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Optimization of Expression and Purification of Recombinant One of Central Nervous System Enzyme (Acetylcholinesterase)

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Abstract

In general, the use of natural enzymes is very limited and they are not economical to use in industry. To solve this problem, the recombinant expression systems approach has been used. Optimization of production processes in heterologous expression systems is one of the most important activities in today's competitive industries. One of the key factors for the commercialization of recombinant proteins is the achievement of a low-cost culture medium available for the production of suitable cell biomass that can be used on an industrial scale. Commercialization of recombinant products requires process economics and cost reduction, and reducing the cost of the product is possible by using a cheaper culture medium, so decided optimization of expression and purification of recombinant acetylcholinesterase (EC.3.1.1.7), because it's one of the body's vital enzymes, that by hydrolyzing acetylcholine, which is a neurotransmitter, terminates neural messages in the area of cholinergic synapses. Because the enzyme acetylcholinesterase produced from natural sources is not suitable for industrial purposes and semi-mass production due to low production efficiency and high costs of enzyme preparation and purification processes, optimizing the growth conditions of microorganisms capable of the production and secretion of the enzyme acetylcholinesterase are very important, both in terms of yield and economic costs. The present study seeks to increase productivity and reduce the cost of producing this enzyme by optimizing the growth medium and expression of pichia pastoris yeast cells that have genes related to this enzyme and also by optimizing the purification and mass production of this enzyme.

Keyword: Acetylcholinesterase, Enzyme, Optimization, Central Nervous System Enzyme.

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Introduction

Acetylcholine is a major neurotransmitter in the cholinergic system, and one of the body's fastest enzymes acetylcholinesterase, breaks down acetylcholine by approximately 15 microseconds, preventing excessive accumulation of the neurotransmitter acetylcholine in synapses. Inactivation of this enzyme by some powerful toxins, such as organophosphorus compounds, can be very dangerous for the body. Due to the fact that dysfunction of this enzyme causes neurological diseases such as Alzheimer's and myasthenia gravis, the study on this enzyme has become very widespread in recent years [1-4].

This enzyme has been observed in all vertebrates, including humans and eels, as well as in some invertebrates such as vinegar flies. In the pre-synapse terminal, acetylcholine neurotransmitter (ACh) formed made from choline and Acetyl coenzyme A which mediated by the enzyme cholinesterase transferase (ChAT) [5,6]. It accumulates in the vesicles carrying acetylcholine (VAChT) in the anterior synaptic membrane. Acetylcholine remains in these vesicles until the nerve endings are activated. After activation of nerve ending acetyl is released into the synaptic cleft and attaches to one of the two types of nicotinic or muscarinic acetylcholine receptors in the postsynaptic membrane. The rest of the acetylcholine in the synapse cleavage is broken down by the acetylcholinesterase enzyme (AChE) into choline and acetate, thus ending the transmission of the neural message [7-10] inhibition of this enzyme by the Organophosphates and carbamates cause the accumulation of ethylcholine in the synaptic cleft. As a result, the acetylcholine receptor loses its sensitivity to new signals, thus blocking the signal transmission pathway [5].

The production of recombinant proteins must have a number of economic and qualitative principles. This depends on the characteristics and application of the compound produced. To produce food additive enzymes, gene technology must compete with and approach the mass production of these compounds from traditional sources. Therefore, the production process must follow the patterns of high efficiency and the tendency to use cheap environmental components in the fermentation process. To produce medicinal products and other compounds for human consumption, its principles must have safe aspects and rely on reliable products [11.14].

Expressive systems, including gram-positive and gram-negative prokaryotes, yeasts, filamentous fungi, mammalian cells, and plant cells, have useful and unique properties, as well as limitations and negative properties. There is a relationship between the complexity of a particular protein and the complexity and capabilities of an expression system [15-18]. Acetylcholinesterase is important because it plays a key role in central nervous system function and on the other hand, it is the biological target of many toxins and insecticides that pose many risks in terms of environmental and food pollution to human health. There for in recent years, the enzyme acetylcholinesterase has received a great deal of attention for the design of biosensors for the detection of chemical contaminants in the environment and food [19,20]. The acetylcholinesterase enzyme is normally extracted from some insects and aquatic animals. The enzyme extracted from these sources is very sensitive and in this regard is suitable for use in the detection of inhibitors in the environment and food, but in terms of the amount of enzyme production are not suitable sources for commercialization of the production of this enzyme. Acetylcholinesterase can also be purified from animal or human blood, but these sources are very limited in inventory and very potential in terms of contamination [20-22]. Therefore, produce of cholinesterase enzyme with high sensitivity and specificity with safe steps and lower costs much needed. Today, the development of genetic engineering methods has made it possible to produce recombinant proteins in different hosts. Due to the very low expression of cholinesterase, a variety of expression systems have been studied to produce this enzyme, but each of them has its drawbacks. This research project intends to achieve semi-mass production of acetylcholinesterase enzyme with special properties using modern methods of protein engineering [23-26].

In a recent study by Yuan Ji et al., Human-engineered enzyme acetylcholinesterase was expressed in the plant species Nicotiana benthamiana p19 system. According to this study, the production of recombinant enzyme in this species is about 180 mg / kg. Despite the very high production interest, the use of this plant species is very costly in terms of downstream processes and is challenging in terms of the genetic instructions required for the recombinant expression of the target protein [27-32]. Recombinant expression of acetylcholinesterases studied in expression systems including human cell lines Hack 293, mouse cells, Chinese hamster ovary cells, baclovirus system and Escherichia coli bacteria [33-35].

Method and Material

PCR reaction was used to confirm the presence of acetylcholinesterase gene in plasmid. About 5 µl of PCR was taken on 1% agarose gel, and the presence of 1698 bp fragment confirmed the presence of AChE gene in plasmid [33].

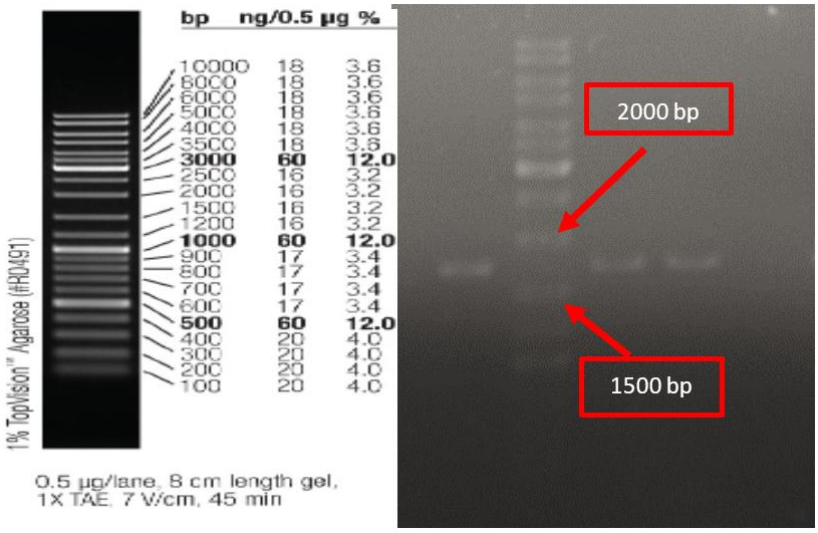


Fig. 1. Confirmation of the Presence of Acetylcholinesterase Gene in Plasmid

Because the protein is secreted out of the cell, the cell suspension was centrifuged at 5,000 rpm for 15 minutes, then the expression supernatant was used as a suitable sample to evaluate the presence of the protein. Cell supernatant was first concentrated by ultrafiltration and then prepared for purification

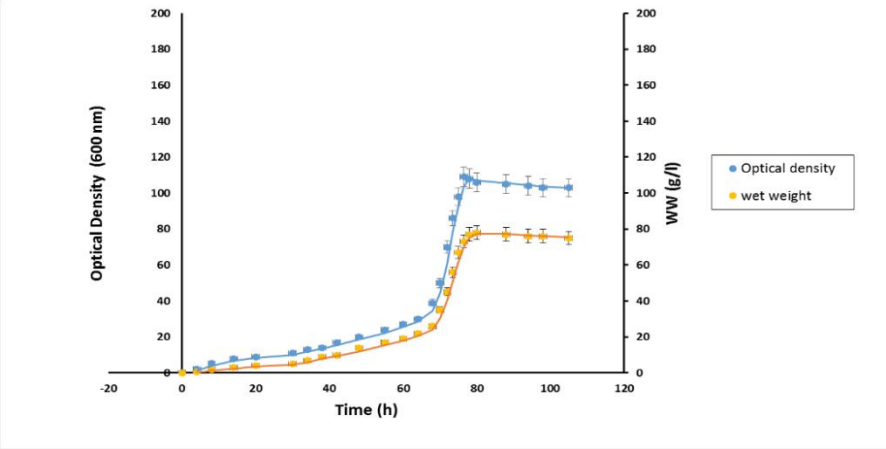


Fig. 2. Graph of the Relationship Between Optical Density and Wet Weight for the BMGY Medium

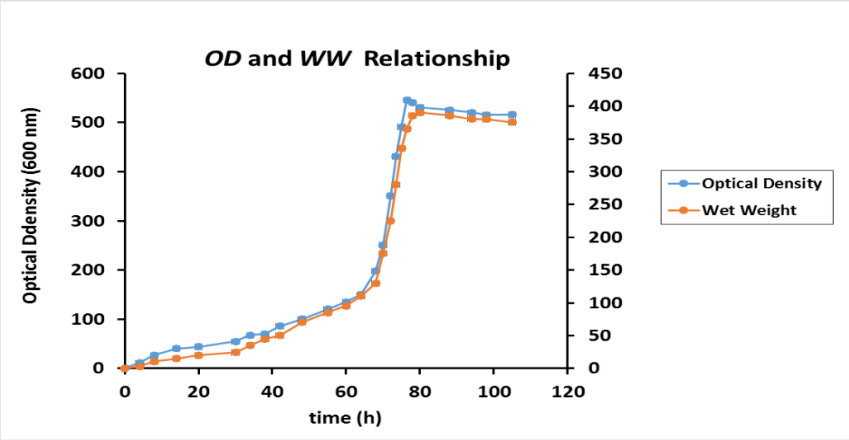


Fig. 3. Graph of the Relationship Between Optical Density and Wet Weight for the Specific New Medium

Examination of the growth in BMGY medium and certain medium shows that reaches its maximum growth in culture medium after about 70 hours and then enters the dormant phase, therefore the sampling time in subsequent experiments after the initial inoculation was the culture medium.

SDS-PAGE technique was used to confirm the presence of target protein and to observe other proteins in certain culture medium and BMGY culture medium and also their presence after purification [32,34].

Since the size of the target enzyme in kilo Daltons is (64.44) approximately equal to the size of the BSA protein in kilo Daltons (66.0), BSA protein was used as a marker to compare the size of the target enzyme. The results of this method confirmed the accuracy of the size of the target enzyme as show in figure 4 [28,30].

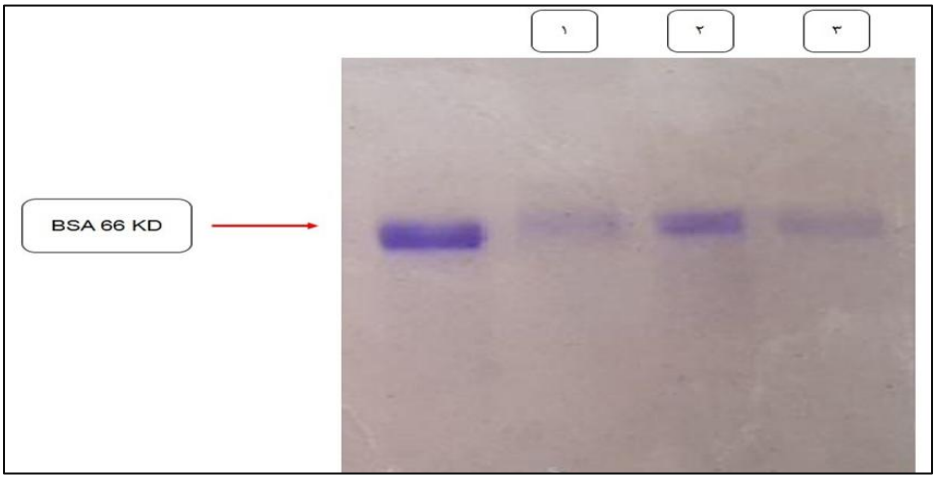


Fig. 4. Confirm the Presence of the Enzyme in Different Concentrations Compared to BSA as a Marker

In this study the new medium prepared by use the material in specific concentration and way to be compared with previous medium BMGY (buffered glycerol-complexed medium). This medium is composed of the basic salts of Basel salt and Pichia Trace Element (PTM). First, the base salt solution was prepared, then the PTM solution was added to it, and the pH of the medium was adjusted using ammonia. Table 1. Materials required to make one liter of the medium.

Table 1: Materials Required to Produce a Specific New Medium

	Quantity	Component
1	26.7 ml	**H3PO4 85%**
2	0.93 g	**CaSO4**
3	18.2 g	**K2SO4**
4	14.9 g	**MgSO4.7H2O**
5	14.13 g	**KOH**

6	40 g	**Glycerol**
7	12 ml	**PTM**

In order to achieve a cheap and suitable medium as well as higher efficiency in terms of product production, the use of certain medium is recommended.

For this purpose, the high-yield medium and the procedures of Taha Recombinant Protein Company were used.

The Aim of Study

The main purpose of this study is the semi-mass production of the enzyme acetylcholinesterase at a low cost for various research applications. Since the import of this protein for therapeutic applications and for the production of specific drugs for the treatment of neurological diseases from foreign companies is not cost-effective, semi-mass production of this protein is considered in this study.

Result

In the present study, pichia pastoris as a heterologous expression system, due to the simplicity of the necessary techniques to genetic rules, the ability to produce new protein compounds in large quantities and the ability to make many post-translational changes including glycosylation and the formation of disulfide bonds for The production of the enzyme acetylcholinesterase in the drosophila melanogaster was used. The yeast used is modified to release the target enzyme into the culture medium, which reduces the enzyme purification process and saves time and money. The specific medium used in this study is superior to the BMGY medium, with features such as economic optimization, lower contamination, higher production and growth efficiency, easier purification, and the use of less harmful compounds.

In the comparison between the two medium, BMGY and specific medium, the growth conditions of pichia pastoris and the expression of the enzyme acetylcholinesterase were studied and according to Table 2, the weight values and optical density of the enzyme sample obtained from the specific new medium were 375 g/L and 515.80 which show those are five times higher than the corresponding values of the enzyme sample obtained from BMGY medium, 75.8 g/L and 103.16, respectively.

Table 2: Growth Characteristics of Yeast in Fermentation Process with BMGY and Specific New Medium

Specific new medium				BMGY medium		
Time (hour)	Average optical density (600 nm)	Average wet weight (g/l)		Time (hour)	Average optical density (600 nm)	Average wet weight (g/l)
0	0.13	0		0	0.13	0
4	11.33	2.95		4	2.30	0.59
8	26.67	10.83		8	5.33	2.17
14	40.33	15.07		14	8.85	3.01
20	43.80	20.12		20	8.07	4.02
30	54.67	24.37		30	10.93	4.87
34	67.33	35.00		34	13.47	7.00
38	69.97	44.95		38	13.99	8.99
42	85.83	50.07		42	17.15	10.01
48	100.07	70.22		48	20.01	14.04
55	120.25	85.10		55	24.05	17.02
60	135.27	95.43		60	27.05	19.09
64	150.21	110.13		64	30.04	22.03
68	197.97	130.03		68	39.59	26.01
70	251.00	175.50		70	50.20	35.10
72	351.13	225.33		72	70.23	45.07
73.5	430.75	280.33		73.5	86.15	56.07
75	491.30	335.52		75	98.26	67.10
76.5	545.97	365.37		76.5	109.19	73.07
78	540.50	385.65		78	108.10	77.13
80	530.75	390.48		80	106.15	78.10
88	525.53	385.55		88	105.11	77.11

94	520.30	380.33		94	104.06	76.07
98	515.53	380.12		98	103.11	76.02
105	515.80	375.34		105	103.16	75.08

Also, the results of Bradford test to determine the protein concentration showed that the protein concentration resulted from the specific medium with a value of 1.3 µg / µl (equivalent to 1300 µg / ml) is more four times higher than the protein concentration obtained from the BMGY medium with a value of 0.3 µg / µl (equivalent to 300 µg / ml).

The results obtained from determining the characteristics of acetylcholinesterase enzyme from a specific medium by examining the optimal pH, maximum activity at different temperatures (range 15 to 70 C) as well as the stability of enzyme activity at different temperatures (range 25 to 70 C) showed that the enzyme has the maximum activity at pH 7.5 and a temperature of 35 C°. Also at a temperature of 35 to 40 C°, the enzyme has the highest temperature stability as showed in figure 5, 6 and 7.

It also shows that the resulting enzyme has a Km of 0.23 mM and a Vmax of 0.48 (µmol / ml.min).

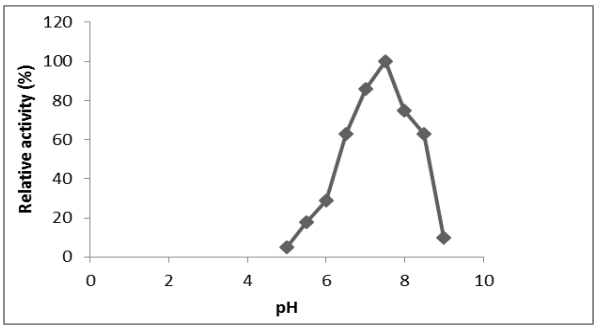


Fig. 5: pH Profile Diagram: Graph as a Percentage of Maximum Activity in the Buffer with Different pHs

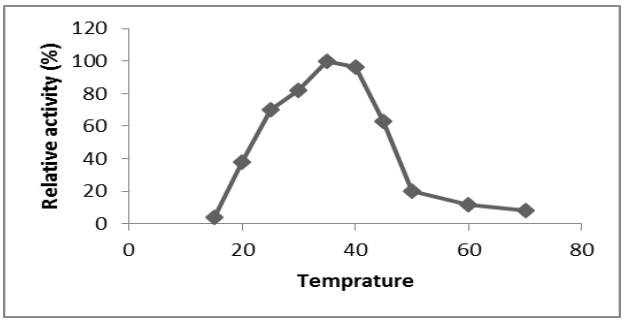


Fig. 6: Temperature Profile Diagram: Graph as a Percentage of Maximum Activity at Different Temperatures (Range 15 to 70 Degrees)

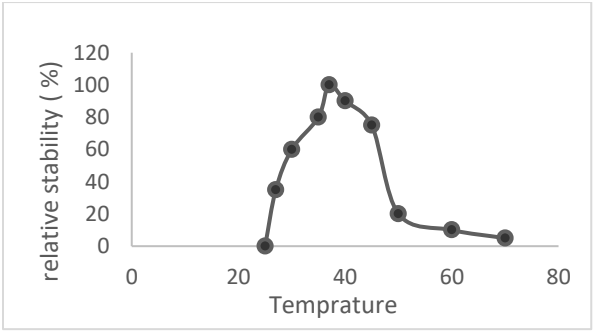


Fig. 7: Temperature Stability Diagram: The Diagram is a Percentage of Maximum Activity at Different Temperatures from 25 to 70 Degrees and at Each Temperature for 10 Minutes the Enzyme is Placed and then its Activity is Measured

Extraction of the specific activity of the enzyme acetylcholinesterase showed that the specific activity of the enzyme produced in a specific medium with a value of 928.3 units per milligram was approximately twice that of its activity

in the BMGY medium with a value of 454.3 units per milligram. Based on the data obtained from this study, an increase in the growth of pichia pastoris and an increase in enzyme production was observed, so in the present study, it was found that optimizing the ingredients of the growth medium leads to an increase of at least fivefold production of acetylcholinesterase enzyme, which is important in terms of mass production of this enzyme. Therefore, optimization of production processes is one of the most important activities in today's competitive industries, and the guide to mass production of biological control agents should be optimized to encourage the industry to trade it commercially.

Conclusion

As can be seen, the results showed that the growth rate of pichia pastoris in a new medium is about 5 times higher than the growth rate of in BMGY medium, so the study medium in terms of biomass production was about five times more than complex medium and its use for industrial production would be reasonable and cost-effective.

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