Original Article

Response Surface Methodology Optimization of Thebaine Biotransformation into Codeine and Morphine Using *Bacillus* sp. FAR

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ABSTRACT

Background: Codeine and morphine are important pharmaceuticals having analgesic characteristics. **Aim:** The current study used response surface methodology to optimize the biotransformation of thebaine into codeine and morphine using *Bacillus* sp. FAR. **Methods:** A central composite design was used to determine the optimal concentrations of buffer and thebaine, pH, time, temperature, and biomass. Improvement in transformation was monitored using HPLC-DAD. **Results:** The optimized conditions employed were pH 7.7 at 36°C for 30 h using a thebaine concentration of 80 μg/mL, 45 mM tris of buffer and 2.9% w/v biomass. The optimal conditions for codeine and morphine production were 2.995 and 1.113 μg, respectively. **Conclusion:** Optimization demonstrated that pH and biomass had the strongest effects on biotransformation.

Key words: Biotransformation, CCD, Codeine, Morphine, Thebaine, *Bacillus*.

INTRODUCTION

The structure and activity of alkaloids are of concern.[1] Morphine and codeine are significant natural pharmaceuticals having powerful analgesic properties.[2-7] The synthesis of morphine alkaloids dates back about 150 years; however their molecular complexity.[8] with five chiral centers[9,10] means that morphine and its analogues have not been investigated from a fully synthetic chemistry approach.[11] The available chemical methods suffer from low yield and produce hazardous waste.[1] Until recently, *Papaver somniferum* ,the opium poppy,[12] has been the only source of production for morphine and its derivatives.[2-9-18] The World Drug Report of 2015 on narcotic drugs states that global opium production reached 7554 tons in 2014 and worldwide consumption is increasing.

Because of illicite use, cultivation of the opium poppy continues[4] therefore, it is necessary to find other sources for morphine and codeine[2-19] The non-narcotic thebaine, the main alkaloid of *P. bracteatum*,[20] has been a focus of attention because it accumulates thebaine with no morphine and codeine.[21] Thebaine is an excellent starting material for synthesis of diverse morphine agonists and antagonists.[1-22] In recent years, microbial production of secondary plant metabolites as an alternative method of synthesis of such effective compounds has been of increased interest.[10-18] Microbial transformation is the modification of certain compounds by enzymatic reactions[22] to achieve specific and useful metabolite accumulation through environmentally harmless and simpler bioprocesses.[23] Selecting microbial reactions directed toward the native plant hosts as alternative to chemical synthesis employed in traditional methods offers benefits such as quick accumulation of biomass, production of main intermediate molecules separately,

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simplicity of purification and accessibility of genetic tools for additional optimization.[8]

Many studies have examined microbial transformation of the morphine alkaloids using biocatalysts[24,25] such as the genus *Trametes,*[22-25-32] *Cunninghamella,*[25-27-34] *Mucor piriformis,*[22-24-26,27-30] and *Cylindrocarpon didymium*[24-30-35] fungi and *Arthrobacter* sp,[25-27-30-32-36] *Pseudomonas testosterone,*[24,25-27-30-32-37] *Pseudomonas putida.*[3-24-28-30,31] *Bacillus,*[24-27-30] *Mycobacterium neoaurum,*[27-29,30] *Streptomyces*[25-27-29-32] and *Nostoc muscorum*[27,28] bacteria. Few reports exist about microbial metabolism of thebaine; the first on thebaine biotransformation appeared in the 1960s.[25] Some reports explained transformation by *Tramete sanguinea*, which converts thebaine into 14-hydroxycodeinone and 14-hydroxycodeine.[22-25] It was also found that the main product of thebaine by *Mucor piriformis* is northebaine.[22] Thebaine transformation using *Cunninghamella echinulata* into northebaine has also been investigated.[22]

In our previous research, we isolated new biocatalyst from *P.bracteatum* microflora to produce morphine and codeine spontaneously.[38] In this study central composite design (CCD) methodology has been applied for improvement the yield of codeine and morphine, in thebaine biotransformationusing whole cell catalyst, *Bacillus* sp. FAR.

MATHERIAL METHODS

Biocatalyst production

A bacterial strain of *P. bracteatum* microflora has the ability to transform thebaine into morphine and codeine which was reported previously. The *Bacillus* sp. FAR (accession number KU746803; GenBank NCBI) has been detailed and completed.[38] For preculture preparation of *Bacillus* sp. FAR, the stock was grown in 100 ml of complex medium containing 15 g/l glucose, 10 g/l pepton, 5 g/l yeast extract, 2 g/l NaCl, 0.5 g/l K_2HPO_4 and 0.2 g/l $MgSO_4$.7 H_2O (pH 6.8) and incubated at 30°C and 140 rpm. Thereafter, 30 ml of cell suspension with an optical density of 0.1 at 620 nm was used to inoculate 270 ml of complex medium, which was then incubated under 140 rpm for 4 h at 30°C. The initial working volume of 2.7 l of complex medium (in a 5 l bioreactor) was inoculated with *Bacillus* sp. FAR (10% v/v). The batch culture was created and the main physical and chemical parameters of agitation rate, air transfer, temperature, pH and foam production were controlled. In brief, the initial stirring frequency was 200 rpm, 1 v/v/min sterile air was a passed through the bioreactor at 30 °C and the pH was maintained at 6.8. Samples were withdrawn every 30 min over a period of 5 h and the biomass turbidity of the culture was monitored at 620 nm.

The main fuction of a bioreactor is to supply high cell biomass concentrations needed for biotransformation. The bioreactor was allowed to proceed until the cells reached the stationary phase of growth and the process ceased at 5 h. The biomass from the early stationary phase of growth in complex medium was harvested (12000 g, 20 min, 4°C) and washed twice with sterile saline solution and stored at -40°C. These pellets were used directly for biotransformation assays.

Optimization by Design Expert

Design Expert 7.0.0 was used for the experimental design and statistical analysis to predict the maximum biotransformation of thebaine into codeine and morphine. The parameters affecting biotransformation at five levels were thebaine concentration, biomass weight, tris-buffer concentration, temperature, incubation period time, and pH. A, B, C, D, E, and F denote pH, temperature, time, substrate, biomass and buffer, respectively. Table 1 shows the actual and coded values of the independent factors over 80 experiments (64 cube point, 12 star point and 4 center point) designed the factors and value shown in Table 1.

Biotransformation and HPLC analysis

The biotransformation reactions were initiated by adding the desired amount of cells and thebaine to 50 ml of tris base buffer according to the experimental design. These cultures were incubated at 140 rpm for different times. Thereafter, the supernatant (centrifugation 8000 g, 20 min, 4°C) were tested for codeine and morphine production by HPLC-DAD.

Each sample was dissolved in aqueous acetonitrile 68% (v/v), and analyzed using an HPLC (Knauer; Germany) equipped with a C18 column (250 mm × 4.6 mm × 5 µm × 100 A°). The flow rate was 1 ml/min and gradient washing using A (H_2O, 0.5% acetic acid, 1% TEA) and B (acetonitrile) was performed. Separation occurred for solvent A and showed the percentage of decrease to 60% and 50% in 10 and 20 min, respectively. Thereafter, solvent A increased to 100% in 7 min and separation ceased by 30 min. An aliquot (20 µl) of sample was injected into the HPLC and analysis was carried out at 30°C at a detection wavelength of 280 nm. The calibration of codeine and morphine was performed at six concentraions (2-10 ng/ml) in three replications. The linear dynamic range equation, LOD and LOQ for codeine were Y = 6114.7x -10769, R^2 = 0.9698, 0.12 and 0.41 ng/g and for morphine were Y = 1635x + 2501.5, R^2 = 0.9699, 0.21 and 0.71 ng/g.

RESULTS AND DISCUSSION

In view of the difficulties associated with synthetic routes for morphine alkaloids, the current project examined the biological transformation of thebaine into codeine and morphine. From Previous results it found that *Bacillus* sp. FAR has the ability to convert thebaine into codeine and morphine (38). This study optimized the factors for codeine and morphine production from thebaine biotransformation using Design Expert software and response surface methodology by resting cells of *Bacillus* sp. FAR. To our knowledge, there have been no reports decribing this transformation functionality.

To determine the growth rate of bacteria, the optical density of the culture and the biomass weight were measured every 30 min. The growth was carried out in a bioreactor to achieve maximum cell concentration in the early stationary phase. A large quantity of resting cells of *Bacillus* sp. FAR (50 g wet cells in l^{-1} over 5 h) in the early stationary phase was harvested and used for biotransformation processes. Approximately 150 g of metabolically active resting cells of *Bacillus* sp. FAR were obtained in the early stationary phase and examined for optimization over the course of the 80 experiments.

Response surface methodology was used to predict the best conditions for thebaine transformation into codeine and morphine**.** Several factors contribute to the reaction, including thebaine concentration, biomass weight, tris buffer concentration, temperature, incubation period time and pH. The experimental design of the data was carried out using Design Expert software 7.0.0. The levels of the factors and the proposed 80 experiments are shown in Table 2. Each experiment was analyzed using HPLC-DAD based on the previous section. The metabolite peaks were identified by comparison with their HPLC retention and on the similarity of UV spectra Figure 1. The results for HPLC retention of morphine and codeine were 10.61 and 14.4 min, respectively. The correlation coefficient (R^2) showed that variations in the experimental response with the regression model are in compliance. The R^2 of biotransformation (0.7502) and the adjusted R^2 (0.7223) indicate that the model and experiment were well-fitted. This model showed a standard deviation (SD) of 0.18 and a mean of 0.71. ANOVA results of the optimization study show the significance of the model at $p<0.001$ Table 3. There is only a 0.01% probability of a model F-value resulting from noise. A value of $F<0.0001$ indicates that the model terms are significant. In this case, interactions between buffer, pH, temperature, biomass and time were significant. For lack of fit, $p=0.4$ and was not significant. A non-significant lack of fit indicates that the experimental and mathematical models fit. These results show sufficient validation of the model Table 3. ANOVA results for the mathematical model equation in Table 3 show significant results for biotransformation of the process variables of pH and biomass. The factors for optimization were set at experimental ranges designed for maximum desirability. The effects of their interaction with thebaine biotransformation were studied by plotting 3D surface curves. Figure 2 shows the curves for codeine and morphine production response surface optimization. Note that, in each plot, two factors were studied and four were held constant at central values.

The software found that optimal conditions were a pH of 7.7 at 36°C for 30 h with a substrate concentration of 80 µg/ml, biomass of 2.29% w/v and tris buffer concentration of 45 mM. Decreases in buffer concentration (F) and substrate concentration (D) increased the production of codeine and morphine. These plots show that morphine alkaloid production increased as temperature (B) increased to 32°C and thereafter decreased

Table 1: Design factor values

Factors	Unit	-2	-1	0	1	2
A: pH	-	5.5	6.5	7.5	8.5	9.5
B: Temperature	°C	28	32	36	40	44
C: Time	h	15	25	25	30	35
D: Substrate	µg/ml	20	40	60	80	100
E: Biomass	g/50 ml	0.5	1	1.5	2	2.5
F: Buffer	mM	25	40	55	70	85

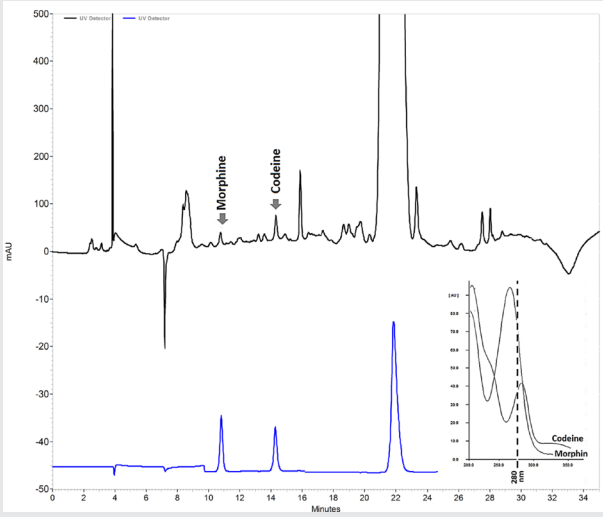

Figure 1: HPLC-DAD analysis of standard (Lower) and optimized sample (Upper) for of codeine and morphine production.

Table 2: Central composite design and response for codeine and morphine production after biotransformation by *Bacillus* sp. FAR. The best codeine and morphine were in runs 32 and 41, respectively

Run	pH	Temperature (°C)	Time (h)	Substrate (µg/ml)	Biomass (g/50 ml)	Buffer (mM)	Morphine and Codeine production (DF)	Run	pH	Temperature (°C)	Time (h)	Substrate (µg/ml)	Biomass (g/50 ml)	Buffer (mM)	Morphine and Codeine production (DF)
1	6.50	40	30	40	1	40	0.000028	41	6.50	32	20	40	2	40	12.531288
2	8.50	32	30	40	2	70	0.002847	42	6.5	40	30	80	2	70	0.591183
3	6.50	40	20	80	2	40	0.002847	43	6.5	40	20	80	1	70	0.162233
4	8.50	32	30	80	1	40	0.446487	44	6.5	40	30	40	2	40	1.043369
5	6.50	32	20	40	1	40	0.102968	45	9.5	36	25	60	1.5	55	0.000028
6	7.50	44	25	60	1.5	55	0.253844	46	8.5	40	30	80	2	70	1.438769
7	6.50	40	20	80	2	70	0.361297	47	8.5	32	20	80	1	40	0.000028
8	8.50	32	30	80	2	70	0.232428	48	7.5	36	25	20	1.5	55	4.612207
9	7.50	36	25	60	1.5	55	0.114936	49	8.5	32	30	80	2	40	3.985770
10	6.50	40	30	80	2	40	4.307277	50	6.5	40	20	40	1	40	0.001040
11	6.50	36	25	60	2.5	55	0.112331	51	8.5	40	20	40	1	70	0.000028
12	6.50	32	30	40	2	70	1.075755	52	5.5	36	25	60	1.5	55	0.001040
13	8.50	32	30	40	1	40	0.229895	53	8.5	32	20	40	1	40	0.001040
14	7.50	36	15	60	1.5	55	2.131466	54	6.5	32	20	80	2	40	0.437404
15	8.50	32	20	80	2	40	0.826197	55	6.5	32	30	80	2	40	0.000028
16	6.50	32	30	40	1	70	0.001040	56	6.5	40	20	40	2	70	0.561739
17	6.50	40	20	40	2	40	0.448894	57	6.5	32	20	80	1	70	1.278751
18	8.50	40	30	40	1	40	1.024827	58	6.5	32	20	40	2	70	0.952792
19	6.50	32	20	40	1	70	0.002847	59	6.5	40	20	40	1	70	0.002847
20	6.50	40	30	40	1	70	0.002847	60	8.5	40	20	80	2	40	3.472798
21	8.50	32	30	80	1	70	0.474166	61	6.5	32	30	40	2	40	0.000028
22	8.50	40	20	40	2	40	2.132454	62	8.5	40	20	80	2	70	0.000028
23	6.50	32	30	40	1	40	0.000028	63	6.5	40	30	80	1	70	0.001040
24	8.50	32	20	80	2	70	0.639461	64	8.5	32	30	40	2	40	0.001040
25	8.50	32	30	40	1	70	0.351788	65	6.5	32	30	80	1	70	0.324141
26	7.50	36	35	60	1.5	55	4.826464	66	8.5	40	30	40	1	70	0.001040
27	8.50	32	20	40	2	70	0.612325	67	7.5	36	25	60	1.5	55	3.149857
28	7.50	36	25	60	0.5	55	2.963731	68	8.5	40	20	80	1	40	0.002847
29	6.50	32	30	80	2	70	0.369130	69	7.5	36	25	60	1.5	55	3.621635
30	8.50	40	30	80	2	40	2.632886	70	7.5	36	25	100	1.5	55	2.527428
31	6.50	32	30	80	1	40	0.337888	71	6.5	32	20	80	2	70	0.001040
32	7.50	36	25	60	1.5	55	18.589700	72	8.5	40	20	40	1	40	0.000028
33	8.50	32	20	80	1	70	0.000028	73	8.5	40	30	80	1	40	0.614602
34	8.50	40	30	40	2	70	1.487322	74	7.5	28	25	60	1.5	55	0.351165
35	6.50	40	20	80	1	40	1.761946	75	6.5	40	30	80	1	40	0.000028
36	6.50	40	30	40	2	70	0.000028	76	8.5	32	20	40	1	70	0.020332
37	6.50	32	20	80	1	40	0.376892	77	7.5	36	25	60	1.5	25	4.601180
38	8.50	40	30	80	1	70	0.790706	78	8.5	32	20	40	2	40	0.830354
39	8.50	40	20	80	1	70	0.002847	79	8.5	40	30	40	2	40	0.001040
40	8.50	40	20	40	2	70	0.000028	80	7.5	36	25	60	1.5	85	2.911799

Table 3: Regression analysis for production of codeine and morphine (ANOVA)						
Source	**Sum of Squares**	**df**	**Mean Square**	**F Value**	**p-value Prob> F**	
Model	2.821705	18	0.156761	5.014425	< 0.0001	**significant**
A-pH	0.003602	1	0.003602	0.115229	0.7354	
B-Temperature	0.007605	1	0.007605	0.243267	0.6236	
C-Time	0.008238	1	0.008238	0.263505	0.6096	
D-Substrate	0.077795	1	0.077795	2.488469	0.1199	
E-Biomass	0.020063	1	0.020063	0.641781	0.4262	
F-Buffer	0.135105	1	0.135105	4.321696	0.0418	
AC	0.223847	1	0.223847	7.16035	0.0096	
AD	0.033293	1	0.033293	1.064952	0.3062	
BD	0.028063	1	0.028063	0.897677	0.3471	
BF	0.027707	1	0.027707	0.886269	0.3502	
CD	0.090442	1	0.090442	2.893025	0.0941	
DE	0.053624	1	0.053624	1.7153	0.1952	
DF	0.036542	1	0.036542	1.168878	0.2839	
EF	0.040975	1	0.040975	1.310688	0.2567	
A^2	0.895994	1	0.895994	28.66073	< 0.0001	
B^2	0.846302	1	0.846302	27.0712	< 0.0001	
E^2	0.36861	1	0.36861	11.79096	0.0011	
F^2	0.051119	1	0.051119	1.635169	0.2058	
Residual	1.906987	61	0.031262			
Lack of Fit	1.846739	58	0.03184	1.585458	0.4019	**not significant**
Std. Dev.	0.017681		**R-Squared**		0.7502	
C.V. %	24.75555		**Adj R-Squared**		0.7223	

(Biotransformation Product)0.1 = -20.58968 +2.17339 pH +0.74271 ×Temperature-0.11341 ×Time -0.34016 ×Substrate +1.63153 ×Biomass +0.039245 ×Buffer+0.011828 ×pH × Time+0.022808 ×pH × Substrate +5.23503E-003 ×Temperature × Substrate -3.46777E-004 ×Temperature × Buffer+7.51839E-003 × Time × Substrate -0.057892 × Substrate × Biomass-1.59299E-003 × Substrate × Buffer-3.37371E-003 × Biomass × Buffer-0.16964 × pH2-0.010304 ×Temperature2 -0.43523 × Biomass2 -1.80087E-004 ×Buffer2

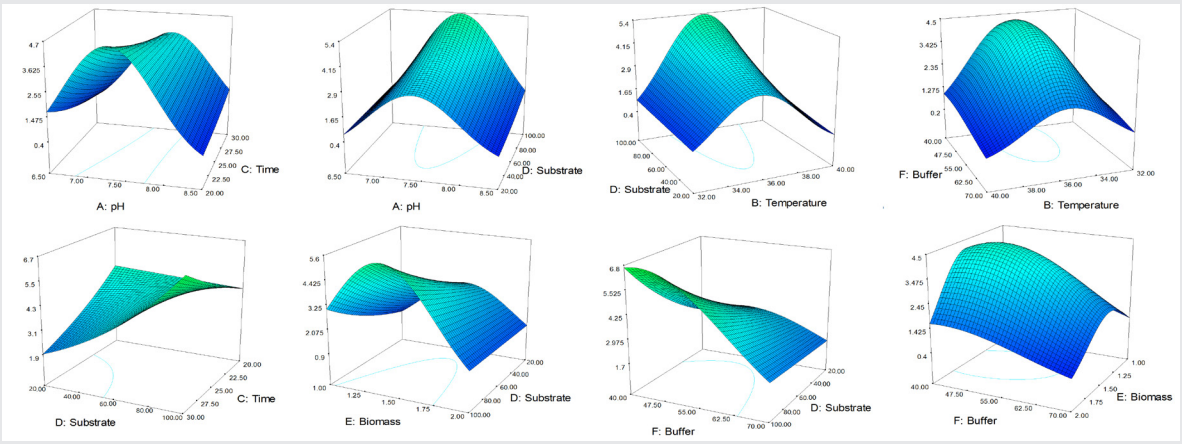

Figure 2: Relationships of each factor within the central terms of other factors for codeine and morphine production vs response rates of other factors (A: pH, B: temperature, C: time, D: substrate concentration, E: biomass, F: buffer concentration).

with a further increase in temperature. The same trend was observed for codeine and morphine production in thebaine biotransformation on acidity (pH) and biomass. An increase in pH from 5.5 to 7.5 and of biomass from 0.5 to 2 g increased codeine and morphine production, but further increases in either factor decreased biotransformation activity. The optimal conditions for pH, temperature and biomass fell in the middle of the ranges investigated. In other words, decreases or increases in these factors decreased codeine and morphine production.

The microbial transformation of thebaine into other morphine alkaloids appears to be a multistep process which requires more than one enzyme. The secondary metabolite biosynthetic pathway in plants is branched and complicated.[10] In *P. somniferum*, morphine alkaloids are produced in sieve elements and then transported to laticifers for storage. In contrast to intact plants, opium poppy cell cultures do not produce morphine alkaloids.[39] In general, thebaine is an extremely metabolically inert compound; out of 230 plants so far tested, it is converted by only two species to metabolites.[40] Despite the complicated and difficult pathway to reconstruction of a microbial production system,[10] it is interesting to note that this biotransformation of thebaine to codeine and morphine was performed in one microbial cell.

The low yield of this transformation may result from the complexity and inert aromatic nature of thebaine, the intricate association of multiple protein subunits and the presence of cofactors and electron donors (NADPH, ATP, etc.),[41] expression of low levels of enzymes,[34] substrate/product inhibited biotransformation,[34] lack of membrane transport processes involved to move the substrate into the site where the enzymes are known to be located,[8-34] and enzymes exhibiting a high tolerance of a substrate.[11] Another factor that may limit conversion is the lack of an intracellular component for storage of products at the same laticifers in *P. somniferum*. It appears that resting cells of *Bacillus* sp. FAR do not fully consume thebaine. Further work on the production of codeine and morphine for yield improvement through protein engineering strategies is proposed.

CONCLUSION

Optimal conditions for codeine and morphine production from thebaine transformation by the gram-positive bacterium belonging to the genus *Bacillus* was investigated. The results of 80 optimization experiments on thebaine transformation using active resting cells of *Bacillus* sp. FAR showed that the optimal yields of codeine and morphine production were 2.995 and 1.113 μg, respectively. The effect of buffer, pH, temperature, biomass and interactional pH and time were significant. Despite its low yield of codeine and morphine, this new biocatalyst offers a source for production that is harmless resource in comparison with the opium poppy.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

ABBREVIATION USED

CCD: Central Composite design; **NCBI:** National Center for Biotechnology Information; **LOD:** Limit of Detection; **LOQ:** Limit of Quantification; **SD:** Standard Deviation.

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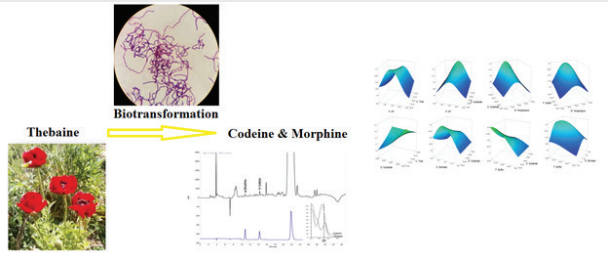
PICTORIAL ABSTRACT



SUMMARY

- Due to dominance of biological methods to the chemical ones for the synthesis of pharmaceuticals, *Bacillus* sp. FAR which has the ability to transform thebaine to codeine and morphine was used as a new biocatalyst.
- Response surface methodology applied for optimization of thebaine transformation in to these analgesic pharmaceuticals.
- The various factors of buffer, PH, temperature, biomass, and interactional PH and time have significant roles in this process.

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