolyticus*) were found, except for aspartic acid, histidine, and cysteine (Table 3). Notably, after bioleaching, amino acids had decreased compared to the initial amino acid content, but they had not disappeared like in the *M. caseolyticus*. It was due to amino acids, peptides, and protein, which isolated strains produced can bind with gold and also showed lower amino acid contents after bioleaching. Then, this is evidence to evaluate that *M. caseolyticus* and *A. calcoaceticus* had capability in gold bioleaching by producing amino acids/peptides or protein. Moreover, from our result, it can be concluded that bacteria and/or amino acids are able to solubilize gold from silicate ore, as shown in Equation (3), because some soluble gold can be dissolved in water under the control condition, as shown in Figure 4. This can be explained by the fact that soluble gold was dissolved from silicate ore to be ions; it is strongly dependent on the value of the soluble product constant (K_{sp}). For this reason, amino acids can enhance solubility by binding to gold ions to become a more stable form, causing a decrease in soluble ion concentration. According to the principle of Le Châtelier, the depletion of ions affects the equilibrium of the dissolution system. Therefore, more insoluble gold needs to be dissolved to maintain equilibrium.



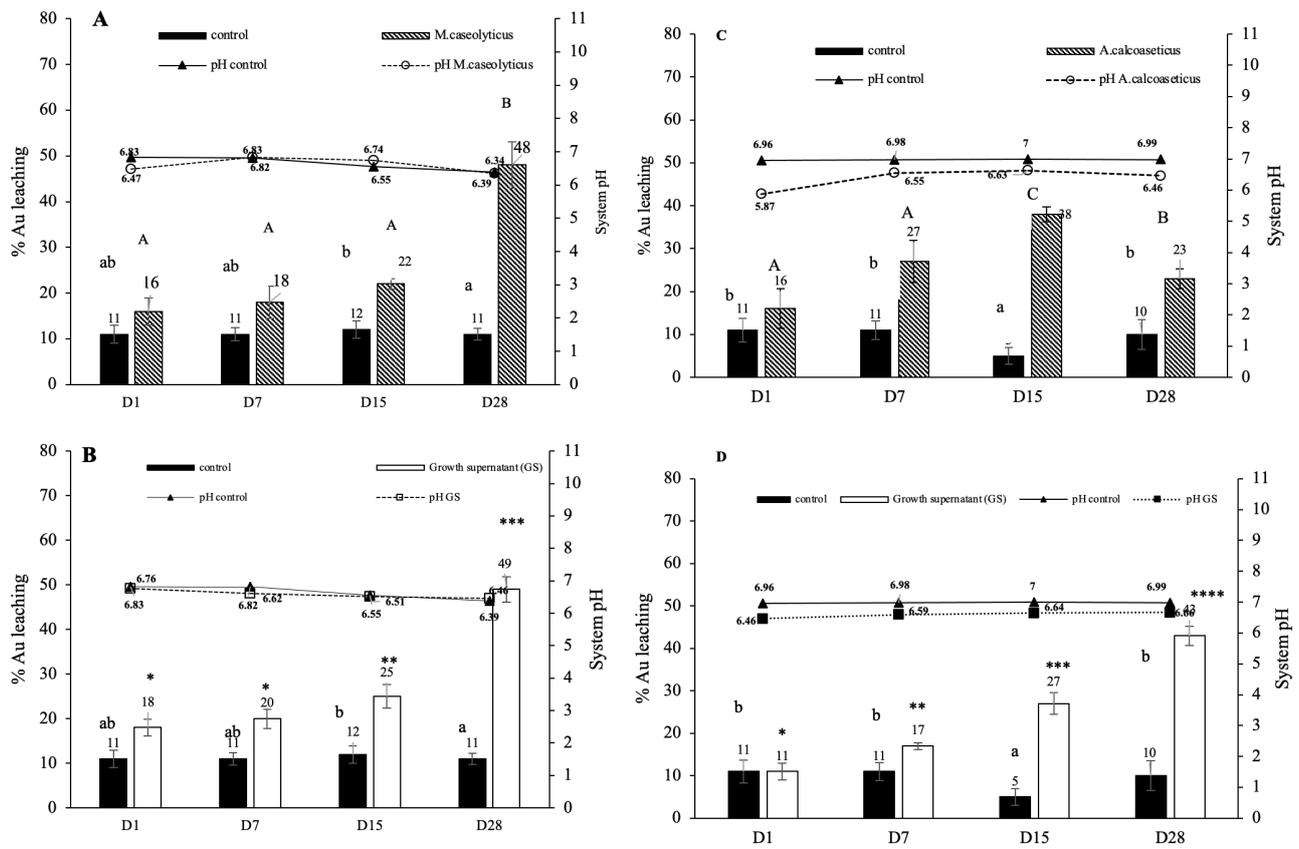


Figure 4. Gold bioleaching from silicate ores by *M. caseolyticus* in the minimal medium under (A) in the presence of microorganisms and (B) growth supernatant conditions and by *A. calcoacetis* in ethanol mineral salt medium under (C) in the presence of microorganisms and (D) growth supernatant conditions. The flasks were shaken at 150 rpm, 30 °C, and the particle size of ores was approximately $\leq 75 \mu\text{m}$. Data were expressed as the mean \pm SD ($n = 3$) and analyzed by Duncan’s test ($p < 0.005$). The lower-case letters, capital letter and asterisks (*) were compared percentage of gold (Au) leaching by medium (control), in the presence of micro-organism and growth supernatant condition at various time.

Table 2. Amino acid content of growth supernatant by *M. caseolyticus* before and after gold bioleaching.

Amino Acid	Minimal Medium	Initial Growth Supernatant of <i>M. caseolyticus</i> (nmol)	Growth Supernatant of <i>M. caseolyticus</i> + Ores at Various Time			
			Day 1	Day 7	Day 15	Day 28
Aspartic acid	nd	nd	nd	nd	nd	nd
Serine	nd	nd	nd	nd	nd	nd
Glutamic acid	nd	nd	nd	nd	nd	nd
Glycine	nd	nd	nd	nd	nd	nd
Histidine	nd	nd	nd	nd	nd	nd
Arginine	nd	3.96 \pm 5.21	nd	nd	nd	nd
Threonine	nd	13.22 \pm 10.11	nd	nd	nd	nd
Alanine	nd	54.15 \pm 39.52	nd	nd	nd	nd
Proline	nd	nd	nd	nd	nd	nd
Cysteine	nd	nd	nd	nd	nd	nd
Tyrosine	nd	7.09 \pm 3.19	nd	nd	nd	nd
Valine	nd	11.89 \pm 4.54	nd	nd	nd	nd
Methionine	nd	nd	nd	nd	nd	nd
Lysine	nd	3.68 \pm 5.2	nd	nd	nd	nd
Isoleucine	nd	7.71 \pm 4.37	nd	nd	nd	nd
Leucine	nd	11.38 \pm 6.01	nd	nd	nd	nd
Phenylalanine	nd	7.33 \pm 4.56	nd	nd	nd	nd

nd = not detected. Growth supernatant = supernatant without cells.

Table 3. Amino acid content of growth supernatant by *A. calcoaceticus* before and after gold bioleaching.

Amino Acid	Ethanol Mineral Salt Medium	Initial Growth Supernatant of <i>A. calcoaceticus</i> (nmol)	Growth Supernatant of <i>A. calcoaceticus</i> + Ores at Various Time			
			Day 1	Day 7	Day 15	Day 28
Aspartic acid	nd	nd	nd	nd	nd	nd
Serine	nd	104.825	nd	nd	21.1	31.925
Glutamic acid	nd	986.7	106.05	45.7	36.2	65.875
Glycine	nd	356.15	81.325	nd	95.125	139.425
Histidine	nd	nd	nd	nd	nd	nd
Arginine	nd	110.525	nd	nd	nd	nd
Threonine	nd	130.475	16.075	nd	nd	nd
Alanine	nd	589.875	nd	nd	nd	nd
Proline	nd	90.375	nd	nd	nd	nd
Cysteine	nd	nd	nd	nd	nd	nd
Tyrosine	nd	88.2	24.45	22.8	nd	35.15
Valine	nd	286.2	nd	nd	nd	nd
Methionine	nd	46.5	nd	nd	nd	nd
Lysine	nd	125.725	nd	nd	nd	nd
Isoleucine	nd	101.025	nd	nd	nd	nd
Leucine	nd	121.125	nd	nd	nd	nd
Phenylalanine	nd	110.85	nd	nd	nd	nd

nd = not detected. Growth supernatant = supernatant without cells.

However, the interaction between gold and the amino acid that is contained in the growth supernatant by *M. caseolyticus* and *A. calcoaceticus* needs to be further investigated. To avoid gold absorption and metal toxicity by microorganisms in the long-term operation, the growth supernatant was selected to further study in order to understand how the microorganism can leach gold from ore.

3.4. FTIR Analysis for Functional Groups on Growth Supernatant of *M. Caseolyticus* and *A. Calcoaceticus* Involved in Gold Bioleaching

FTIR was used to understand the interaction of gold and amino acids in gold bioleaching by isolated strains. Growth supernatant of *M. caseolyticus* and *A. calcoaceticus* were used to confirmed by FTIR analysis. Then, the spectra of growth supernatant as produced by both strains before and after bioleaching were compared. The results showed that the growth supernatant of *M. caseolyticus* showed a strong peak at 1316 cm^{-1} , which was assigned to the amine group, as represented by NH_2 in amino acids. Interestingly, after the bioleaching process, this peak had disappeared (Figure 5A). It might be possible that the amine group of amino acids/peptides/proteins was involved in gold bioleaching. In addition, the growth supernatant of *A. calcoaceticus* also showed similar results with *M. caseolyticus*. The result showed that the peak of NH_2 occurred at 1607 cm^{-1} before gold bioleaching, and it had disappeared after 28 days of cultivation (Figure 5B). Moreover, other peaks presented the carboxylic group of the growth supernatant, as produced by both strains at position 848. The height of this peak (COOH) had also decreased after the gold bioleaching process. It was demonstrated that this position could be related to gold bioleaching. Consistent with Reith, Jingrong (15) (26) proposed that amino acids could react with gold through the nitrogen atom of the amine group and the oxygen atom of the carboxylic group. Therefore, this evidence clearly showed that the functional groups of growth supernatant had an influence on the leaching of gold from ores, particularly carboxylic groups and amine groups, which are found in amino acids/peptides/proteins. However, the detailed mechanism of the gold leaching by the amino acids/peptides/proteins in these strains needs to be further investigated.

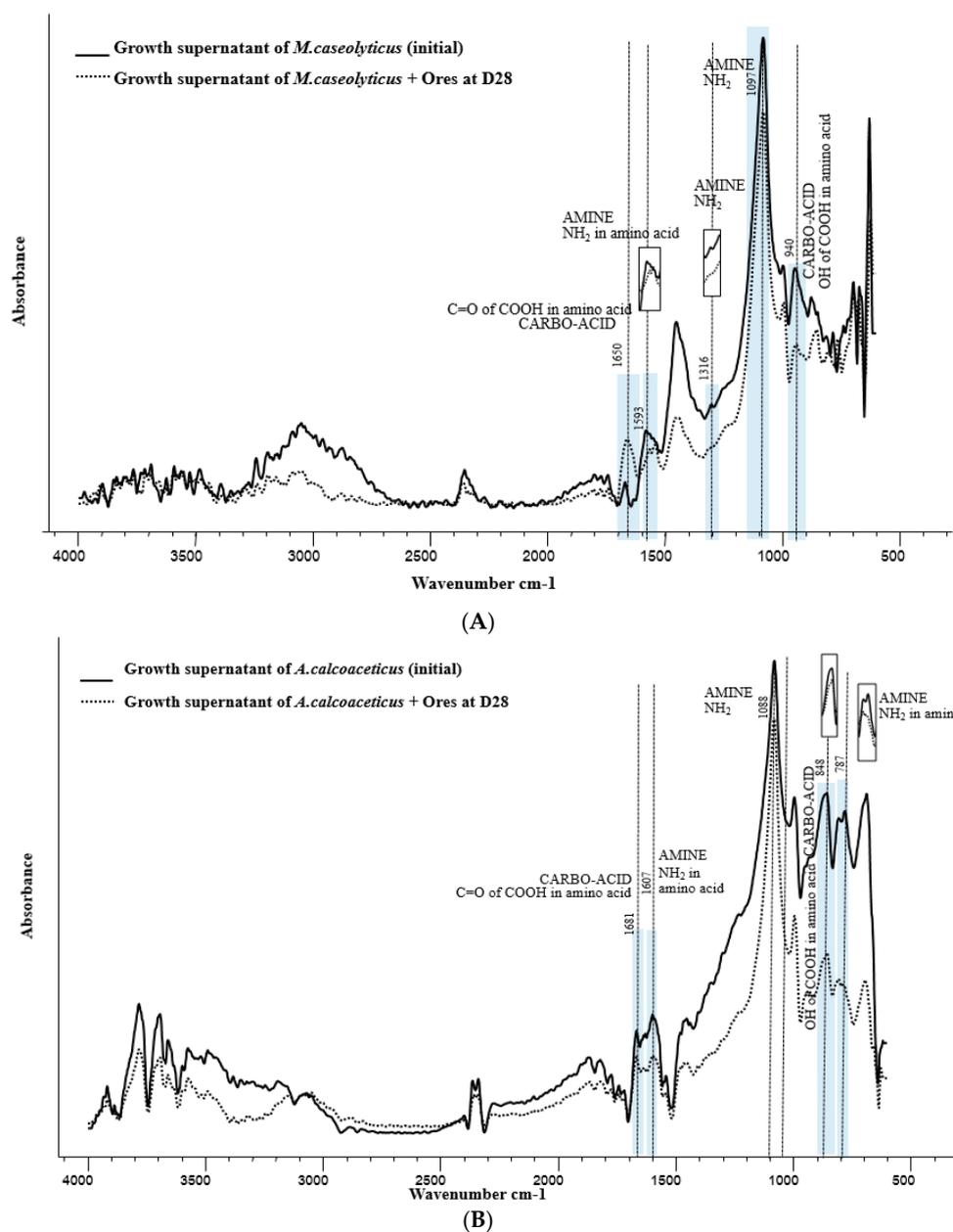


Figure 5. FTIR spectra of growth supernatant (GS) of *M. caseolyticus* (A) and *A. calcoaceticus* (B) before and after gold bioleaching.

3.5. Comparison of Gold Bioleaching Efficiency by Growth Supernatant of Isolated Strains and Mixed Amino Acids

It was proven that isolated strains had capability in gold bioleaching by producing amino acids/peptides or protein. Then, the efficiency of gold bioleaching by synthetic mixed amino acids (at the same concentration with growth supernatant) was compared with the growth supernatant of *M. caseolyticus* and *A. calcoaceticus*. The result showed that the growth supernatant of *M. caseolyticus* also had the highest efficiency of gold bioleaching compared with mixed amino acid (Figure 6A). A similar trend was also found in the growth supernatant of *A. calcoaceticus* (27%); it had significantly higher gold bioleaching than mixed pure amino acid (Figure 6B). This suggested that not only pure amino acids could leach gold, but other peptides, polypeptides, and proteins might be contributing to the gold bioleaching. Therefore, this evidence was to confirm that compounds produced

by isolated strains, especially amino acids/peptides or proteins, had a higher ability to leach gold than mixed pure amino acid.

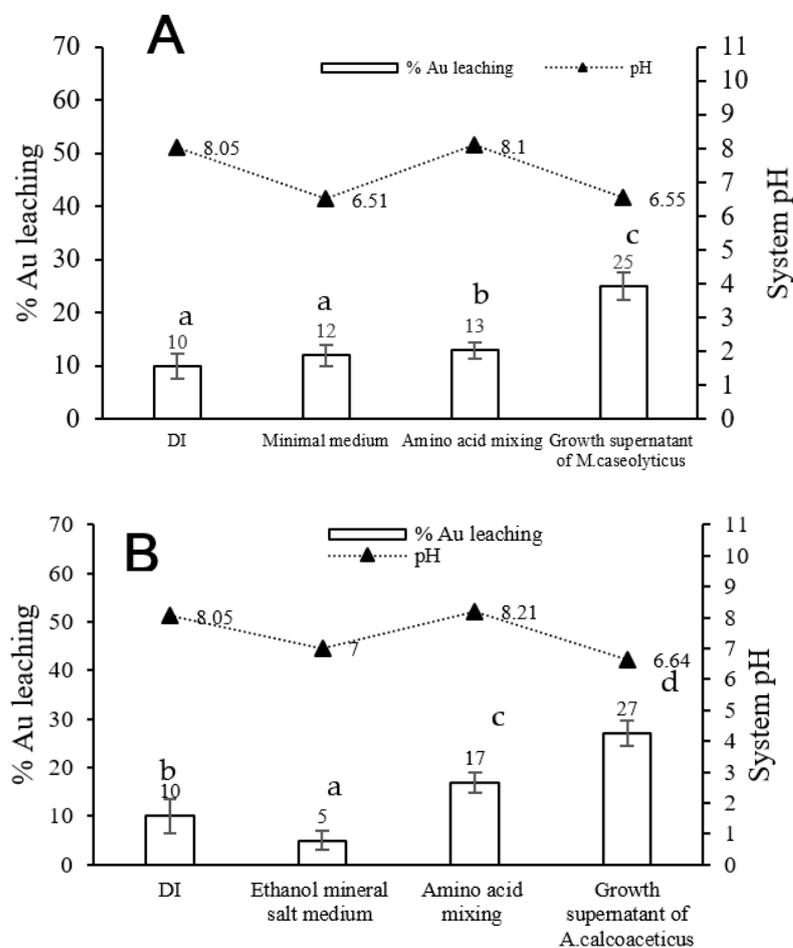


Figure 6. Comparison of gold bioleaching efficiency by growth supernatant of isolated strains (A) *M. caseolyticus* and (B) *A. calcoaceticus* with amino acid mixing (at the same concentration with growth supernatant) within 15 days. The flasks were shaken at 150 rpm, 30 °C, and the particle size of ores was approximately $\leq 75 \mu\text{m}$. Data were expressed as the mean \pm SD ($n = 3$) and analyzed by Duncan's test ($p < 0.005$). The lower case letters compared the percentage of gold bioleaching by control (DI water), medium, amino acid mixing and growth supernatant by (A) *M. caseolyticus* and (B) *A. calcoaceticus*.

4. Conclusion

M. caseolyticus and *A. calcoaceticus* as isolated strains from gold silicate ore had the ability to leach gold from ore. Both strains were able to produce compounds, especially amino acids/peptides/proteins, which can bind with gold. The possible interaction of gold and growth supernatant involved the nitrogen atom of amine groups and the oxygen atom of carboxylic groups was confirmed by FTIR. Moreover, the growth supernatant of both strains was more suitable for the extraction of gold from ores than in the presence of microorganism conditions, due to the fact that growth supernatant had no effect on metal toxicity, while, in the presence of the microorganism condition, gold leaching can be re-absorbed through the organism and cause lower leaching. In addition, in a long-term operation, metals can be toxic to the cell and affect bioleaching directly. Our study also confirmed that amino acids/peptides/proteins as produced by microorganisms had higher efficiency than mixed pure amino acid. Therefore, growth supernatants of gold bioleaching by *Macrocooccus caseolyticus* and *Acinetobacter calcoaceticus* can be applied for use in gold bioleaching; they are safe, environmentally friendly, and not corrosive compared to the

traditional cyanidation method. Furthermore, an investigation should be conducted on how to decrease the leaching time and increase the percentage of gold leaching by growth supernatant in order to make leaching constant and more economic.

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