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Enhancement the Antimicrobial Activity of Disinfectants by the Purified Lipase from *Coronobacter Dublinensis*

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Abstract

There are many different commercial disinfectants purchased, choosing of effective product is a prime target to ensure its decontamination efficiency. Thus we screened *Coronobacter dublinensis* isolates to produce lipase enzyme with using of vegetable oil like olive oil as inducer of lipase production in the medium. The lipase was purified by using two-step purification procedure consisting of ethanol precipitation and ion exchange chromatography with a yield of 48.3%. The lipase has an important role in enhancement of disinfectant activity. Since the combination between this disinfectant and lipase led to increase the activity of disinfectant to higher levels and the width of inhibition area enlarged to 28 mm against *Acinetobacter* sp. followed by *P. aeruginosa* and *S. aureus* with 25mm, respectively. So that we can conclude that lipase has excellent activity for enhancement of the commercial disinfectants purchased in decontamination activities in the laboratory and hospital environments.

Keywords: *L*ipase, *Coronobacter dublinensis*, disinfectants

Introduction

Cronobacter genus composes of a different groups of Gram negative and facultative anaerobic rods that belongs to Enterobacteriaceae family and closely related to *Enterobacter* and *Citrobacter* [1,2]. It is includes seven opportunistic pathogenic species: *Cronobacter sakazakii, Cronobacter malonaticus, Cronobacter muytjensii, Cronobacter turicensis, Cronobacter dublinensis, Cronobacter universalis* and *Cronobacter condimenti* [1,3].

The organism has association with severe blood infections (sepsis), neonatal infections; necrotizing enterocolitis, septicaemia and meningitis which may be lead to fatal effect. However, these infections with *Cronobacter* are rare and occur in all age groups [2,3].

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) catalyze the hydrolysis of ester bonds in water insoluble lipid substrates, acting at the interface between the aqueous and the organic phases [4]. They are produced by animals, plants and microorganisms. Microbes have an ability to produce of lipases with high yields in comparison with the animal and plants, this return to that they are more common than animal and plant sources, easier in extraction and more commercialization in their involvement in enzymatic reaction [5,6]. Lipases from microorganisms (bacterial and fungal) are the most used as biocatalysts in biotechnological applications and organic chemistry [7,8].

Disinfectants are antimicrobial agents that are applied to the surface of non-living objects to destroy microorganisms that are living on the objects. They are an essential part of infection control practices and aid in the prevention of laboratory and nosocomial infections [9]. Many gram negative bacteria, gram positive bacteria and yeasts leads to nosocomial infections [10]. As there are many different commercial disinfectants purchased, choosing of effective product is a prime target to ensure its decontamination efficiency.

The aim of this research was to detect the lipolytic activity in *Coronobacter dublinensis* and purification of lipase from *Coronobacter dublinensis* isolated from hospital environment clinical samples and using of lipase for enhancement of the commercial disinfectants purchased in decontamination activities in the laboratory and hospital environments.

Materials and Method

Bacterial isolates and Identification

In this study, we obtained 3*Coronobacter dublinensis* isolates from blood samples of patients. All the laboratory procedures were performed at central public health laboratories of Teaching Laboratories / Medical city, Baghdad during July/2015. The phenotypic identification was confirmed by using of the morphological testes and by Vitek-2 system (Bio-Merieux, France).

Lipolytic activity on media

Lipase indicator plates were prepared in 1 liter by addition of 10 g peptone, 5g NaCl, 0.1g $CaCl_2$, 1ml olive oil, 10g agar-agar and 0.5g Congo red. The lipase activity was indicated by the formation of clear halos around the colonies [11].

Purification of lipase

Coronobacter dublinensis extracellular lipase was purified by a modification of the method [12]. The cells were grown in the mineral growth medium (MGM) contained (in g/L): NaH2PO4 12, KH_2PO_4 2, MgSO4·7 H2O 0.3 and CaCl2 0.25. Ammonium sulphate at 1% (w/v) and castor oil at 2% (v/v) were used as nitrogen and carbon sources, respectively [11] and incubated at 30°C in shaking incubator for 18-24h.The supernatant was carefully removed after centrifugation at 10000xg for 30min at 4°C and filtered through 0.22µm Millipore filters then lipase activity in supernatant was assayed. The supernatant was treated with ethanol at ratio of saturation 60% then the mixture was centrifuged and the lipase activity was assayed for the precipitate after dissolving in 20mM Tris buffer, pH=8.The supernatant was dialyzed against the same buffer.

The supernatant was loaded on diethyl aminoethyl (DEAE)- sephadex A-25 column (2.5 by 25cm) that has been equilibrated with 20mM Tris buffer, pH=8.The column was washed with 5 to 10 volumes of the above buffer. The lipase was eluted with a gradient elution from 0.1-0.5M NaCl solutions. The fractions (5ml) were collected and assayed for lipase activity.

Lipase assay

Lipase assay was conducted by a modified method based on (13). About 30mg of *p*-nitrophenyl palmitate (*p*NPP) was dissolved in 10ml of 2-propanol at 60°C and added to 90ml of 100mM potassium phosphate buffer (pH= 7.0) containing 10mM $MgSO_4$ to yield a final concentration of 1mM *p*NPP.50µlof sample was added to the substrate solution to give a final volume of 2.5ml, the solution was incubated for 15min at 37°C and the absorbance was measured at 410nm. One unit of lipase activity was defined as the amount of enzyme that produced an absorbance at 410 nm equivalent to 1µmol of *p*-nitrophenol in one min under the assay conditions.

Estimation of protein content

The protein content of the enzyme was determined by using Bradford dye method with BSA as a standard [14].

Effect of lipase on the efficiency of disinfectants

Two types of purchased commercial disinfectant were examined to determine their inhibition zone against different types of gram negative bacteria (*Acinetobacter* sp., *P. aeruginosa* and *Salmonella typhimurium*), gram positive bacteria (*Staph. aureus*) and yeast (*Candida albicans*).Two fold serial dilutions of each disinfectant was mixed with distilled water to give concentrations ranging (16 – 128µg/ml). A 0.1ml of $1.5x10^8$ cfu/ml bacterial suspension was spread on the surface of Mueller-Hinton agar plates, left to dry for 15 minutes at room temperature. About 50µl from every dilution was put in wells (7 mm in diameter) with Mueller-Hinton agar plates. In the other hand, 5.6 mg/ml purified lipase was combined with each concentration of each disinfectantseparatelythen50µl from the mixture was placed in wells on Mueller-Hinton agar plates. After that the plates were incubated at 37°C for 18 to 24hour. The diameter of an inhibition zone for the mixtures and disinfectant alone was measured [15].

Results

Bacterial isolates and identification

In this study, three *Coronobacter dublinensis* isolates were collected from blood samples of patients. These isolates were diagnosed by using many morphological and biochemical tests.

Lipolytic activity on media

The aims of this study was to screen the capability of *Coronobacter dublinensis* isolates to produce lipase enzyme. Therefore, we used olive oil as vegetable oil for determination of *Coronobacter dublinensis* harboring

lipase activity. so the first step of the screening process, we analyzed for the hydrolytic action of *Coronobacter dublinensis* isolates. It is an easy, fast and economy test to act on solid media created on the optical examination of plates having one of the vegetable oils and presence of a clearing zone around the colony edges. The results revealed that all 3 *Coronobacter dublinensis* isolates have the ability to hydrolyze the olive oil but in different levels as shown in (figure-1). Therefore, Thus we can conclude that *Coronobacter dublinensis* had an ability to produce lipase in the medium by using olive oil as inducer.

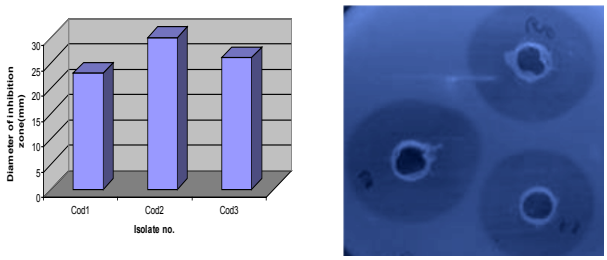

Figure 1: Lipolytic activity for *Coronobacter dublinensis* isolates

Purification of lipase

Mineral growth medium (MGM) with 2%castor oil was chose as the best medium for lipase productionbecause it composed of only inorganic nitrogen sources and some minerals. In *Coronobacter dublinensis* 2, the castor oil was used as a sole carbon source for lipase production. A two-step purification program consisting of the precipitation by ethanol and ion exchange chromatography by DEAE-sephadex A-25 column were used to obtain a highly purified lipase from *Coronobacter dublinensis*2. Table (1) showed the details for each purification step. 60% saturation for cooled ethanol solution was added to the crude extract and led to rise of the specific activity to 4.8 U/mg and revealed 2.5 fold of purification with 55.7% lipase recovery before the dialysis. Ion exchange chromatography by DEAE-sephadex A-25 column was the second purification step. When lipase solution was passed through DEAE-sephadex A-25 column and eluted with NaCl solutions(0.1 - 0.5M), two peaks of protein appeared in the eluted fractions with one peak of lipase activity located in the second protein peak (figure-2). Fold of purification was 11.2 in this step with 48.3% recovery.

Table 1: Purification of lipase from *Coronobacter dublinensis*

Purification step	Size (ml)	Protein conc. (mg/ml)	Lipolytic activity (U/ml)	Specific activity (U/mg)	Purification (fold)	Total activity	Total Recovery(%)
Crude extract	60	32	62	1.9	1	3720	100
Ethanol precipitation	25	17	83	4.8	2.5	2075	55.7
DEAE-Sephadex A- 75	15	5.6	120	21.4	11.2	1800	48.3

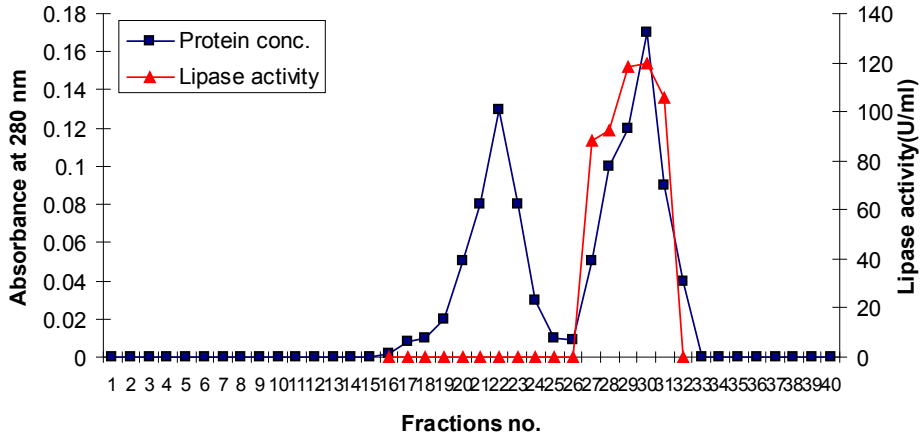

Figure 2: Purification of lipase from *Coronobacter dublinensis2* using ion exchange on DEAE-Sephadex A- 75 column.

Effect of lipase on the efficiency of disinfectants

The results showed that any one of each type of disinfectant had antimicrobial activity against all the tested microorganisms.The type one of disinfectant reveald higher effectiveness toward *Acinetobacter* sp. with diameter 23mm followed by 22mm for *P. aeruginosa* while lower effectiveness against *Candida albicans*. But in the combination with the purified lipase the effect increased and showed higher level toward *Acinetobacter* sp. followed by *P. aeruginosa* and *S. aureus* since the diameter of inhibition zone was 26mm for *Acinetobacter* sp. and 25mm for *P. aeruginosa* and *S. aureus* (table 2).

In the case of using the second disinfectant, this disinfectant had stronger effect against gram positive and vegative bacteria and lower effect toward *Candida albicans.* On the other hand, the combination between this disinfectant and lipase led to increase the activity of disinfectant to higher levels and the diameter of inhibition zone became 28 mm against *Acinetobacter* sp.(figure 3) followed by *P. aeruginosa* and *S. aureus* with 25, respectively, and lower diameter was22 mm against *Candida albicans.* According to these results we can conclude that the lipase has an important role in enhancement of disinfectant activity.

Table 2: Diameter of inhibition zones for different strains in plates in presence of disinfectants with and without lipase

Organism	Diameter of inhibition zone for disinf.1 (mm) with different con. (mg/ml)				Diameter of inhibition zone for disinf.1 (mm) at different con. + 5.6 (mg/ml)				Diameter of inhibition zone for disinf.2 (mm) with different con. (mg/ml)				Diameter of inhibition zone for disinf.2 (mm) at different con. + 5.6 (mg/ml)			
	16	32	64	128	16	32	64	128	16	32	64	128	16	32	64	128
***Acinetobacter* sp.**	23	20	16	14	26	24	21	18	23	20	19	17	28	25	21	20
P. aeruginosa	22	19	17	16	25	23	19	17	21	19	16	14	24	22	19	18
Salmonella typhimurium	21	20	18	17	23	20	19	18	23	22	19	17	25	22	21	20
S. aureus	21	19	17	16	25	21	18	14	22	19	17	15	24	21	19	17
Candida albicans	19	18	17	14	22	19	16	15	18	17	15	14	21	19	17	14

Figure 3: Inhibition zones for *Acinetobacter* sp. in plates in presence of disinfectants with and without lipase

Discussion

Most *Cronobacter* spp. have been associated with human infections in newborn and infant infections, causing meningitis, necrotizing enterocolitis (NEC) and bacteraemia or sepsis [16,17].

There is distribution of Lipolytic germs in flora and about 20% of them are lipase creators [16]. Lipase production from *Microbacterium* sp. showed that the bacterial growing and lipase creation got to upper level after 48 hours at the start of stationary phase[17] and lipase activity in a late stationary phase was decreased and this can be owed to the presence of proteases in media of the culture. In contrast, *Staphylococcus warneri* produced higher level of lipase after 24 hours and at the beginning of the stationary phase [18]. the parameters of growth such as optimum temperature, pH and enzyme specificity affected on lipase production [19].

Ethanolic precipitation with alginate led to rapid and almost completely harvest of lipase from the cell-free culture medium by (about 95% of the original exolipase activity was coprecipitated) [20]. An ammonium sulphate precipitation trailed with Sephadex G-100 column chromatography led to clean of an extracellular lipase from *Microbacterium* sp. with a complete harvest of 20.8 % and fold purification equal to 2.1 [21].

The isolation source, the type of culture medium that was used for growth of bacteria and the procedure that was used for purification of enzyme have effect on the enzyme activity, fold of purification and the recovery of purified enzyme [22]. Lipases are used generally in house, dishwashers and modern laundry. They generally working as additives to cleaners, as they are added to the cleaners mostly in mixture with proteases and cellulases [9], besides to other enzymes such as amylases, peroxidases and oxidases. The hydrolysis of fatty stains by lipase to 15 hydrophilic parts that make these stains easily removed in comparison with non-hydrolyzed stains [21]. Lipases should be both thermophilic (30- 60°C) and alkalophilic (pH 10-11) add to a wide substrate specify to be able to hydrolyze fats of numerous compositions and a suitable chemical addition in cleaners and keeping their action in the presence of the numerous workings of clothes wash powder formulations like surfactants and proteases. In addition, they should have [10]. The combination of genetic and protein engineering enhanced these properties of lipases in addition to their application in laundry, dish washing, contact lenses cleaning, degradation of organic wastes on the surface of exhaust pipes and toilet bowls, etc.[23].

Conclusion

Disinfectants have limited levels in decontamination. The lipase has an important role in enhancement of commercial disinfectants purchased activity to achieve acceptable level of decontamination.

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