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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%