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Biocatalysts screening of *Papaver bracteatum* flora for thebaine transformation to codeine and morphine

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ABSTRACT

The aim of this study was to find biocatalyst which uses thebaine and extract of different parts of *Papaver bracteatum* to synthesize morphine alkaloids. The thebaine-resistant strains were obtained from microbial flora of different parts of *P.bracteatum*. They were purified and treated utilizing different concentrations of thebaine. Those that can grow at a concentration of over 500 μg/ml were chosen for the biotransformation experiments. Biotransformation experiments were carried out utilizing selected cells in the medium containing thebaine and/or the extract of *P.bracteatum*; the products of such biotransformation were extracted and the profiles of metabolites were evaluated using HPTLC, and LC/ESI-MS methods. Thereafter, the effective isolate for thebaine transformation was characterized by physiological, biochemical and biomolecular methods. The results show that among 67 isolates, 12 strains were selected using the HPTLC screening as candidates that can transform thebaine into codeine and morphine. Among them, 5 strains were identified to transform plant extract, among which, using LC/ESI-MS, a candidate was selected and identified as *Bacillus* sp. FAR. It can be concluded from this study, that this microbial flora candidate can transform thebaine into important narcotic drugs and it will be a valuable step in biotechnology.

1. Introduction

Morphine alkaloids are important class of pharmaceutical substances because of their powerful analgesic (Bruce et al., 1990; Lister et al., 1999; Niknam et al., 2010), antitussive and narcotic antagonist characteristics (Kyslíková et al. 2013). *Papaver somniferum* (opium poppy), a traditional source of morphine alkaloids (Kyslíková et al. 2013; Nyman, 1978), with secondary metabolites accumulate at low level in plant as a sole commercial resource (Nakagawa et al., 2011; Nyman, 1978). Syntheses of these compounds were difficult and time consuming due to their complexity and strict regulation of biosynthesis morphine alkaloids pathways (Nakagawa et al., 2011). Finding an appropriate way to produce the natural pharmacological compounds is preferable because it results in more purified and qualified substances. (Bruce et al., 1995; Nakagawa et al., 2011; Rathbone and Bruce, 2002; Rinner and Hudlicky, 2012). Notwithstanding the efforts of chemists for the chemical synthesis of morphine alkaloids, these compounds are still produced by isolation from the opium poppy (Boonstra et al., 2001). An alternative approach is biotransformation; via the use of enzyme or whole cell biocatalysts, this approach has benefits over the conventional chemical processes (Bruce et al., 1995), including non-extreme pH and temperature, low levels of toxic waste products (Rathbone and Bruce, 2002), high chemo, regio and enantiosis selectivity under ecologically compatible conditions (Brunati et al., 2004).

Transformation of morphine alkaloids utilizing plant cells and tissue cultures has been reported (Corchete and Yeoman, 1989; Furuya et al., 1984; Tam et al., 1982; Wilhelm and Zenk, 1997). Unfortunately, the amounts of desired metabolites produced by plant *in-vitro* cultures are usually lower than the content in intact plant (Muffler et al., 2011; Rao et al., 1999). In addition to plant, microorganisms have ultimate potential to operate selective biochemical transformations (Rathbone and Bruce, 2002). There are many reports on the production of morphine alkaloids from fungi and bacteria including the genus *Trametes* (Boonstra et al., 2001; Bruce et al., 1990; Hailes and Bruce, 1993; Kunz et al., 1985; Kyslíková et al., 2013; Long

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et al., 1995; Madyastha et al., 2000; Niknam et al., 2010), *Cunninghamella* (Abel et al., 2002; Asha and Vidyavathi, 2009; Boonstra et al., 2001; Bruce et al., 1990; Hartman et al., 1964; Kunz et al., 1985; Kyslíková et al., 2013; Niknam et al., 2010), *Mucor piriformis* (Kyslíková et al., 2013; Madyastha et al., 2000; Abel et al., 2002; Chaudhary et al., 2009), and *Cylindrocarpon didymum* (Boonstra et al., 2001; Rathbone and Bruce, 2002; Stabler et al., 2001); in a group of fungi and *Arthrobacter* sp. (Boonstra et al., 2001; Hailes and Bruce, 1993; Kunz et al., 1985; Kyslíková et al., 2013; Liras and Umbreit, 1975; Long et al., 1995; Niknam et al., 2010), *Pseudomonas testosteroni* (Boonstra et al., 2001; Hailes and Bruce, 1993; Kunz et al., 1985; Kyslíková et al., 2013; Liras et al.,1975; Rathbone and Bruce, 2002), *Pseudomonas putida* (Boonstra et al., 2001; Bruce et al., 1990; Hailes and Bruce, 1993; Kyslíková et al., 2013; Lister et al., 1999; Madyastha et al., 2000; Niknam et al., 2010; Rathbone and Bruce, 2002), *Bacillus* (Boonstra et al., 2001; Kyslíková et al., 2013; Rathbone and Bruce, 2002), *Mycobacterium neoaurum* (Niknam et al., 2010), *Streptomyces* (Bruce et al., 1990; Niknam et al., 2010; Kyslíková et al., 2013; Boonstra et al., 2001; Long et al., 1995; Hartman et al., 1964; Chaudhary et al., 2009) and *Nostoc muscorum* (Niknam et al., 2010; Kyslíková et al., 2013) in the case of bacteria.

It has been reported that *P. bracteatum* which contains thebaine but not codeine and morphine (Hodges et al., 1977) may be considered as a source of thebaine which cannot be altered in illicit drugs (Laane et al., 1988; Seddigh et al., 1982). Thebaine is an ideal starting material that can be transformed into several opiates (Chaudhary et al., 2009). This study deals with how local microorganisms isolated from natural habitat of *P.bracteatum* or Iranian poppy (Sharafi et al., 2013) will be able to transform thebaine into codeine and morphine. This study aims at finding the desired isolate for renewing thebaine through conversion into valuable products.

2. Experimental

2.1. Isolation and purification of thebaine-resistant microorganism

P.bracteatum was collected in June, from Damavand mount, Iran (geographical location; Alt: 2344–2640, Latitude 35°51′118″N, Longitude 52°04′073″E). Microorganisms were isolated from *P. bracteatum* and its surrounding soil utilizing nutrient agar (NA) and Sabouraud Dextrose Agar (SDA). It is hypothesized that there are strains more adapted to thebaine-containing environment.

For this experiment, 10 g of the samples were shacked for 1 h in 90 ml sterile normal saline (0.85%). Serial dilutions up to 10^{-9} and 10^{-6} were prepared from soil and plant suspensions on NA and SDA and were incubated at30°C for two weeks. Microorganisms were isolated according to their colony appearance and microscopic morphology. Isolated bacteria and fungi were purified via the culturing on NA and SDA, respectively, and stored at −80 °C. The microorganism screening was carried out using the method of Bicas et al., (Bicas and Pastore, 2007) with some modifications. In summary, a group of limonene resistant microorganisms were selected for biotransformation trials. All isolated microorganisms were treated with thebaine in two replicates, (TEMAD Co. Tehran, Iran), at a concentration of 0, 10, 20, 50, 100, 200, 500, 1000 μg/ml. in Mueller Hinton Agar. The turbidity of inoculums was adjusted to McFarland 0.5 standard; thereafter, these suspensions were diluted 100-fold, approximately at 10^6 cfu/ml, to yield final inoculums suspension. Plates were inoculated with the lowest concentration and were incubated at 30 °C for 24–48 h. The effect of thebaine on the growth of microorganisms was monitored. Microorganisms which grow at a thebaine concentration of 500 μg/ml or higher were selected for the next microbial screening tests.

The complex medium containing 15g/L glucose, 10g/L peptone, 5g/L yeast extract, 2g/L NaCl, 0.5g/L, K_2HPO_4 and 0.2g/L, $MgSO_4 \cdot 7H_2O$ (pH 6.8) was sterilized and inoculated with selected microorganism. The turbidity of the culture was monitored at 620 nm for the absorbance of 0.1. Thereafter, 100 ml of complex medium was inoculated with the cell suspension and incubated for 24–30 h at 30 °C and 140 rpm. The obtained cells (8000g, at 4 °C, for 15 min) were washed (10 ml of sterile saline solution) and stored for further analysis at −80 °C.

2.2. Preparation of plant extracts

The leaves, shoots, petals and capsule of *P.bracteatum* were shade dried and powdered. Extraction of the leaves, capsules, stems and flower petals was performed with several solvent including ethanol, acetone, methanol, ethyl acetate and chloroform. The extracts were filtered (0.45 μm) and dried under reduced pressure. The resulted extract was kept at 4 °C for further analysis.

2.3. Microbial transformation using thebaine and plant extracts

Selected strains were subjected to thebaine biotransformation test of 8 and 16 h period. In the first experiment (Set.1.), 1 mg of thebaine was added as a substrate and 1g (wet weight) of the obtained cells was re-suspended in 50 mM Tris-base; pH 8. The samples were incubated at 30 °C, 140 rpm for 8 or 16 h. The supernatant was collected (8000g, 15 min,4 °C) and extracted with methanol and chloroform. Methanol and chloroform extracts were directly monitored for a thebaine transformation by HPTLC analysis.

In order to study the potency of the plant extract for thebaine transformation, selected strains (Set.1) were subjected to plant extract treatments (Set.2). The treatment condition and analysis process were the same as those in Set.1.

2.4. Methods of analysis

Isolates were screened for codeine and morphine production using different analytical techniques including HPTLC, HPLC/PDA and LC/ESI-MS.

For HPTLC assay, stock solutions of three standards (thebaine, codeine and morphine; TEMAD Co. Tehran, Iran) were separately prepared by dissolving the thebaine standard in chloroform and the codeine and morphine in methanol in a final concentration of 0, 10, 20, 50, 100, 200, 500, 1000 μg/ml. A volume of 15 μl of standard solutions were loaded into a HPTLC aluminium Silica 60 F254 plate (6 mm in width; 10 mm apart) utilizing a Linomat 5 (CAMAG Co., Muttenz, Switzerland) sample applicator (Ahamad, Amin and Mir, 2014), in triplicate. Samples from Set.1 and Set.2 were loaded on a plate in the same conditions. Different solvent mixtures were examined to develop the standards and samples including ethyl acetate, ethanol, toluene, acetone, toluene:acetone (50:50), toluene:acetone:ethanol; (40:40:20), acetone:ammonia; (95:5), toluene:ethanol:acetone:ammonia; (40:40:15:5), toluene:ethanol:acetone:ammonia; (45:45:7:3) and toluene:ethanol:acetone:ammonia; (44:46:7:3). The plates were scanned at 285 nm and the individual Rf values of peaks were obtained. Thereafter, the plates were visualized using sulphuric acid containing 3% v/v formaldehyde, thus visualized spots and the presence of natural metabolites were confirmed.

The HPLC analysis resulted in some samples probably having more codeine and morphine which were subjected to HPLC/PDA and LC/ESI-MSMS analysis. These methods were utilized to identify and approve the transformation process more accurately. Samples were dissolved in aqueous solution containing 68% (v/v) acetonitrile. Separation was carried out on HPLC/PDA and LC–ESI/MS analyses utilizing an Agilent HPLC system (Waldbronn, Germany) equipped with C4 column, (250 mm×4.6 mm×5 μm×100 A°). The flow rate was 1 ml/min and the gradient mobile phase was Solvent A (H_2O:0.5% acetic acid:1% TEA) and B (acetonitrile:0.5% acetic acid). The separation started with Solvent A and its percentage was reduced to 60% and 50% in 10 and 20 min, respectively. Thereafter, Solvent A rose to 100%

in 7 min and the separation was finalized in 30 min. An aliquot (25 µl) of sample was injected into the HPLC system and analysis was performed at 30 °C, at a detection wavelength of 280 nm. The HPLC system comprised a FinniganTM LCQTM DECA instrument equipped with an ion trap mass spectrometer (mass range m/z 10–2000) and an electro spray ionization (ESI) source. The solvent system in positive ion (PI) mode was similar to HPLC. Nitrogen was utilized as sheath gas at 40 ml/min and auxiliary gas at 20 ml/min. The ESI-MS/MS instrument was operated with the capillary temperature at 300 °C. The electro spray voltage was set at 5 kV, the capillary voltage at −15 V, and the tube lens was offset at −30 V. All operations, data acquisition, and processing were controlled by the Xcalibur 2.0 SR2 software (copyright Thermo Electron Corporation 1998–2006).

2.5. Biochemical and biomolecular experiments

The phenotypic related properties of the selected isolate, including shape, size, the characteristics of colony and gram staining were investigated and the strain was identified by physiological and biochemical tests (DEMELİ et al., 2014; Shivaji et al., 2009). Genomic DNA of native isolate was extracted using polymerase chain reaction (PCR) template purification kit (Kiagene Co.) and was utilized as a template DNA for PCR amplification. In order to identify the bacterial isolate for phylogenetic studies, 16S rDNA gene was amplified utilizing PCR and universal primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) (Souza et al., 2014). The 25 µl of PCR reaction mixture contain 1 µl of template DNA, 0.25 µl of each primer (10 mM), 0.5 µl deoxynucleoside triphosphate dNTP (10 mM), 0.75 µl $MgCl_2$ (50 mM), 0.25 µl Taq DNA polymerase (5 u/µl), 2.5 µl PCR buffer (10X) and 19.5 µl double distilled H_2O. The PCR process was carried out as follows: primary denaturation for 5 min at 95 °C, 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 45 s, and extension at 72 °C for 1 min, and additional reaction at 72 °C for 10 min. The PCR product was checked on a 1% agarose gel and the purified product was sequenced directly by ABI 3730XL DNA sequencer at Macrogen (Seoul, South Korea).

The sequence was identified through its similarity, using the EzTaxon-e web server (http://eztaxon-e.ezbiocloud.net/) and aligned utilizing Clustal X2. To study the phylogenetic relationship among the strain and other species, the neighbor-joining (NJ) method was applied utilizing the MEGA5 software. Confidence levels for the phylogenetic tree were evaluated by bootstrapping with 1000 replicates (Kashi et al., 2014).

3. Results

3.1. Isolation and production of microorganism

Several parts of the plants including stems, capsules, leaves, and petals, except roots were collected and 67 various isolates containing 62 bacterial and 5 fungi strains were isolated. Majority of the isolated microorganisms were Gram positive bacilli (36 strains), followed by Gram negative bacilli (22 strains), and some cocci (4 strains), as well as fungi strains (5 strains).

3.2. Screening of plant extracts for thebaine

In order to find the part of the plant that have the maximum amount of thebaine as well as qualified extracting solvents, each part of the plant was extracted utilizing five solvents. Thereafter, 10 solvent mixtures were examined for development of thebaine, codeine and morphine on HPTLC plates. The best developing mobile phase was toluene:ethanol:acetone:ammonia (44:46:7:3). The metabolites profiles were evaluated by HPTLC method (Fig. 1A), with the optimized mobile phase. The dark spot was considered as a positive evidence for thebaine in samples. Results reveal that the methanolic extract of capsule was the appropriate extract and was used for the rest of the analysis (Fig. 1A).

3.3. Screening of thebaine-resistant microorganism and transformation process

The microorganisms collected from natural habitat of *p. bracteatum* were tested using various concentrations of thebaine to find the most thebaine tolerated microorganisms. The results showed that the surviving isolates were not affected by the low concentration of thebaine. When thebaine concentration is higher than 500 µg/ml, they were considered to be lethal; 48 of 67 isolates (about 72%) are capable of growing on high concentration (≥500 µg/ml) of thebaine. Thereafter, surviving microorganisms were selected to estimate their ability to transform thebaine. Forty eight (48) isolates with high resistancy values were selected for biotransformation purposes.

As earlier mentioned, selected microorganisms were treated using 96 independent biotransformation tests in comparison with control condition with no microorganism. All biotransformation reactions were monitored utilizing HPTLC and HPLC/PDA as earlier mentioned. Biotransformation products were identified using their Rf values in comparison with standard Rf values. Results showed that, 12 microbial strains are known to transform thebaine into desired products (Fig. 1B).

3.4. Microbial transformation process with plant extract

Both thebaine and plant extract were evaluated for their transformation potency. Based on this, in addition to pure thebaine, plant extracts were utilized for thebaine transformation. The results revealed that, five isolates can use plant extract like thebaine, for the production of codeine and morphine. This was demonstrated using HPTLC and LC/ESI-MS analysis. Since the HPTLC and HPLC/UV–vis absorption pattern was not enough to properly identify the compounds, the mass spectrometric pattern was studied. In each case, fragmentation was utilized to interpret the chemical structure. In the case of codeine (at a retention capacity of 60.44; Fig. 2A), the identified m/z based on its 300.47 Da belong to $[M+H]^{o+}$ and 338.07; $[M+K]^{o+}$,317.93; $[M+NH_4]^{o+}$, 248.07; $[M+H-3H_2O]^{o+}$, 263.93; $[M+H-2H_2O]^{o+}$,282.80; $[M+H-H_2O]^{o+}$(Fig. 2B). In morphine (at a retention capacity of 66.84; Fig. 2A), the identified m/z based on its 285.00 Da belong to $[M+H]^{o+}$ and 308.20; $[M+Na]^{o+}$,323.33; $[M+K]^{o+}$,303.13; $[M+NH_4]^{o+}$, 257.33; $[M+H-3H_2O]^{o+}$, 268.73; $[M+H-2H_2O]^{o+}$,(Fig. 2C). The results revealed that one of these isolates could transform thebaine into both codeine and morphine products (Fig. 1B, No.6). For Identification validation the MSMS fragmentations with the highest intensity were monitored. The selected fragments in the case of codeine and morphine and their MSMS spectrum are shows in Fig. 3.

3.5. Identification of Bacillus sp. FAR

Among the various microorganisms isolated from *P. bracteatum*, the most capable strain for biotransformation of thebaine is the one isolated from the stems and was characterized by experimental methods. The phenotypic confirmation demonstrated that the mentioned microorganism is rod shaped and Gram positive bacteria. On the nutrient agar, this bacterium was formed as circular, white color, having medium size and flat colonies. It also have positive response to endospore, catalase, Voges-Proskauer, starch hydrolysis, urease, producing acid from glucose, lactose, fructose, arabinose, galactose, maltose and mannitol analysis and it is tolerant in 6% NaCl and at 45 °C temperature. On the other hand, it has negative response to oxidase, haemolysis, methyl red, indole, citrate, H_2S, lysine decarboxylase and DNase.

The 16S rDNA gene sequence retrieved has been deposited at Genbank (www.ncbi.nlm.nih.gov), and the accession number is

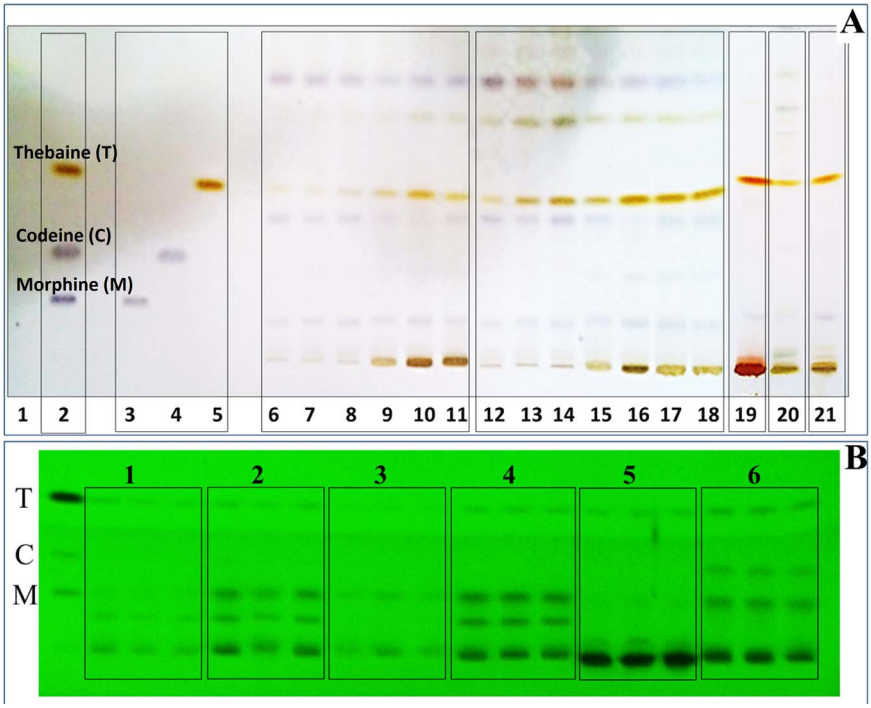

Fig. 1. (A) Metabolites profiles of several parts of *Papaver bracteatum* with TLC by different solvent; 1: Blank, 2: mixture of codeine, morphine and thebaine, 3–5: morphine (M), codeine (C), thebaine (T) standard, 6–11: stems, 12–18: capsule, 19: petal, 20: leaf, 21: capsule. (B) Metabolites profiles of six samples tested for thebaine biotransformation with HPTLC. Sample No.6 produced both codeine and morphine.

KU746803. Finally, the partial 16S rDNA sequence and phylogenetic analysis demonstrated that this microorganism, named *Bacillus* sp. FAR, has more than 99% similarity to *Bacillus aryabhattai* B8W22 (T), (Fig. 4).

4. Discussion

The present study is a part of a long and detailed project for the production of morphine alkaloids utilizing microbial biotransformation. Several studies have noted the potency of microorganism in converting morphine alkaloids into other products. There are few studies on microbial transformations of thebaine. In the early 1960s, Iizuka et al. and Yamada et al. described how *Trametes sanguinea* converts thebaine into 14-hydroxycodeinone and 14-hydroxycodeine (Hailes and Bruce, 1993; Kunz et al., 1985; Madyastha et al., 2000). Northebaine, as indicated by Madyastha et al. (2000), was the main product formed during the bioconversion of thebaine by *Mucor piriformis* (Madyastha et al., 2000). In previous reports, various fungi were utilized for thebaine transformation. There is no report on the use of bacteria for biotransformation of thebaine into codeine and morphine. Despite the complex polycyclic nature of the morphine alkaloids, microbial process may able to synthesize these compounds through several useful ways (Boonstra et al., 2001; Rathbone and Bruce, 2002).

In this study, agar dilution with various concentrations of thebaine was utilized to (i) induce the production of enzymes for the transformation of thebaine, and (ii) select the thebaine tolerate microorganisms. The initial substrate resistancy dose does not guarantee a better biotransformation activity. But it is an important characteristic for a bio-transforming agent (Bicas and Pastore, 2007). Therefore, surviving microorganisms having higher resistancy values were selected for biotransformation.

The results indicate that plant capsule has higher amount of thebaine which was previously reported as well (Seddigh et al., 1982). Thebaine is an abundant constituent (98% of alkaloids) in *P.bracteatum* (Brochmann-Hanssen and Wunderly, 1978; Seddigh et al., 1982). Although it was stated that the usage condition of thebaine is restricted (Bruce et al., 1995); it is the habitat plant in Iran and an ideal target for biotransformation. Moreover, the use of these species for illicit drugs is difficult when compared to *P. somniferum*, (Seddigh et al., 1982). Therefore as earlier mentioned, in addition to pure thebaine, selected microorganisms were treated with plant extracts for thebaine transformation.

A major challenge for the desired biotransformation reaction is to find the appropriate microorganism. Thus, classical screening of a series of microbial strains is still the most widely used method (Muffler et al., 2011). Fundamental studies on alkaloids metabolism in microorganisms can reveal new enzymes for the biological synthesis of the morphine alkaloids (Boonstra et al., 2001). It can be concluded that this study introduced a bacterium which can transform thebaine into both codeine and morphine. Physiological and morphological studies and phylogenetic analysis followed by 16S rDNA sequencing identified *Bacillus* sp. as the best microorganism for biotransformation of thebaine. To the best of our knowledge, there is no previous study on thebaine biotransformation using this class of bacteria which has more than 99% similarity to *B. aryabhattai* B8W22(T). Moreover, previous studies have shown that *B.aryabhattai* is an upper atmospheric bacteria existing at altitudes of 40 and 41 km, (Shivaji et al., 2009; Tanamool et al., 2013). *Bacillus* sp. FAR was also isolated from microbial flora of *P.bracteatum* or Persian poppy at altitudes of 2–3 km.

In conclusion, despite the low conversion recovery, codeine and morphine, important compounds for pharmaceutical industries, were produced from thebaine. It is certain that optimization of different factors would be necessary and useful so as to increase the yield of biotransformation process. Ongoing research will be continued based on this result.

In this study, 67 microorganisms were isolated from *P. bracteatum* and its surrounding soil. The extraction process and HPTLC, HPLC/PDA and LC/ESI-MS were optimized for thebaine, codeine and morphine evaluation. Screening tests on 67 isolates resulted in the selection of 48 strains for biotransformation processes in order to select the microorganism capable of transforming thebaine into

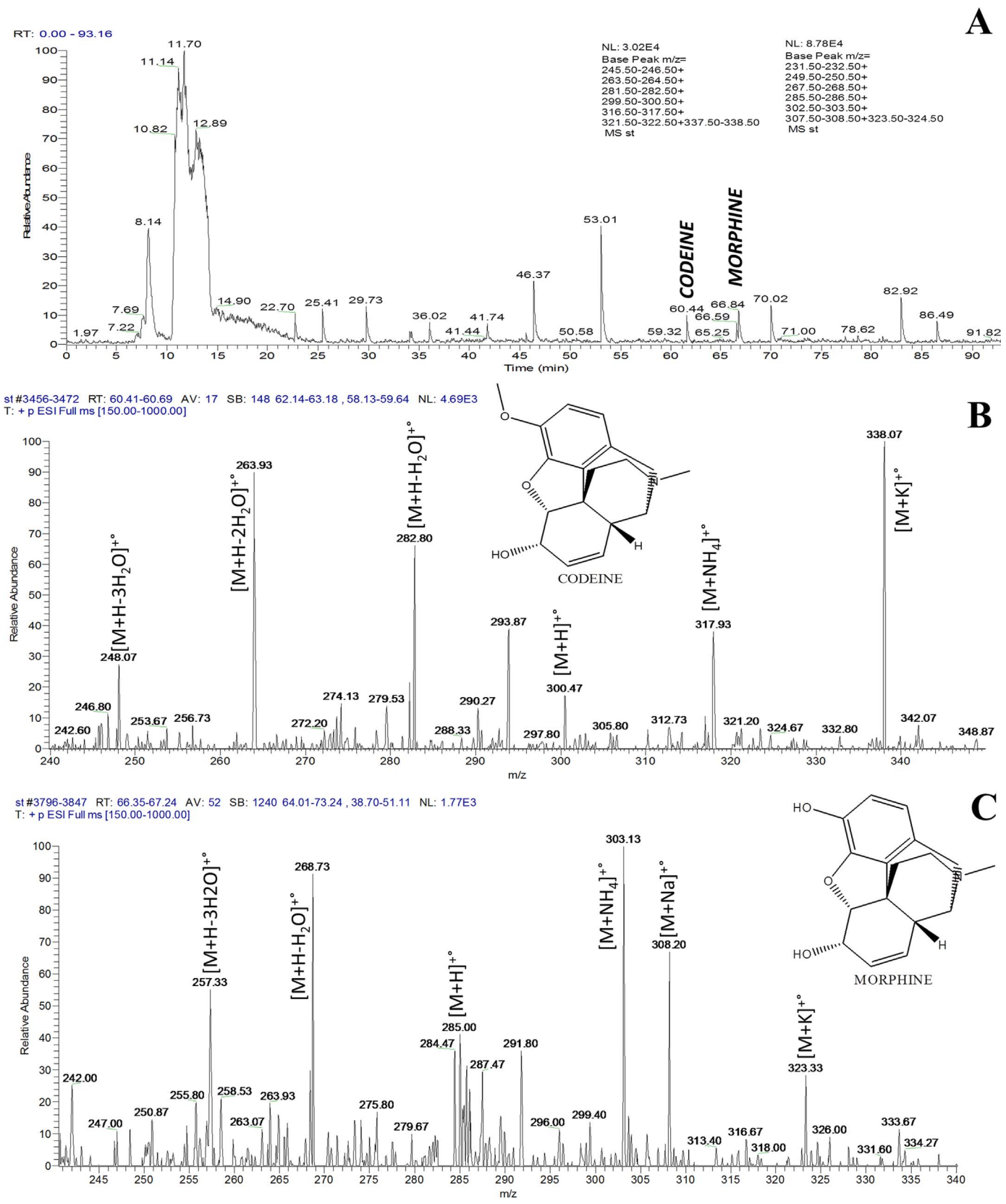

Fig. 2. LC/ESI-MS of bio-transformed product of codeine and morphine; (A) Chromatogram of UV–vis absorption pattern of Sample No. 6. Spectrum of bio-transformed product of codeine (B) and morphine (C).

codeine and morphine. HPTLC results revealed that among 48 isolates, 12 strains have the ability to produce codeine and morphine from thebaine. Based on HPTLC developed chromatogram results, 12 isolates can transform thebaine. Among them, five isolates were monitored for their transformation potency using plant extract. Finally, it was founded that *Bacillus* sp. FAR has the potency to

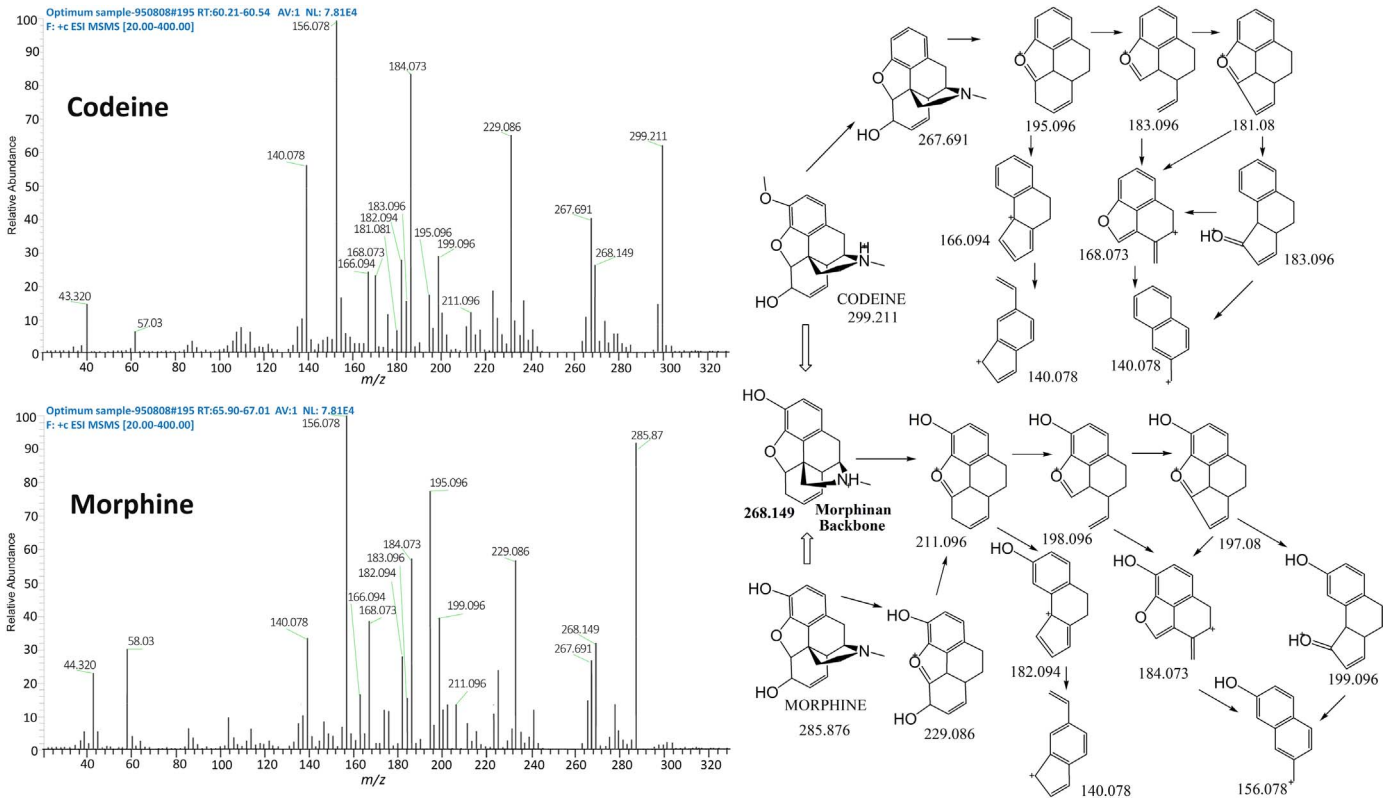

Fig. 3. MS/MS of the codeine (up) and morphine (down) ion trap tandem mass spectrum and the fragmentation pattern.

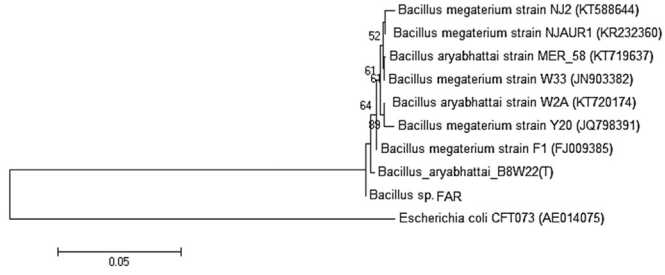

Fig. 4. Phylogenetic tree inferred from comparison of the 16S rDNA sequence. The bar represents 0.05 estimated changes per nucleotide position.

biotransform thebaine into codeine and morphine.

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