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ORIGINAL ARTICLE

Isolation and Characterization of Alkaliphilic and Thermotolerant Bacteria that Reduce Insoluble Indigo to Soluble *Leuco*-indigo from Indigo Dye Vat

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Abstract Indigo dye has been used in the textile dye industry for long period. Insoluble indigo are reduced to soluble *leuco*-indigo before dying textiles. A traditional process for solubilization of indigo using microbial reduction metabolism has been considered as environmentally benign method and as alternative to faster chemical reactions. Thus, fermentation liquor aged for 6 years with *Polygonum tinctorium* (indigo plant) extracts was used to isolate bacteria able to reduce insoluble indigo. Two bacterial isolates, A1 and G5, showed indigo-reducing activity, and were identified as *Alkalibacterium* sp. and *Pseudomonas* sp. respectively, with 99% sequence similarity by 16S rDNA sequence analyses. Based on the concentrations of *leuco*-indigo reduced from indigo, *Alkalibacterium* sp. A1 and *Pseudomonas* sp. G5 showed alkaliphilic and thermotolerant charactertistics, optimally functioning at pH 10.0 and 50°C. Isolation of alkaliphilic and thermotolerant bacterial strains, which can reduce insoluble indigo into *leuco*-indigo, from Korean traditional fermentation liquor could provide a biological tool to enhance efficiency in the traditional indigo dye by an environmentally friendly manner.

Keywords *Alkalibacterium* · indigo · *Pseudomonas* · reduction · textile dye

Introduction

Indigo has been used as a dye agent for long time and is considered to be one of the most well-known industrial dyes due to continuing popularity of blue jeans and other blue denim. Indigo dye has been extracted from plants such as *Indigofera* spp. (*I. tinctoria, I. leptostachya, I. anil,* etc.), *Isatis tinctoria, Polygonum tinctrorium,* and *Lonchocarpus cyanescens* (Song et al., 2011). Among these plants, *P. tinctorium* has been cultured and used as a main source of indigo in Far East countries including Korea and Japan for thousands of years (Kim et al., 1996). In contrast, *Indigofera* spp. has been the main source of indigo dye in India and Egypt since the seventeenth century (Domenech et al., 2007).

Since indigo is insoluble in water, ether, and alcohol, it is essential to convert indigo into water-soluble *leuco*-indigo before dying cloths. Once soluble *leuco*-indigo is formed, it can be easily oxidized to indigo when exposed to air. Due to this property, the dipped textiles in reduced dye solution are exposed to the air in order to dye clothes through oxidation of soluble *leuco*-indigo back to insoluble indigo.

In Korea, this process is commonly performed using crushed plant leaves of *P. tinctorium*, which are immersed in the water for extraction of glucoside indican in a fermentation indigo dye vat for 2 days. Indican is changed to indoxyl when the leaves are smashed and exposed to the air. The liberated indoxyl from the precursors is spontaneously oxidized by oxygen and is converted to indigo, which is then mixed with lime ($Ca(OH)_2$) to form the blue indigo mud. Plant debris and water are removed from the dye vat, and 4–5 volumes of hot lye solution (at approximately pH 10.0–11.5 and above 50°C) are added to the vat for further fermentation at 25–30°C for 10 days (Kim, 2010). Finally, the soluble *leuco*-indigo are formed from the blue indigo mud in the alkaline solution through bacterial reduction processes (Fig. 1).

Compared to the fast chemical reduction of indigo to *leuco*-indigo, microbial fermentation processes for production of *leuco*-indigo is considered to be difficult to apply to commercial dyeing due to time-consuming and the difficulty of managing consistent

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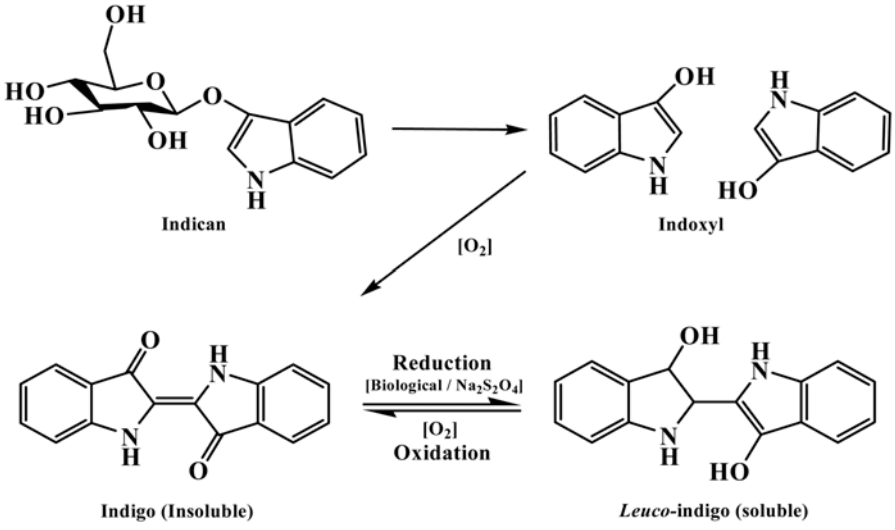

Fig. 1 Formation process of indigo and *leuco*-indigo.

maintenance of the fermentation systems (Clark, 1993; Schmidt, 1997). Therefore, application of fast chemical reactions using reducing agents, sodium dithionite (or sodium hydrosulfite, $Na_2S_2O_4$) or sodium sulfide (Na_2S), has gained popularity in the textile industry (Bozic et al., 2009). However, as expected, this abiological process can also cause environmental problems due to production of toxic compounds such as sodium sulphate, sulphate ions, and thiosulphate from oxidation reaction of sodium dithionite (Aino et al., 2010), and the strong reducing power of remaining sodium dithionite (Bozic and Kokol, 2008).

Compared to synthetic dye products, the public considers natural dye products to be more comfortable, to have better delicate natural color tones, and to be produced from pollution-free dyeing processes. Along with a new fashion trend toward pursuance of functionality, comfortableness, and practicality, cloths with natural fabric dyed with natural indigo by traditional methods are becoming popular in the Korean market, fetching higher prices. Considering these facts, the microbial processes for the reduction of indigo could be a better method for dying clothes if a solution to decrease time required for indigo reduction with increased production yields of *leuco*-indigo is found.

In the present study, we isolated two bacteria from a traditional indigo fermentation dye vat to possibly apply traditional indigo dye methods in an industrial scale, through characterization of the bacterial reduction mechanism of indigo to *leuco*-indigo. Two isolated bacteria, A1 and G5, identified as *Alkalibacterium* sp. A1 and *Pseudomonas* sp. G5 respectively, were shown to be facultative alkaliphilics and able to reduce indigo into *leuco*-indigo at pH 10.0 under anoxic conditions. In addition, these bacteria showed the optimum temperature for indigo reduction activity at 50°C, which has been known as an optimal temperature condition for dying indigo.

Materials and Methods

Bacterial cultivation and isolation from a fermented indigo dye vat. Fermented indigo liquor sample was obtained from a dye vat in Hwasun-gun, Jeonnam, Korea. This fermented indigo liquor has been used for 6 years for dyeing cotton textiles and was maintained by adding boiled rice wine, with stirring, once a day. Ten milliliters of indigo fermentation liquor was added to 30 mL of minimal medium containing indigo (0.01%) at 30°C, and incubated for 60 days. The minimal medium contains $NaHCO_3$ (25 g), $CaCl_3$ (6 g), NH_4Cl (10 g), $MgCl_2(H_2O)$ (20 g), KCl (10 g), NaCl (10 g), HEPES (7.2 g), yeast extract (0.1 g), glycophosphate (0.1 g), and glucose (10 mM) per liter in 100 mM $NaHCO_3$/Na_2CO_3 buffer (pH 11.0). In this study, peptone/yeast extract/alkaline (PYA) agar medium containing indigo-carmine (0.2% (w/v)), peptone (8 g) and yeast extract (3 g) per liter in 100 mM $NaHCO_3$/Na_2CO_3 buffer was used for isolating and screening indigo-reducing bacteria (Aino et al., 2010). The culture from the fermentation liquor was spread on PYA agar medium and incubated at 30°C for 3 days. Twenty bacterial candidates were isolated and inoculated into PYA broth medium containing indigo (0.01% (w/v)) to determine their ability for reducing indigo to *leuco*-indigo. All bacterial cultivation processes were performed in an anaerobic chamber filled with N_2 gas (Padden et al., 2000).

16S rDNA sequence analysis for phylogenetic analysis. Two candidate bacteria were incubated in PYA agar medium containing 100 mM $NaHCO_3$/Na_2CO_3 buffer (pH 11.0) at 30°C. Genomic DNA was extracted from the candidate bacteria using a GeneAll^R Exgene^{TM} Cell SV kit (GeneAll Biotech., Seoul, Korea) according to the manufacturer's instructions. The 16S rDNA gene was amplified by PCR using the following universal primers: 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGY

TACCTTGTTACGACTT-3'). For the PCR reaction, an initial denaturation step of 3 min at 94°C was followed by 35 cycles of amplification consisting of 30 s at 94°C, 30 s at 55°C, 1 min at 72°C, and a final extension step of 7 min at 72°C. The PCR product was sequenced using ABI PRISM 3730XL Analyzer with a BigDye™ Terminator Cycle Sequencing Kits (Applied Biosystems., Foster City, CA). The assignment of determined sequence was performed by alignments and database identification of consensus sequences using BLASTIN 2.2.18. A phylogenetic tree was constructed with the neighbor-joining method using the CLUSTAL W program (Saitou and Nei, 1987). Similarities between the sequences were calculated using the MEGA 4 software program (Kumar et al., 2007).

Effects of pH and temperature on bacterial growth. To investigate bacterial alkaliphilicity and thermotolerance, bacterial strains were grown at various pH and temperature conditions under aerobic conditions. The isolates pre-grown in Luria-Bertani (LB) medium at 30°C and 180 rpm for 24 h were harvested by centrifugation at 4,620 × g for 15 min and washed twice with phosphate buffer (pH 7.0). After adjusting cell density to OD_{600}=0.6, 100 μL culture of bacteria (1%, v/v) was inoculated into 100 mL of LB. Bacteria were incubated aerobically at pH 7.0 or 10.0, and at 30, 50, and 70°C with shaking at 180 rpm. Growth was measured at 600 nm by using a UV/Visible spectrophotometer (UV-1601PC, Shimadzu, Kyoto, Japan).

Determination of indigo-reducing activity. Due to instability of *leuco*-indigo in the air, the indigo-reducing activity of isolates was determined by measuring the concentration of indigo which was reoxidized from *leuco*-indigo in the supernatant as follows. The bacterial culture (300 mL) previously grown in PYA broth medium containing 0.01% (w/v) indigo was transferred to minimal medium with 10 mM of glucose and 100 μM of indigo. Isolated bacteria were grown in 100 mL of LB broth at 30°C with shaking at 180 rpm until OD_{600} reached 0.5–0.6, and then harvested by centrifugation at 4,620 × g for 10 min. During the incubaction, 1 mL of bacterial broth was sampled and centrifuged at 13,226 × g for 10 min under the anoxic condition. The supernatant containing soluble *leuco*-indigo was then exposed to the air for 10 min to allow oxidation of *leuco*-indigo to indigo. After oxidation of the supernatant, this supernatant was centrifuged at 13,226 × g for 30 min to precipitate any formed indigo. The supernatant was discarded and precipitant insoluble indigo was dissolved in 1 mL of pure DMSO. The absorbance of the dissolved indigo was measured using a UV/Visible

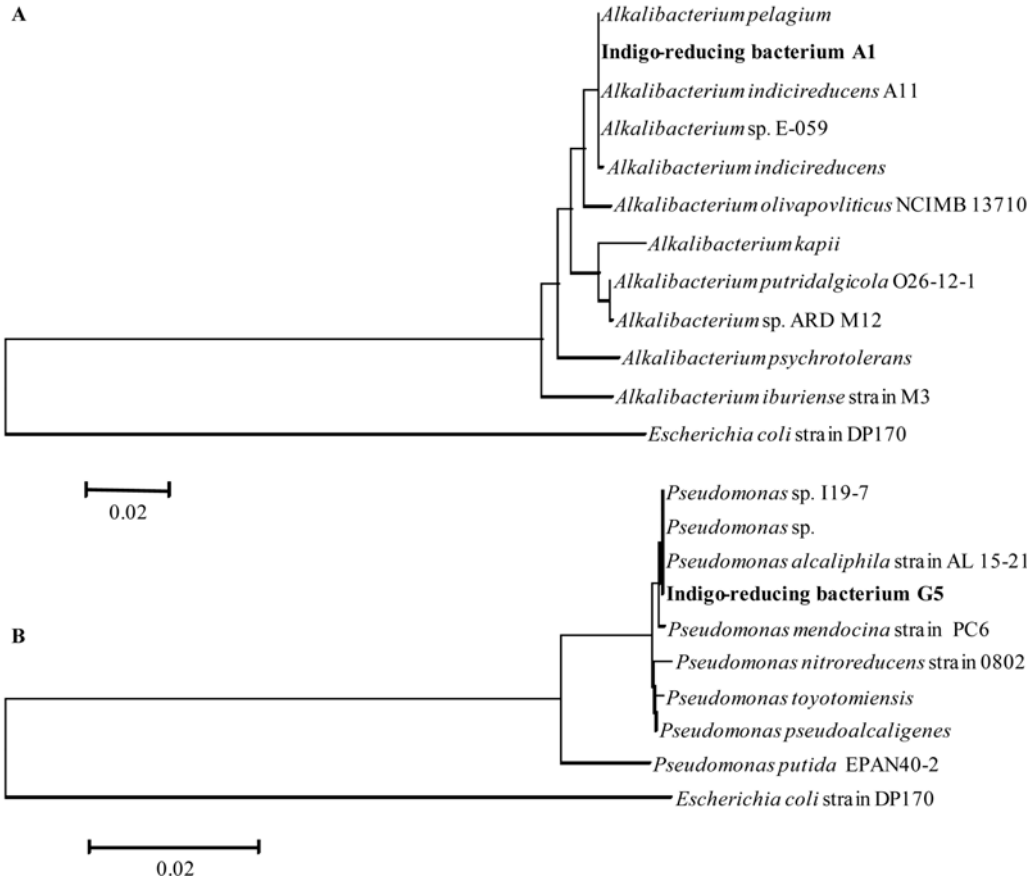

Fig. 2 Phylogenetic tree of *Alkalibacterium* sp. A1 (A) and *Pseudomonas* sp. G5 (B) and other related organisms using neighbor-joining method based on 16S rDNA sequences. The scale bar indicates 0.02 substitutions per nucleotide position.

spectrophotometer (UV-1601PC, Kyoto, Japan) at 610 nm (Kawahito and Yasukawa, 2009). Indigo reduction activity by isolated bacteria was determined under various pH (at pH 6.0, 8.0, and 10.0) and temperature conditions (at 30, 50, and 70°C) for identifying optimal indigo reduction activity.

Results and Discussion

Identification of indigo-reducing bacteria. Based on the full sequence analysis of 16S rDNA of indigo-reducing bacteria obtained from fermented liquid containing 0.01% (w/v) indigo aged for more than 60 days, strain A1 showed high similarity (99%) to *Alkalibacterium* sp. and the sequence of strain G5 best matched *Pseudomonas* sp. with similarity of 99% (Fig. 2). *Alkalibacterium indicireducens* has been previously reported as an indigo-reducing bacterium under anoxic and alkaline conditions (above pH 10) (Yumoto et al., 2008). These two isolates showed yellow color around the colonies on agar plates containing indigo carmine through the reduction of indigo carmine at 30°C under anoxic conditions. These two isolates were further tested for indigo reduction activity in indigo-containing broth. The reason of using indigo carmine (5,5'-indigosulfonic acid) in agar plates as the first step of screening for indigo-reducing bacteria is due to its better solubility in water than indigo. Indeed, indigo carmine has been used as a pH indicator due to its sensitivity in color change to pH above 13 (Ammar et al., 2006). After confirmation of reducing activity with indigo carmine, water-insoluble indigo was used to further test indigo-reducing activity of the isolates. Figure 3 shows the aerobic growth of *Alkalibacterium* sp. A1 and *Pseudomonas* sp. G5 in LB at various pH and temperature conditions. *Alkalibacterium* sp. A1 showed the optimal growth condition at pH 10.0 and 30°C. Interestingly, *Alkalibacterium* sp. A1 did not die-off at the growth condition of pH 10.0 and 50°C, indicating this strain is likely to be alkaliphilic and theromotolerant. However, growth of *Alkalibacterium* sp. A1 at pH 7.0 and 30°C only reached to an OD_{600} of 0.17, which was similar to the growth at pH 7.0 and 50°C. *Pseudomonas* sp.G5 showed the optimal growth condition at pH 7.0 and 30°C, similar to the growth pattern at pH 10.0 and 30°C, suggesting strain G5 appears to be alkalitolerant.

Indigo-reducing activities of *Alkalibacterium* sp. A1 and *Pseudomonas sp.* G5. Reduced *leuco*-indigo was quantified based on growth of strains under anoxic conditions at pH 8 and 30°C (Fig. 4); the *leuco*-indigo concentration produced by *Alkalibacterium* sp. A1 increased to approximately 12.1 μM for 30 days, whereas

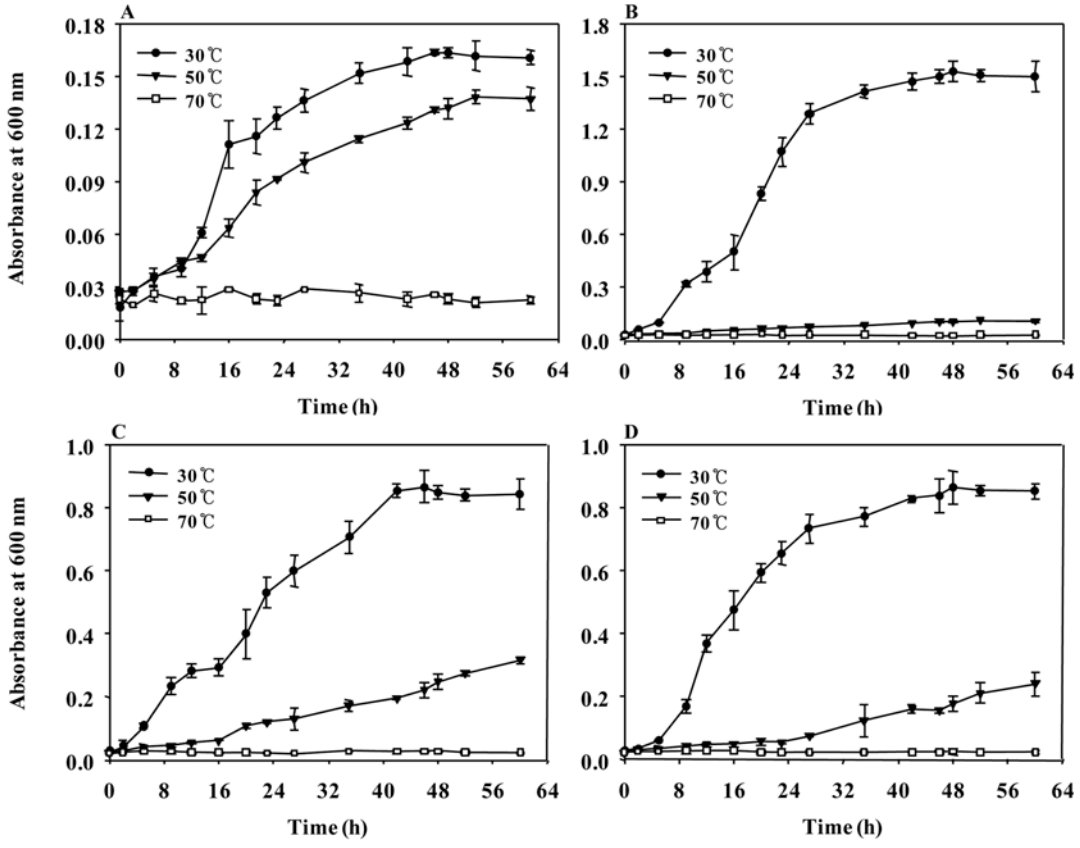

Fig. 3 Growth curves of *Alkalibacterium* sp. A1 at 30 (●), 50 (▼), and 70°C (□) under pH 7.0 (A) and pH 10.0 (B) and *Pseudomonas* sp. G5 at 30 (●), 50 (▼), and 70°C (□) under pH 7.0 (C) and pH 10.0 (D).

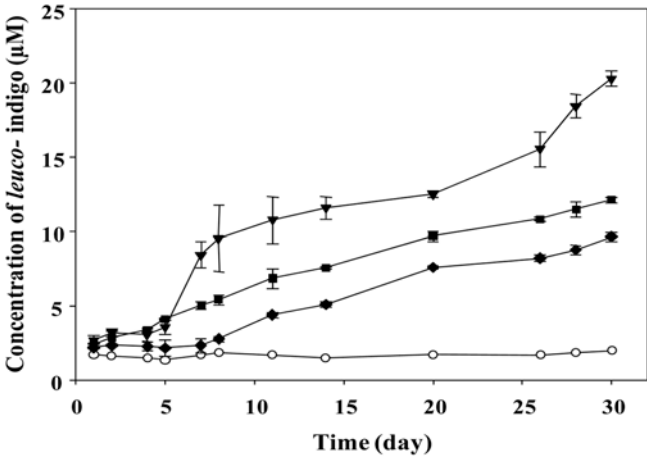

Fig. 4 Concentration (μM) of *leuco*-indigo produced from indigo by isolates under the anoxic conditions at 30°C and pH 8.0. Control (no bacterial inoculation) (○), *Alkalibacterium* sp. A1 (■), *Pseudomonas* sp. G5 (◆), and *Alkalibacterium* sp. A1 and *Pseudomonas* sp. G5 together (▼).

production of *leuco*-indigo by *Pseudomonas* sp. G5 increased to 9.6 μM. *Alkalibacterium* sp. A1 were able to reduce indigo into *leuco*-indigo by 1.3-fold more than *Pseudomonas* sp. G5. However, when these two bacteria were cultured together in the same bottles, the concentration of *leuco*-indigo increased to 20.3 μM for 30 days, which accounted for approximately 20% of the initially added 100 μM of indigo. This result suggested that co-culture of two bacterial strains exert a synergistic effect on indigo reduction.

pH and temperature effects on indigo reduction. In order to investigate the optimal condition for indigo reduction, concentration of *leuco*-indigo formed from indigo by the isolates were measured at various pH (6.0, 8.0, and 10.0) and temperature (30, 37, and 50°C) conditions (Fig. 5). Fig. 5A shows that concentration of *leuco*-indigo by *Alkalibacterium* sp. A1 increased at all applied ranges of pH at 30°C. The final concentration of *leuco*-indigo at pH 10.0 (26.7 μM) was 4.7-and 2.4-fold higher than at pH 6.0 and 8.0, respectively. In the case of *Pseudomonas* sp. G5, *leuco*-indigo concentration at pH 10.0 was 11.2 μM, which was 1.8 and 1.2

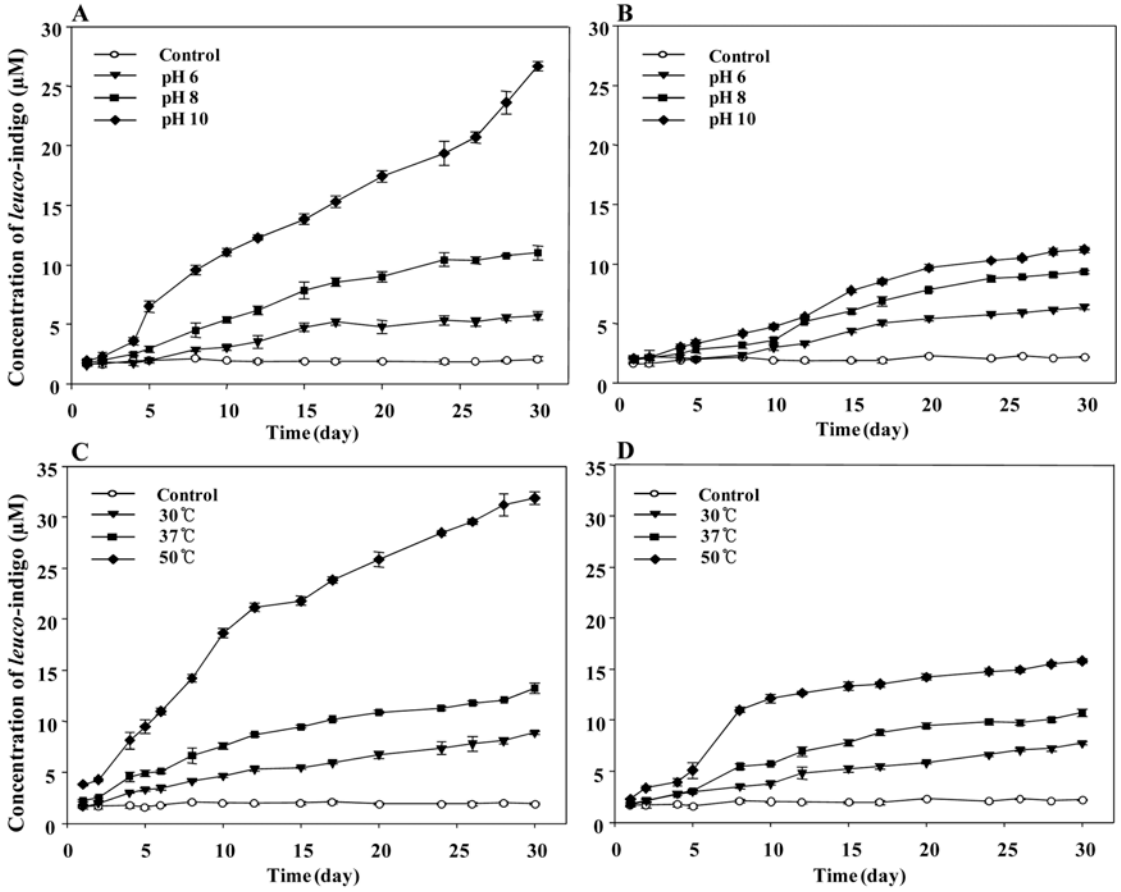

Fig. 5 Concentration (μM) of *leuco*-indigo under the different pH and temperature conditions. Control (no bacterial inoculation) (○), pH 6.0 (▼), 8.0 (■), and 10.0 (◆) at 30°C by *Alkalibacterium* sp. A1 (A) and *Pseudomonas* sp. G5 (B); Control (no bacterial inoculation) (○), 30 (▼), 37 (■), and 50°C (◆) at pH 10.0 by *Alkalibacterium* sp. A1 (C) and *Pseudomonas* sp. G5 (D).

times higher than at pH 6.0 and 8.0, respectively (Fig. 5B). Both isolates showed the highest concentration of *leuco*-indigo at pH 10.0. Temperature effect on bacterial indigo reduction reaction was tested at 30, 37, and 50°C with pH 10.0. Concentration of *leuco*-indigo reduced by *Alkalibacterium* sp. A1 at 50°C reached approximately 42 μM, which was 3.6 and 2.4-fold higher than at 30 and 37°C, respectively (Fig. 5C). On the other hand, *Pseudomonas* sp. G5 produced 15.8 μM of *leuco*-indigo from indigo at 50°C, 2 and 1.5 times higher than that at 30 and 37°C, respectively (Fig. 5D). These results indicate *Alkalibacterium* sp. A1 exerts optimal indigo-reducing activity at pH 10.0 and 50°C under anoxic conditions and stronger activity than *Pseudomonas* sp.G5. In addition, both strains *Alkalibacterium* sp. A1 and *Pseudomonas* sp. G5 have higher indigo reduction activities at 50°C under the alkaline conditions. Considering the traditional indigo reduction process, in which indigo mud is treated with hot lye solution (above 50°C) at the start of fermentation (Aino et al., 2010), two newly isolated strains *Alkalibacterium* sp. A1 and *Pseudomonas* sp. G5 are likely to be effective when inoculated at the beginning stage of indigo-reducing processes. Although various types of bacteria have been isolated and characterized as indigo-reducing bacteria, their indigo-reducing mechanisms are not well known. There are several studies on the explanation of indigo reduction mechanism. For example, Compton et al. (2000) reported *Clostridium isatidis* can interact directly with insoluble indigo particles without a redox mediator, and require direct contact with insoluble indigo particles. In other hand, Takahara et al. (1962) repored that indigo reduction is undertaken as a result of electron and proton transportation system. Up to now, scientific data are limited for explaining bacterial indigo reduction. Therefore, more studies on bacteria capable of reducing indigo will provide clues to the microbial reduction process for the industrial application (Sarethy et al., 2011). Indigo reduction by microbial reaction, without using harmful chemicals, such as sodium dithionite (Na_2SO_4), could be environmentally friendly. In addition, microbial reduction has been regarded as a better colored-dying process than the chemical reduction (Kim, 2010). In the present study, two bacterial strains were isolated from the indigo dye vat and identified as alkaliphilic and thermotolerant *Alkalibacterium* sp. A1 and *Pseudomonas* sp. G5. In addition, both strains showed optimal indigo-reducing activities at pH 10.0 and 50°C under anoxic conditions. Considering the begining stage of traditional indigo reduction processes at pH 10.0–11.5 and 50°C, and the best dying temperature of *leuco*-indigo at 50°C (Kim, 2010), *Alkalibacterium* sp. A1 and *Pseudomonas* sp. G5 may be valuable bacteria for industrial applications.

Despite several advantages of biotechnological application for reducing indigo to *leuco*-indigo, there are still a number of unsolved problems including unproved mechanisms of bacterial indigo reduction and absence of enzyme or gene information related to indigo reduction. Further studies should be focused on increasing the indigo-reducing activity of isolates through in-depth biochemical and molecular characterizations of bacterial reduction of indigo at alkaline pH and 50°C under anoxic conditions.

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