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Production of peroxidase from *Providencia* spp. bacteria

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Abstract

Twenty isolate within four species of bacteria were studied to produce of peroxidase enzyme, involved *Pantoea dispersa*, *B. subtilis*, *Pseudomons aeruginosa* and *S. aureus*. The isolates were inoculated in production media under optimal conditions in order to produce peroxidase enzyme. The results of biochemical tests indicated that the *P. aeruginosa* species (PA3) was the most efficient in the production of enzyme (1.634 U/ml), the isolate with the highest production was selected for further studies of production, purification, DNA profile, identification by 16 S rRNA gene. The results of amplification for 16 Sr RNAgene indicated that the PA3 isolate was not *P. aeruginosa* but belonging to *Providencia* spp. The results of amplification were similar to these of NCBI data base for *Providencia* spp., the MW the amplicon was 1500 dalton, while the results of the purification appeared that the final purification step by using ion exchange chromotography of DEAE-cellulose given enzyme activity, protein concentration, specific activity, total activity, fold of purification and yield of protein 162.3 U/ml, 0.162 mg/ml, 1001 U/mg, 486.9U, 20.5, 221% .

Keywords: Peroxidase, Providencia sp., Production, Purification, 16Sr RNA gene.

INTRODUCTION

The peroxidase enzyme is found in plants, animals and bacteria, a group of compounds are oxidizes by using peroxidase like hydroquinones, flavonoids, benzidine derivatives, phenols, and lignin. The peroxidase its usefulness as a catalyst in the industeries of textile, laundary, pulp and paper. In addition to use it as biosensor and for the decomposition of pollutants [1]. The official EC number to peroxidase is 1.11.1.7., which is represent the enzymatic classification for this enzyme and peroxidases belong to oxidoreductases enzymes. The enzyme its electron acceptor by catalyze the transport of oxigen of hydrogen peroxide. The microbial peroxidase has importance, because it abble to degreds various compounds like dyes, dioxins nitro aromatics and other compounds [2]. Peroxidase enzyme is a globular protein which bind through its active site to substrates in order to helps in supporting of oxidation reactions. The peroxidase includes enzymes like NAD peroxidase, fatty peroxidase, glutatione peroxidase also agroup of non specific enzymes from various sources simply known as peroxidase. The peroxidase is oxidoreductase enzyme which used hydrogen peroxide as electron acceptor for catalyze oxidative reactions [3]. Peroxidases playing role in the defenses against plant pathogens [4]. Peroxidase is use for treatment of the industrial wastes in waters, like phenols, which are represents pollutants, can be removed by enzyme-catalyzed polymerization using
peroxidase enzyme, the treatment of peroxidase to waste is less sensitive from others of bacterial treatments because the later sense to pH, concentration of phenol or other toxic substances and temperature. The phenols are oxidized by using peroxidase enzyme to phenoxy radicals, that are less toxic than phenols. There are many of studies in future about the use of peroxidase in the manufactors like adhesives, computer chips, car parts, linings of drums and cans. The peroxidases perhaps utilized in the polymerization from anilines and phenols in organic solvent matrix [5]. The study aims to find isolate with high level of the production of peroxidase enzyme in order to produce this enzyme and identify this isolate exactly by using molecular fashion.

MATERIALS AND METHODS

Bacterial isolates and cultural conditions

The isolates for peroxidase production of *Pantoea agglomerans*, *P. aerogenosa*, *Bacillus subtilis*, *S. aureus* were supplied from laboratories of biological department in Baghdad university. The bacteria were preserved in nutrient agar at 4 C° and subcultured weekly. Bacteria were grown in 100 ml flasks containing 25 ml of nutrient broth medium in shaker incubator at 150 rpm / 37 C° for 24 hr. To estimate peroxidase activity of tested bacteria, they were grown in broth contained 10 g/L of $NaNO_3$ and 1 g/L of KH_2PO_4 [1].

Screening of bacteria for production of peroxidase enzyme

One milliliter of overnight broth culture from the tested bacteria was taken in a test tube, then add H_2O_2 till the color it turn into pink as a positive result [6].

Peroxidase assay

The enzyme activity was studied spectrophotometrically to all isolates at 420 nm, in order to determine the increasing in the absorbance by using 100 mM of citrate phosphate buffer, 1.7 mM ABTS (azino bis 3 ethyl benzo thiazolin 6 sulphonic acid), 2.5 mM H_2O_2 in 1ml of reaction mixture. The reaction contained 0.1 ml of supernatant, 0.9 ml of 1.7 mM ABTS and 25 µl of 2,5 mM H_2O_2 was added and the OD was read at 420 nm for one minute. 0.1 ml of distilled water, 0.9 ml of 1.7 mM ABTS and 25 µL of 2.5 mM H_2O_2 were used as blank. Heat denatured enzyme sample served as control. One unit of peroxidase enzyme was know as the alteration in absorbance of 1.0 ml/min at 420 nm [6, 19].

Peroxidase enzyme purification

Three steps was applied in order to purify peroxidase enzyme of *Providencia* spp. bacteria. Ammonium sulfate precipitation [20]**.** It's the first step of purification by

using this salt, in order to precipitate peroxidase enzyme, which produced by *Pesudomonas* bacteria under optimum conditions, diyalysis step and ion exchange chromotography method step, the protein was estimated by bradford's method [8, 9].

DNA extraction

The DNA extraction was completed according to protocol of genomic DNA purgation kit of promega.

DNA quantification

The purity and concentration of DNA was determined by using nanodrop spectrophotometer, by taking 2 µl of DNA solution into the photocell of the nanodrop, then the sample was measured at 260 and 280 nm. The DNA concentration was measured according to the following formula [11]:

DNA conc. (ng/µl)= Abs (260 nm)×50 µg/ml.

PCR amplification and analysis of the 16S rRNA sequence

The amplification of 16S rRNA gene of *Providencia* spp. was performed by using a primers that reported in table -1 [12]. The reaction mixture of PCR was applied according to the instruments of the supllied company, that reported in table-2. The PCR was performed as it mentioned in table-3, and the product of PCR was preserved at 4 Cº. 5 µL of the production was exercised in agarose gel electrophoresis, thereafter the amplicon of 1.5 kb was sequnced in the automated DNA sequencer by Sanger sequencing using ABI3730XL of Macrogen Corporation-Korea. The comparasion in the similarity of 16S rRNA was performed by using NCBI Blast database.

RESULTS AND DISCUSSION

Identify of isolate by employ 16S rRNA gene

Providencia sp. Isolate was identified as confirmation identify by using specific 16S rRNA gene primers as shown in table-2, which were designed and used for amplification of this gene, the amplicon was isolated on 1% agarose gel, the results indicated in figure-1 were appeared that the DNA of 16S rRNA gene band has 1500 bp in size. The results were agreements with these obtained from NCBI data base in 100% of compatibility. The scores obtained from the alignment was 1707/ 1707 which equal to 100% as it reported in figuer 2 as compelet similarity between the sequences of the tested bacteria with the recorded data in NCBI.

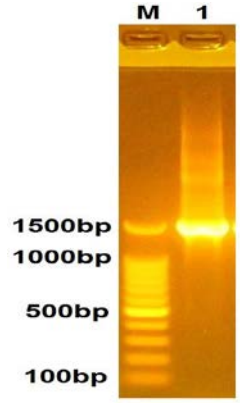

Figure (1): Agarose gel electrophoresis for amplification of 16S rRNA gene of *Providencia* spp. bacteria on (1%) agarose gel, 70 voltage for 1hr. Lane (M): DNA marker (500 bp), Lane (1): Amplicon of the gene.

Table (1):- The primers were used in the identification of *S. mutans* bacteria and fluoride resistance gene amplification.

The primer	Primer name	The sequence (5' → 3')	Size (bp)
Forward primer of 16S rRNA gene	27 F	AGAGTTTGATCTTGGCTCAG	1500
Reverse primer of 16S rRNA gene	1492 R	TACGGTTACCTTGTTACGACTT	

Table (2): The reaction mixture (25 µl) for amplification by PCR was consisted of the following components

Components	Final Conc.	Volume (µl)
Go Taq-Green master mix	1x	12.5
Upstream primer, 10 µM	0.1-1.0 µM	1
Downstream primer, 10 µM	0.1-1.0 µM	1
DNA template	10 ng	2
Nuclease-free water	N.A.	8.5
Total volume		25

Table (3): Amplification procedure.

Steps	Temperature (Cº)	Time	Cycle
Initial denaturation	95	5min.	1
Denaturation	95	30sec.	35
Annealing	60	30sec.	
Extension	72	1min.	
Final extension	72	7min.	1
Hold	10	10min.	

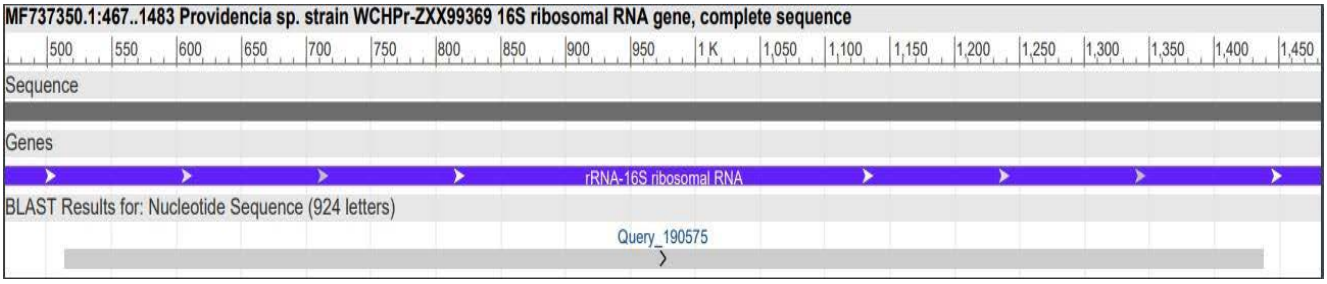

Figure (2): Similarity between the tested bacteria and NCBI data base for *Providencia* spp. strain.

Screening production of peroxidase by the tested isolates

The results of detection the ability of the isolates to producing peroxidase enzyme indicated positive results, if the color of reaction mixture it became pink, therefore the results were similar to these mentioned by [6] who repored that the positve test for producing of peroxidase represents by forming pink color.

Peroxidase enzyme activity

Twenty isolate of different four species five from all one of *Pantoea dispersa, Pseudomonas aerogenosa, Bacillus subtilis, Staphylococcus aureus* bacteria were undergo to define their capability to manufactor peroxidase enzyme. The results indicated that these products were given activity in various ability. And the results appeared that *Pseudomonas aerogenosa* P1 bacteria was most efficient than other isolates table-4.

Table 4- Peroxidase enzyme activity for crude products of tested bacteria.

Symbol of isolate	Bacteria	Activity U/ml
P1	***Pseudomonas aerogenosa***	**1.634**
P2	*Pseudomonas aerogenosa*	1.0
P3	*Pseudomonas aerogenosa*	1.0
P4	*Pseudomonas aerogenosa*	0.50
P5	*Pseudomonas aerogenosa*	0.59
B6	*Bacillus subtilis*	0.7
B7	*Bacillus subtilis*	1.0
B8	*Bacillus subtilis*	1.1
B9	*Bacillus subtilis*	0.7
B10	*Bacillus subtilis*	1.501
S11	*Staphylococcus aureus*	1.383
S12	*Staphylococcus aureus*	1.3
S13	*Staphylococcus aureus*	0.9
S14	*Staphylococcus aureus*	1.2
S15	*Staphylococcus aureus*	0.6
PA16	*Pantoea agglomerans*	0.8
PA17	*Pantoea agglomerans*	1.337
PA18	*Pantoea agglomerans*	1.2
PA19	*Pantoea agglomerans*	0.9
PA20	*Pantoea agglomerans*	0.7

The results were agreements with others results of [13, 14, 6, 15 and 16] which indicated that *Pseudomonas aerogenosa***,** *Bacillus subtilis, Staphylococcus aureaus* and *Pantoea agglomerans* inaddition to *Providencia* spp. are produce peroxidase enzyme.

Peroxidase enzyme purification

Four steps were used to purification of peroxidase enzyme which produced by locally isolated *Providencia* spp. bacteria**,** these steps listed in the following (table -5) :

Ammonium sulfate precipitation

The step was performed by using gradual saturation ratios ranged between 60 and 80% of ammonium sulfate salt to precipitate crude enzyme, then the precipitate was estimated, in order to determine peroxidase activity. The results showed that 90% of ammonium sulfate was given completely precipitation to enzyme and appeared there were increase in the specific activity (80.66 U/mg) between the various steps of purification table-5. While the higher ammonium sulfate saturation ratios decreased the enzyme specific activity. The results were similar to these reported by [10, 15] those mentioned the peroxidase enzyme its purify by successive ammonium sulfate fractionation. Ammonium sulfate is a salt used in precipitation of enzyme due to its high solubility, therefore, it was used in precipitation of diffirent enzymes [18]. After the deposition with ammonium sulfate, the sediment was redissolved in 0.2 M phosphate buffer (pH 6.5) and dialyzed contra the same buffer. The results showed that after dialysis step, peroxidase specific activity and activity increased to 16.6 U/ml, 83 U/mg respectively.

Ion exchange chromatography

Two protein peaks showed in the wash step, whereas there were one protein peak in the elution by employ gradient condensation of sodium chloride extended among 0.5 and 1% (figure 3). Three protein peaks were discovered by measuring the absorbance at 280 nm of every fraction, one protein peak was fixed peroxidase leverage in elution step at 1% of sodium chloride of concentration. Protein fractionation distributed in fraction numbers 25 to 50 and the peak at fraction tube 43 with a peroxidase activity reached 1001 U/mg and activity reached to 162.3 U/ml. These fractions were pooled and concentrated to 3 ml, then protein concentration, peroxidase activity and specific activity were measured. The results indicated that peroxidase particles were negatively charged, since they were bound with anionic ion exchange of DEAE-cellulose. There were no activities in the washing protein peaks while there were peroxidase activity in the elution protein peak as shown in table-5 and figure-3. Results

indicated in table-5 showed that protein concentration, peroxidase activity and specific activity in this step were 0.162 mg/ml, 20 U/ml, 123.4 U/mg protien respectively, with purification fold of 2.52 and enzyme yield of 27.2 %. The results were in agreements with [8, 19,21] those reported that enzyme has more activity, specific activity, fold of purification and yield (%) after using ion exchanger chromatography step in the purification of enzyme.

Table -5: Purification steps for peroxidase produced by *Pesudomonas aeruginosa*.

Purification Steps	Volume (ml)	Enzy. Activity (U/ml)	Protien conc. (mg/ml)	Specific Activity (U/mg)	Total Activity (U)	Purification fold	Yield (%)
Crude enzyme	50	4.4	0.09	48.8	220	100	1
Ammonium sulfate (80%)	10	12.1	0.15	80.66	121	1.65	55
Dialysis	5	16.6	0.2	83	83	1.7	37.7
Ion exchange chromatography	3	20	0.162	123.4	60	2.52	27.2

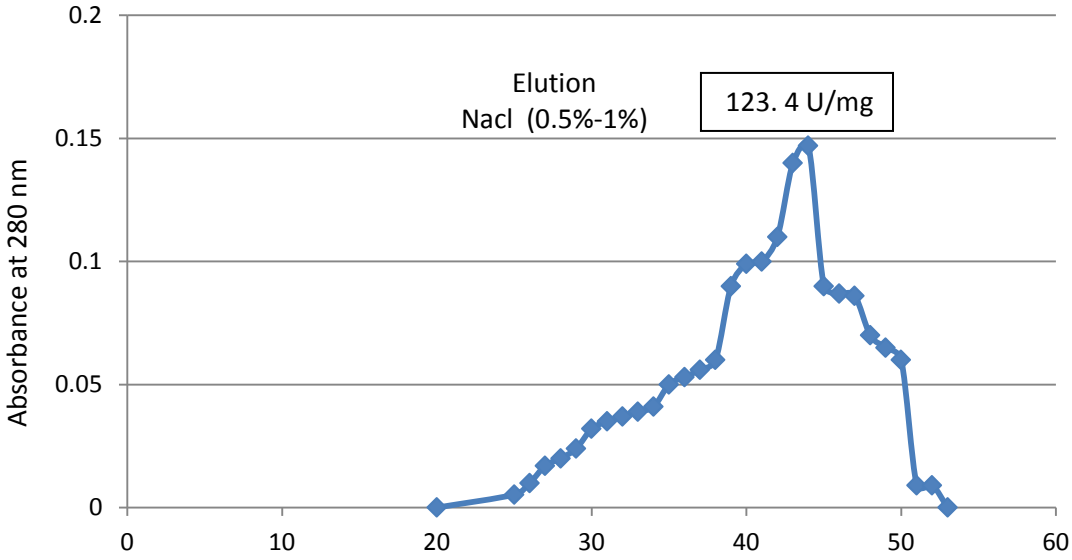

Figure (3): Ion exchanger chromatography of cellulose produced by the locally isolated *Providencia* spp. using DEAE-Cellulose column (1.75×20 cm) with a flow rate of 30 ml/hr.

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