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Research Article

Transformation of Morphine Alkaloid by a Novel Indigenous *Pseudomonas* Strain Isolated from Wastewater of a Pharmaceutical Plant in Iran

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ABSTRACT

Morphine alkaloids represent a class of pharmaceuticals characterized by their potent analgesic properties. In recent years, researchers have employed microbial biotransformation of alkaloids as a strategy to generate more effective analgesic compounds. Evidence suggests that biotransformation is an efficient and cost-effective method for the production of novel substances. In this study, *Pseudomonas* sp. was isolated from wastewater samples obtained from a pharmaceutical manufacturing facility and cultured in a mineral medium. The medium was supplemented with 5 mM morphine as the sole carbon source. The isolated strain was identified using morphological and biochemical methods. For genetic identification, 16S rDNA PCR sequencing was conducted. The capabilities of the isolated strain to biotransform morphine alkaloids were evaluated using High Performance Liquid Chromatography (HPLC). The results demonstrated that the isolated strain exhibited over 99% genetic similarity to *Pseudomonas putida*. Following an 8-day incubation period, the concentration of morphine in the growth medium decreased from 1000 to 616.2 µg/mL, while a concentration of 387.2 µg/mL of hydromorphone was synthesized. These findings indicate that the isolated strain can efficiently and effectively convert morphine into hydromorphone for pharmaceutical applications. Further investigations are warranted to elucidate the specific proteins and enzymes involved in this biochemical pathway.

1. Introduction

Alkaloids are a group of nitrogenous organic compounds that are produced by a wide variety of organisms, including animals, plants, fungi, and bacteria. These compounds are used in the treatment of various medical complaints, including anticancer (Kittakoop et al., 2014), antibacterial (Cushnie et al., 2014), antiarrhythmic, and analgesic (Sinatra et al., 2011) activities. They are an important group of chemical substances that have diverse physiological effects on the human body. As a result, they are commonly utilized as the initial stages for drug discovery (Cushnie et al., 2014). Morphine alkaloids belong to a subgroup of alkaloids and possess potent analgesic properties. Currently, a significant number of analgesic drugs available in the market are either morphine alkaloids or their chemical derivatives, and they are widely utilized in various medical settings worldwide (Reed, 2015). As a result, numerous efforts have been made in the past few decades to discover novel biological catalysts for the conversion of morphine alkaloids. Scientists are not only interested in identifying

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new drugs but also in finding new intermediate compounds for the synthesis of these drugs (Rathbone, 2002). Despite the extensive use of morphine alkaloids and their derivatives in various medical settings, they are primarily obtained through extraction from plants. However, these traditional production methods are not as efficient as required due to the low concentrations at which most of these substances accumulate in plant cells (Nakagawa et al., 2011). Furthermore, the methods of plant metabolic engineering and the utilization of chemical synthesis for the production of these chemicals were also unsuccessful. This can be attributed to the intricate and chiral nature of these chemical substances, as well as the complexity of biosynthetic pathways in plants (Glenn et al., 2013). Due to the aforementioned reasons, the development of a new biological catalyst or biosynthetic route capable of competing with traditional production methods would guarantee a steady supply of morphine alkaloids to meet the global demand for this valuable drug. Microbial transformation systems offer numerous advantages over plant systems. Firstly, microbial genetic systems are well-understood and various genetic tools and approaches are available for the genetic engineering of microorganisms. Secondly, their culture and growth are much more feasible in a laboratory compared to plants. Lastly, microbial transformations enable the production of sufficient quantities of useful intermediates or metabolites, allowing for the identification and examination of catalytic mechanisms that may be unknown in synthetic organic chemistry (Pervaiz et al., 2013). The objective of this study is to isolate native *Pseudomonas* strains capable of efficiently degrading morphine from wastewater generated by the Behansar pharmaceutical factory, with the aim of developing pharmaceutical derivatives of this compound.

2. Materials and Methods

2.1. Chemicals

All of the culture media, organic and inorganic compounds, and reagents utilized in HPLC analysis were procured from Sigma. Taq DNA polymerase was acquired from Fermentas. The chemicals and solvents employed were of the utmost purity obtainable.

2.2. Isolation of the bacterium

Samples were collected from the wastewater of a pharmaceutical company called Behansar, located in Saveh, Iran. The collected samples were diluted and then cultured in a mineral medium containing $(NH_4)_2SO_4$ (0.5 g), K_2HPO_4 (0.2 g), KH_2PO_4 (0.2 g), and $MgSO_4$ (0.05 g) per liter. Additionally, trace elements were also added to the solution. To serve as the only carbon source, the medium was further supplemented with 5 mM morphine. The inoculated flasks were incubated in a shaking incubator (180 rpm) at 30 ℃ for 48 hours (Bruce et al., 1990). After that, diluted samples of the liquid culture were inoculated onto the agar plates. Single colonies from these plates were transferred to Luria-Bertany broth medium. One of the morphine-consuming strains was selected for further analysis.

2.3. Identification of the isolate

Some diagnostic tests were performed on the isolated bacterial strain. These tests included gram staining, catalase test, oxidase test, cultivation in TSI medium, methyl red and Voges Proskauer tests. The genomic DNA of the selected strain was extracted using the boiling method. The 16SrRNA gene was amplified using universal primers 8F (AGA GTT TGA TCC TGG CTC AG) and 1541R (AAG GAG GTG ATC CAG CCG CA). The PCR was conducted using an Applied Biosystem 9700 thermal Cycler for 30 cycles. The first denaturation step was carried out at 94 ℃ for 3 minutes. Each cycle consisted of denaturation at 94 ℃ for 30 seconds, annealing at 57 ℃ for 1 minute, extension at 72 ℃ for 1 minute, and a final extension at 72 ℃ for 10 minutes. After the PCR reaction is completed, the PCR product is subjected to electrophoresis to confirm the success of the reaction. Subsequently, the PCR product is purified and sent to Macrogen for sequencing. The sequencing results were edited using Chromas and Bioedit software. The phylogenetic relationship of the isolate was determined by comparing the sequencing data with sequences of some members of the *Pseudomonas* genera available through the Gen Bank. The evolutionary history was inferred using the Maximum Likelihood method based on the Tamura-Nei model (Tamura, 1993).

Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016).

2.4. Determination of the growth curve of isolated bacteria

The growth curve of the isolated strain was measured using the spectrophotometric turbidity method for a period of 10 days. The optical absorption of the medium at a wavelength of 600 nm was measured daily, and the results were used to draw the growth curve (Hirsch, 1988).

2.5. Morphine transformation Process

The bacterial samples, which had turbidity equal to 0.5 of McFarland at a volume of 10% V/V, were added to 90 mL of mineral medium. This medium contained 1000 µg/mL of morphine as the sole carbon source. The flasks, which had a volume of 250 mL, were then incubated at 30 ℃ and 180 rpm for 8 days. The cell-free extracts were prepared by centrifuging at 5000×g. Alkaloids were extracted by adjusting the acidity to 7.8 and adding sodium bicarbonate and sodium hydroxide. Ethyl acetate and chloroform were then added. After removing the solvent under vacuum, the alkaloids were dissolved in a small amount of methanol. This solution was used for chromatography (Korkmaz, 2006).

2.6. HPLC analysis

HPLC analysis was conducted using a Cecil model Adept CE4900 chromatograph that was equipped with a Cecil model CE4200 UV detector set at 218 nm. To achieve separation for analytical purposes, an oven column model CE4601 and a lichrosorb C18 column with a 4.6mm inside diameter and 25cm height were utilized. The solvent system employed was the one described by Umans (Umans et al., 1982). The morphine alkaloids from the culture medium were extracted on days 2, 4, 6, and 8. Each sample was then injected into the HPLC device. A variable known as the response factor was employed to quantitatively assess the amount of compound present in the sample. The response factor was computed using Equation 1.

$$Response\ Factor = \frac{Peak\ Area\ (Standard)}{Standard\ Amount} \quad (1)$$

$$Sample\ amount = \frac{Peak\ Area\ (Sample)}{Response\ Factor}$$

3. Results

3.1. Isolation, determination of the growth curve, and identification of the bacterium

Bacterial strains that were capable of utilizing morphine as the only carbon source were isolated. Among them, the strain that exhibited the highest rate of growth in the presence of morphine was chosen for further investigation. The findings indicated that this particular strain enters the stationary phase after 8 days (Figure 1).

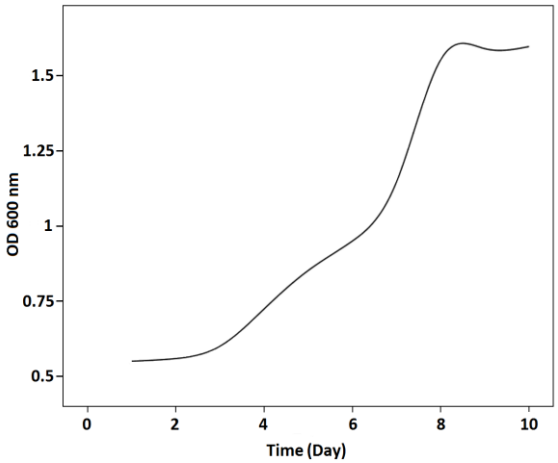


Figure 1. The Growth Curve of Bacterial Strain PAD-116 Over 10 Days

The results from the phenotypic tests of the selected strain are displayed in Table 1.

Table 1. Phenotypic characteristics of *Pseudomonas* sp. strain PAD-116

Line	Diagnostic tests	Result
1	Shape	Rod
2	Gram Staining	-
3	Catalase	+
4	Oxidase	+
5	Reaction in TSI* Agar	K/K**
6	Methyl Red	-
7	Voges–Proskauer	-

* Triple Sugar Iron
** Alkaline/Alkaline

According to the phenotypic characterization of the strain and in comparison to other studies, the strain was tentatively named as *Pseudomonas* sp. The DNA extraction of the selected strain and the amplified 16SrRNA gene are shown in Figure 2. The results of the

comparison between the strains in the gene bank showed that it has more than 99% similarity with *Pseudomonas putida*, and the isolate was identified as *Pseudomonas* sp. strain PAD-116. The phylogenetic tree is shown in Figure 3.

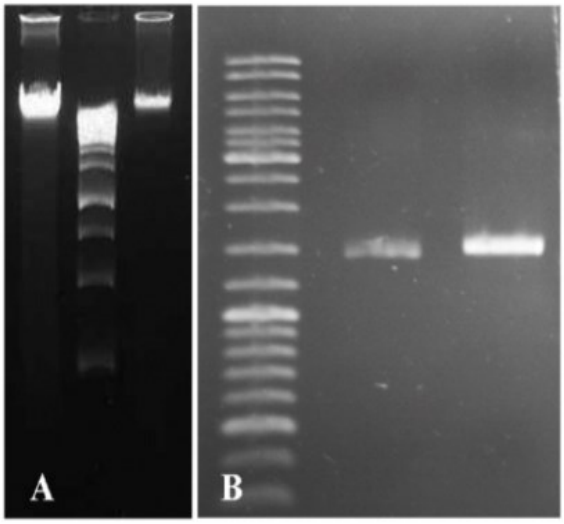


Figure 2. Electrophoresis on Agarose Gel A: DNA Extraction of the Selected Strain (Middle: Ladder; Left: Positive Control; Right: PAD-116) B: Amplified 16S rRNA Gene (Left: Ladder; Middle: PAD-116; Right: Positive Control)

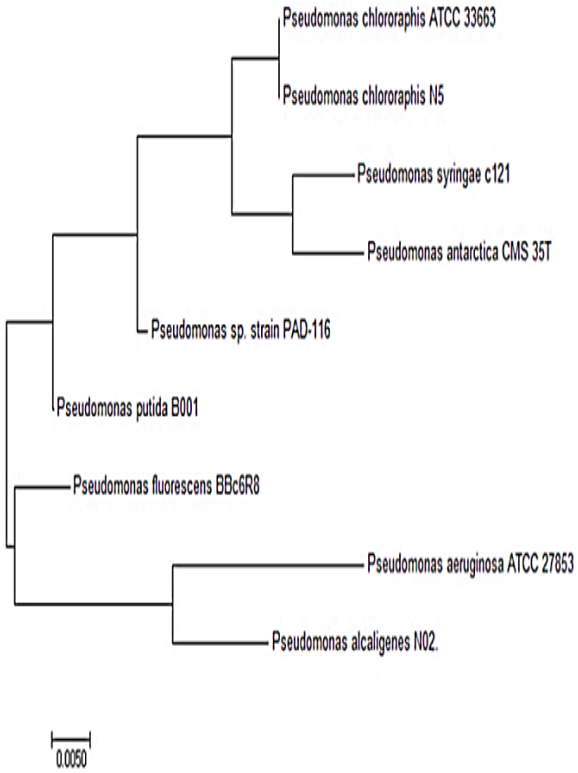


Figure 3. Phylogenetic tree of selected strain *Pseudomonas* sp. strain PAD-116

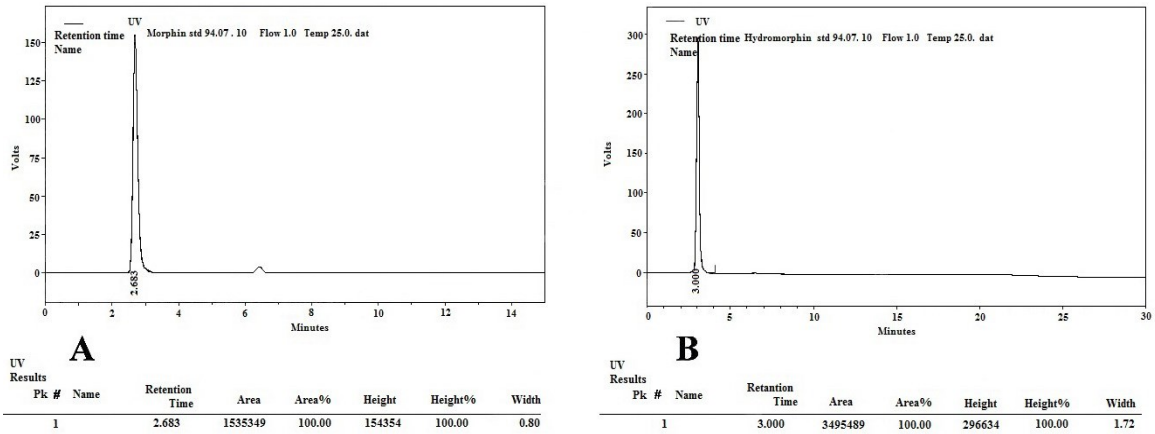
3.2. Results of Morphine transformation Process

The ability of PAD-116 to biotransform morphine was evaluated through HPLC analysis. By comparing the peak retention times in the samples, two significant peaks were identified as morphine and hydromorphone. The amounts of morphine and hydromorphine in the growth medium were evaluated for eight days. The results showed a significant decrease in the amount of morphine after 8 days of incubation, while the amount of hydromorphine experienced a substantial increase during the same period. The chromatogram obtained by injecting standard morphine (1000 µg/mL) and hydromorphone samples into the HPLC device is shown in Figure 4.

The average inhibition times for morphine and hydromorphone are 2.683 and 3.005 seconds, respectively, with corresponding standard deviations of 0.016 and 0.009 seconds. The results revealed that the isolated strain efficiently transformed morphine into hydromorphine. Figures 5 and 6 displayed the chromatogram of morphine alkaloids extracted from the culture medium after 2, 4, 6, and 8 days of incubation. By analyzing the area under the peak diagram for morphine and hydromorphine, the concentration of each compound in the culture medium was calculated. After two and four days, the concentration of morphine was 930.3 and 874, and the concentration of hydromorphine was 88.5 and 133 µg/ml respectively.

Based on the peak diagram, the concentration of morphine and hydromorphine in the culture medium was calculated. After six and eight days, the concentration of morphine was 803.8 µg/mL and 616.2 µg/mL, while hydromorphine was 203.6 µg/mL and 387.2 µg/mL, respectively.

The amount of morphine in the growth medium decreased from 1000 µg/mL to 616.2 µg/mL after eight days, and 387.2 µg/mL of hydromorphine was produced. Figure 7 shows the quantities of morphine and hydromorphine at different time intervals.



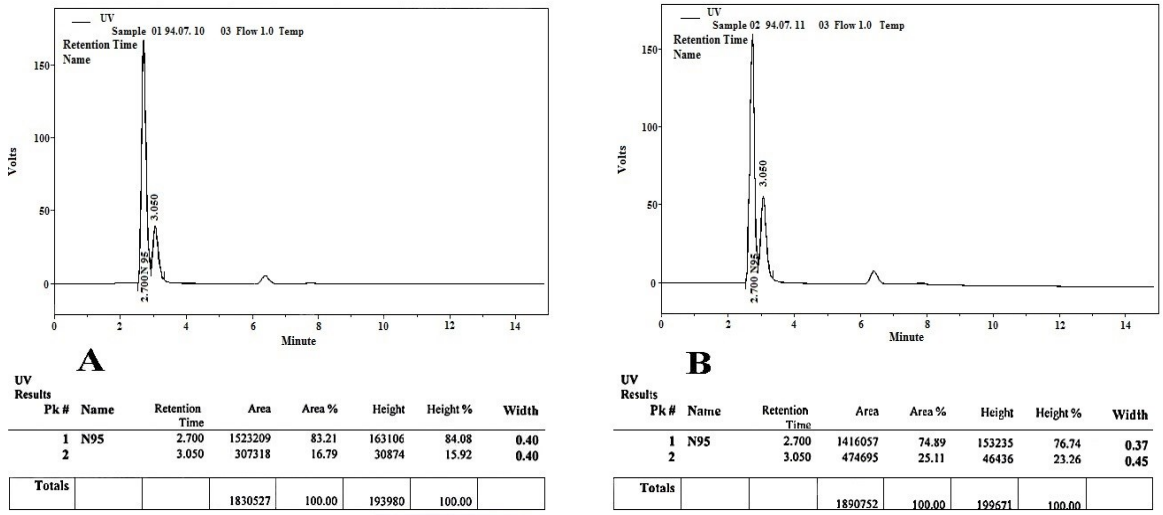
UV Results

Pk #	Name	Retention Time	Area	Area%	Height	Height%	Width
1		2.683	1535349	100.00	154354	100.00	0.80

UV Results

Pk #	Name	Retantion Time	Area	Area%	Height	Height%	Width
1		3.000	3495489	100.00	296634	100.00	1.72

Figure 4. Chromatogram obtained by injecting standard A (morphine) and B (hydromorphone) samples into an HPLC device



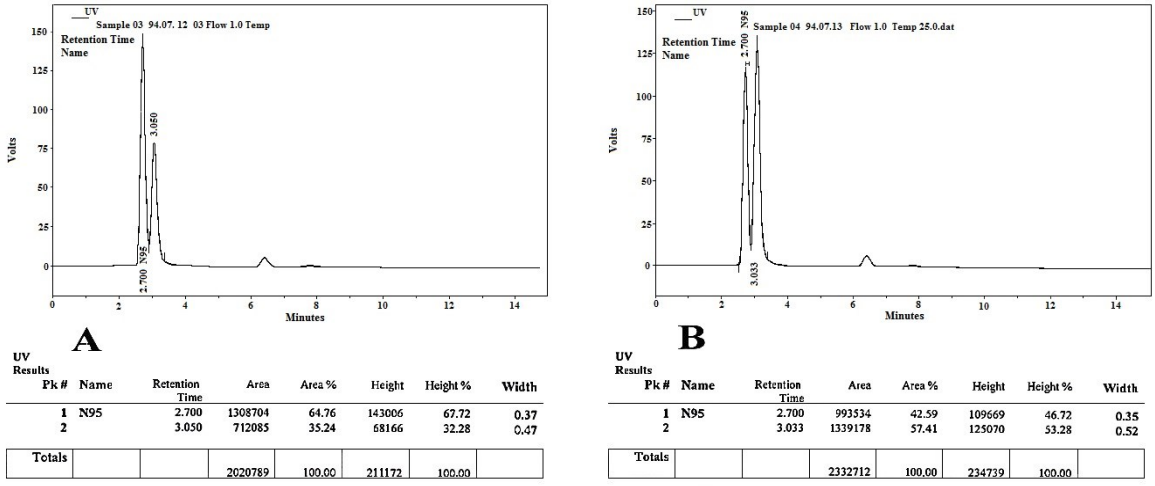
UV Results

Pk #	Name	Retention Time	Area	Area %	Height	Height %	Width
1	N95	2.700	1523209	83.21	163106	84.08	0.40
2		3.050	307318	16.79	30874	15.92	0.40
Totals			1830527	100.00	193980	100.00	

UV Results

Pk #	Name	Retention Time	Area	Area %	Height	Height %	Width
1	N95	2.700	1416057	74.89	153235	76.74	0.37
2		3.050	474695	25.11	46436	23.26	0.45
Totals			1890752	100.00	199671	100.00	

Figure 5. Chromatogram of morphine alkaloids extracted from the culture medium after A) two and B) four days of incubation



UV Results

Pk #	Name	Retention Time	Area	Area %	Height	Height %	Width
1	N95	2.700	1308704	64.76	143006	67.72	0.37
2		3.050	712085	35.24	68166	32.28	0.47
Totals			2020789	100.00	211172	100.00	

UV Results

Pk #	Name	Retention Time	Area	Area %	Height	Height %	Width
1	N95	2.700	993534	42.59	109669	46.72	0.35
2		3.033	1339178	57.41	125070	53.28	0.52
Totals			2332712	100.00	234739	100.00	

Figure 6. Chromatogram of morphine alkaloids extracted from the culture medium after A: six and B: eight days of incubation

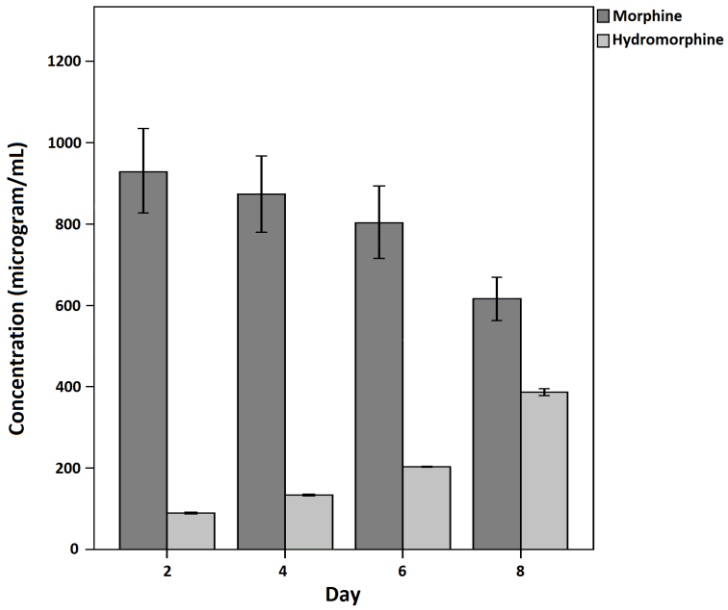


Figure 7. Changes in the concentration of morphine and hydromorphine in the medium at different time intervals

4. Discussion

Alkaloids are a group of chemical compounds present in nature, which contain alkaline nitrogen in their structure. They are produced by a wide range of organisms, including bacteria, fungi, plants, and animals. These compounds can be extracted and purified from the organisms' extracts. Currently, plants serve as one of the primary sources for the production of these substances, although their production by plants tends to be low. Alkaloids possess a broad spectrum of pharmaceutical applications. Thus far, these chemical compounds have been identified to possess medicinal properties such as antimalarial, anti-asthma, anticancer, antiarrhythmic, antidiabetic, analgesic, and antibacterial properties (Pelletier, 1999). Morphine alkaloids are a group of substances that are the most potent painkillers currently employed in the treatment of chronic pain. These compounds hold significant medical and economic value, prompting numerous endeavors to produce derivatives through chemical and biological reactions. *Papaver somniferum* is one of the oldest medicinal plants utilized by humans. This plant serves as the primary provider of morphine and codeine pain relievers, as well as approximately 80 other alkaloids (Weid, 2004). Presently, numerous compounds derived from morphine are commercially available, including oxycodone, hydrocodone, naloxone, naltrexone, and nalmefene. They are used in medical centers as sedatives (codeine and hydrocodone), as drugs for opium and alcohol addiction (naltrexone and nalmefene), and for the treatment of drug overdose (naloxone) (Kreutzmann, 2007). In recent years, microbial transformation has become an essential tool in the production of natural compounds. Scientists have long been aware of the metabolic capacity of microorganisms, and in recent decades, there has been a significant increase in the utilization of this capacity. Biotransformation refers to the conversion of a specific chemical compound into a similar one, facilitated by biological catalysts like microorganisms (Rathbone, 2002). Microbial transformation offers numerous advantages. Its processes are conducted at ambient temperature, atmospheric pressure, and neutral acidity, unlike chemical reactions that necessitate extreme conditions like high temperature and pressure, making them economically and environmentally inefficient. Additionally, biocatalysts exhibit high specificity for their substrate, enabling differentiation between various steric and enantiomeric forms of the substrate (Collins, 1999). Corrected: Over the past few decades, extensive studies have been conducted on the transformation of morphine alkaloids and the

production of more effective analgesics from natural compounds. These studies have evaluated the ability of various groups of microorganisms to produce new products. As a result, dozens of microorganisms from different phylogenetic groups have been isolated. The most promising groups were bacteria, particularly the *Pseudomonas* genus, probably due to its metabolic diversity. For instance, the strain *Pseudomonas testosteroni* has been reported to undergo morphine transformation. This organism was capable of oxidizing morphine and codeine. Morphine was converted into 14 hydroxymorphinone with a relatively low yield (Liras et al., 1975). In another study, the researchers evaluated the ability of *Pseudomonas putida* M10 to biotransform morphine alkaloids. This bacterium was isolated from the industrial wastewater of a poppy processing plant, and it was discovered that it could only use morphine and codeine as sources of growth. Experiments conducted with this strain indicated that the organism could utilize both morphine and codeine, but not thebaine (Bruce et al., 1990; Boonstra et al., 2001). Later on, Lister and his colleagues reported that the organism was capable of hydroxylating morphine alkaloids. This chemical alteration holds industrial significance as even a slight modification in the chemical composition can lead to a substantial variation in the potency and efficacy of the drug. In this particular scenario, the addition of the 14-hydroxyl group significantly enhances the pain-relieving properties of morphine, making it a challenging task to incorporate this functional group through chemical means. Additionally, studies have demonstrated that this organism hydroxylates codeine, resulting in the formation of 14-hydroxycodeinone, which is subsequently converted into 14-hydroxycodeine. Moreover, this organism has the capability to produce hydrocodone, dihydrocodeine, and oxycodone (Lister et al., 1999). It is important to note that the transformation of alkaloids among bacteria is not limited to the *Pseudomonas* genus. In one study, researchers isolated *Rhizobium radiobacter* R89-1, which is capable of biotransforming morphine at concentrations up to 24g/L. This strain was able to convert codeine and morphine into a C-14-hydroxy derivative (Kyslíková et al., 2013). Additionally, strains from *Bacillus* (Madyastha et al., 1998), *Mycobacterium* (Zhang et al., 2005), and *Streptomyces* (Harder, 1989) exhibited activity in transforming morphine alkaloids. In nearly all of these instances, the biotransformation was catalyzed by enzymes called morphine dehydrogenase and morphinone reductase (Rathbone, 2002; Boonstra et al., 2001). During this research, a recently discovered bacteria strain was obtained from the wastewater of an Iranian pharmaceutical company. This strain has demonstrated the ability to efficiently convert morphine into hydromorphine. This company is recognized as a manufacturer of pharmaceutical raw materials. It holds the distinction of being the largest producer of mineral raw materials and the second largest producer of narcotics raw materials in the Middle East. Behansar product range includes codeine, morphine, thebaine, and papaverine, all of which are derived from the poppy plant. Additionally, other compounds such as codeine phosphate, morphine sulfate, oxycodone, and hydrocodone are also produced through chemical modifications of precursor compounds. These characteristics make it a suitable environment for isolating bacteria with the ability to utilize morphine alkaloids. In this study, a bacterial strain that could solely rely on morphine as its carbon source was successfully isolated. The capability of the isolated strain to transform morphine was analyzed using the HPLC method. The results indicated that this organism was able to produce a significant amount of hydromorphine after four days. In the growth medium, a total of 387.2 µg/mL of hydromorphine was produced during this period. Hydromorphine is a crucial medication prescribed for patients suffering from moderate to severe chronic pain, and its production holds great importance in medical settings. It is most commonly prescribed when morphine is no longer effective in treating patients with severe pain, as it is nearly three times stronger than morphine and carries a lower risk of dependence (Schuler, 2015). Further studies should be conducted to identify the genes and proteins involved in order to enhance the production efficiency of this valuable drug.

Conclusion

In conclusion, alkaloids, particularly those derived from the morphine group; represent a significant source of potent medicinal

compounds with diverse therapeutic applications. The exploration of microbial transformation, especially using strains like Pseudomonas and Rhizobium, has emerged as a promising method for producing enhanced derivatives such as hydromorphine. This approach not only offers an environmentally friendly and efficient alternative to traditional chemical synthesis but also holds the potential to improve pain management in clinical settings. Given the increasing demand for effective analgesics, further research into the genetic and enzymatic mechanisms of these microorganisms is essential to optimize production processes. Ultimately, harnessing microbial capabilities may lead to the development of more effective and safer pain relief options in modern medicine.

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Conflict of Interest:

The author states that there is no conflict of interest.

Refereces

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