PURIFICATION OF INULINASE FROM KLEBSIELLA PNEUMONIAE AND STUDY THE ANTIBACTERIAL EFFECT OF COMBINATION OF INULINASE AND CEFTAZIDIME

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ABSTRACT

Introduction: *Klebsiella pneumoniae* are Gram-negative which cause many diseases such as urinary tract infections, respiratory tract infections and septicemia. Inulinase is an enzyme used in food manufacture and pharmaceuticals. Inulinase is used in decreasing lipid ratio and, cholesterol in blood and considered as a prebiotic factor inside intestine. Many microorganisms can produce inulinase, such as yeast, fungi and bacteria; among such bacteria: *Bacillus* spp., *Arthrobacter* spp*., and Pseudomonas* spp. but there are no studies about inulinase production by *K. pneumoniae* have been reported. So the current study aims at investing the ability of producing and purification inulinase by *K. pneumoniae*. **Method:** *K. pneumoniae* were isolated from many hospitals and screened for the production of inulinase. Isolation percentage was 32%. A combination between the enzyme and the ceftazidime were assayed for detecting the antibacterial activity agonist Gram positive and Gram negative bacteria were done. **Result**s: It is found that *K. pneumoniae* K4 isolate is the best producer of this enzyme. Inulinase, purified with ammonium sulfate at 70% saturation with specific activity 7.01 U/mg protein. As well, it's found that inulinase had increased the activity of ceftazidime against bacteria when combination between this enzyme and the antibiotic had done. **Conclusion:** This study proves for the first time that *K. pneumoniae* can produce inulinase which can be used in tremendous applications and also proves the broad spectrum bioactivity of inulinase against microbial pathogens. Ceftazidime antimicrobial activity against bacteria, is increased when a combination between inulinase and ceftazidime had done.

Keywords: inulinase, inulin, *Klebsiella pneumoniae*, Urinary tract infection, ceftazidime.

Introduction

Klebsiella pneumoniae are Gram-negative, motile rod-shaped bacteria. Typical *K. pneumoniae* are opportunistic bacteria that cause nosocomial infections, urinary tract infections, respiratory tract infections and septicemia (Vasaikar et al., 2017). These bacteria can be found in the skin, mouth and intestines, as well as in hospital settings and medical devices. Inulinase (2, 1 - b - D - fructano - hydrolases EC 3.2.1.7) is a microbial enzyme hydrolyzes inulin to fructose and inulooligosaccharides, which are used in food manufacture and pharmaceuticals. Beside its important role in decreasing the ratio of lipid and total cholesterol in blood (Nascimento et al., 2012).

Inulin stored in the tubers and roots of plants like Jerusalem artichoke Jicama, dahlia, Garlic and Chicory (Fawzi, 2011). Inulin and fructooligosaccharides (FOS) are indigestible food that widely beneficial to human body health (Angel et al., 2012, Bradford., 1976) and considered as prebiotic factors inside intestine (Bharathi et al., 2011). Fructose, very important food and drink manufacture (Gao et al., 2007) (Dilipkumar et al., 2011).

Microorganisms are important producers sources of inulinases, many studies among which (Wang et al., 2003) (Sahin et al. 2004) (Singh et al.,2017) showed the successful production of inulinase from various filamentous fungi, yeast and bacteria strains like *Bacillus* spp., *Arthrobacter* spp*.,* and *Pseudomonas* spp. have been studied as high productivity producers of inulinase. The bacteria *K. pneumoniae* have been rarely used as producers to this microbial enzyme. To our knowledge there is no previous study about inulinase production by these bacteria have been reported.

There is a need for developing substrate in cheaper and more effective process, thus this study aims to detect the ability of producing inulinase from *K. pneumoniae* in addition to partial purification of this microbial enzyme and study its antibacterial activity with antibiotic ceftazidime.

Methods

Samples collection

During a period of two months from September to November, 2017. Twenty-five diverse clinical samples, which included: blood, urine and burns, were collected from several hospitals in Baghdad – Iraq.

Bacteriological analysis

The bacterial diagnosis, including morphological characters and biochemical tests, were done (Holt, 1994) the *Klebsiella* spp. isolates were identified by characteristic colonies (mucoid, fermentation of lactose, hemolysis), indole test (-) Methyl red test (+) Voges – Proskauer test (+) Citrate utilization test (+) urease (+), followed by the complementary API 20E test that carried out according to directives of the company (BioMeriux/France).

Primary screening for inulinase activity

For detecting inulinase production by *Klebsiella pneumoniae*, all *K. pneumoniae* were cultured in to the inulin agar media, which contained (per liter) the following: inulin 20g as (carbon source), yeast extract agar 20g, MgCl2 0.5g, NH_4NO_3 2g, pepton 2 g, KH_2PO_4 5g and of agar 20g. All components were dissolved in 1 liter of distilled water and pH was adjusted at 7. The mixture was autoclaved at 121°C for 15 min. This medium was used for the screening the capability of bacteria to produce inulinase. After 24 hrs. of incubation periods at 37 ℃, bacteria showed growth with clear inhibitions zones round bacterial colonies that had measured (Jenny et al., 2012).

Secondary screening for inulinase activity

K. pneumoniae isolate was transferred into inulin 20g, yeast extract 20g , MgCl2 0.5g, NH_4NO_3 2g, pepton 2 g, KH_2PO_4 5g, pH fixed at 7. *K. pneumoniae* isolate put on rotary shaker incubator at 100rpm at 30℃ for 24 hrs. Centrifugation had done at 10000 xg for 20 min, cells removed, specific activity and the protein content were checked (Angel et al., 2012).

Assay of inulinase

Enzyme solution (2 with ml) was incubated 2% of inulin, with citrate phosphate buffer 10 mM and pH 7 at 35 ° C for 50 min and then the reaction was terminated by putting reaction

tubes in water bath at 100℃ for 10min so that inactivate the reaction of enzyme. After that enzyme was cooled at room temp. Then in order to reduce fructose sugar, mixture tested by using method of DNS that described by (Jenny et al., 2012). By spectrophotometer, reaction was measured at (575 nm) and standardized against the fructose standard. One unit of the enzyme activity was defined as the amount of inulinase needed to produce 1µ mole of fructose from inulin per minute under the standard condition of the assay.

Estimation of protein content

The estimation of total protein content was carried out according to Bradford dye method with bovine serum albumin as a standard (Bradford., 1976).

Inulinase purification

The selected isolate that secreted inulinase was purified by the method explained by (Fawzi, 2011). After incubation, centrifugation had done so cells detached in centrifuge at 10000 rpm for 20 min under cooling conditions. The supernatant which is the crude extract, was precipitated by ammonium sulfate with gradient concentrations saturations (40-80%). The precipitate was dissolved in 5ml of buffer at pH 6.5, the protein concentration was distributed for the measurement of volume and specific activity.

Determining the minimum inhibitory concentration (MIC)

Ceftazidime was used as antimicrobial against *S. aureus* and *E. coli* by using macro dilution method (Bharathi et al., 2011). For determining MIC, ceftazidime was solved in distilled water for giving 10000 µg/ml of stock concentration, stock concentration of ceftazidime was filtered by millipore filter with 0.22 µm. Two fold serial dilutions of ceftazidime were made inside the nutrient broth in order to give the rang of concentrations (1 - 5000) µg/ml. one hundred microliter of 10^4 cfu/ml bacterial suspension that consists of *S. aureus* and *E. coli* was added to test tubes, separately. 50µl of each dilution entered through the wells of Mueller-Hinton agar media. The plane tubes and plates put in incubation for 24 hrs at 37°C. Lowest antibiotic concentration can inhibit the growth of 50% or more of microorganisms that showing inhibition zone in plates, no growth in tubes, was determined and recorded as minimum inhibitory concentration (MIC).

Determination the interaction between inulinase and Ceftazidime

A combination between the enzyme and the antibiotic were assayed in the same way mentioned above with *S. aureus* and *E. coli*, as 100 microliter of inulinase and the bacterial suspension were added in secluded to two fold serial dilutions of ceftazidime. 50µl of each dilution entered through the wells of Mueller-Hinton agar media. The plane tubes and plates put in incubation for 24 hrs at 37°C. Lowest antibiotic concentration can inhibit the growth of 50% or more of microorganisms that showing inhibition zone in plates, no growth in tubes, was determined and recorded as the minimum inhibitory concentration (MIC).

Ethical statement

All experiments that included human blood, urine and burns samples, bacterial collection and isolation were ethically approved according to biosafety conditions in Biology department-College of Science -Mustansiryiah University-Iraq and according to the ethics Committees in Ministry of Health, Baghdad, Iraq

Results

Isolation of *Klebsiella pneumoniae*

To determine the distribution of *K. pneumoniae*, Twenty-five diverse clinical samples of *K. pneumoniae* were collected from several hospitals in Baghdad – Iraq. Eight isolates of *K. pneumoniae* (32%) were gained of 13 *K.* spp. isolates. *K. pneumoniae* was assayed and diagnosed through biochemical tests and API 20E test as shown in figure (1).

Screening the production of nulinase from *Klebsiella pneumoniae*

Eight isolates of *K. pneumoniae* were tested and screened for producing the inulinase in agar plates. 5 isolates of *K. pneumoniae* grew in inulin production medium and showed the inulinase activity. All five isolates of *K. pneumoniae* were submitted to the next step of screening for producing the inulinase in broth media. Results detected that *Klebsiella pneumoniae* K3 was the best inulinase producer as shown in figures (2).

Inulinase Purification

Klebsiella pneumoniae K3 was selected for inulinase purification by culturing in broth media that contains inulin. Table (1) show results of purification. Precipitate of the enzyme done by

Ammonium sulfate at saturation of 70% from the supernatant with higher specific activity.

Antibacterial activity of ceftazidime

Table (2) detected a little antibacterial activity of ceftazidime with the tested isolates. The minimum inhibitory concentration value for. *S. aureus* was (256 µg/ml) and for *E. coli* was (1024 µg/ml). Furthermore, a mixture of inulinase and ceftazidime detected high activity to the antibacterial to these bacteria. The (MIC) of ceftazidime decreased to the (64 µg/ml) for *S. aureus* and (16 µg/ml) for *E. coli*.

The same results were obtained when agar diffusion method used, since in this method at (16 µg/ml) concentration the diameter of inhibition zone for *S. aureus* was (19 mm) and at a (64 µg/ml) concentration the diameter of inhibition zone was (22 mm) for *E. coli* (figure 3 and figure 4). Through these result we can prove that G+ve bacteria have more sensitivity than G-ve bacteria in combination of inulinase enzyme and ceftazidime antibiotic by comparison with the control. These results proved that β-lactam antibiotics had increased in the inulinase presence, this will be useful agent for treatment many infections of *S. aureus* with the β-lactam antibiotics.

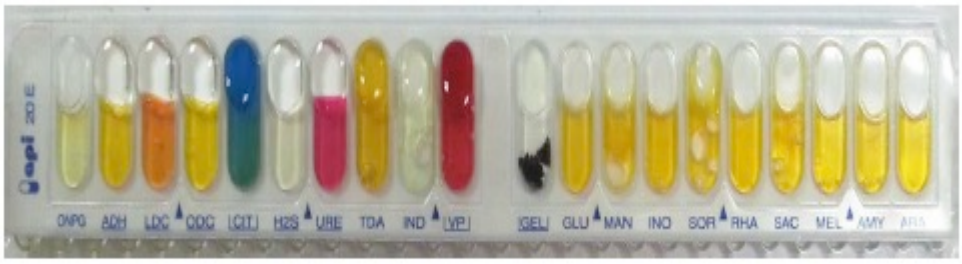

Figure 1: API 20E system for characterization of *K. pneumoniae*

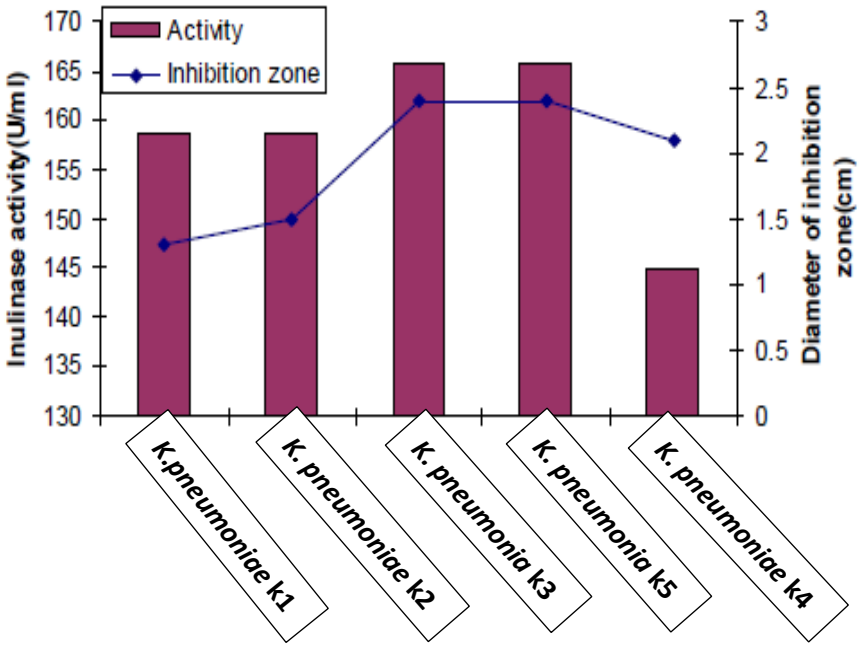

Figure 2: Diameter of inhibition zone and inulinase activities for *K. pneumoniae* isolates shows that isolate. K3 was the best inulinase producer.

Table 1: Treatments used for inulinase partial purification from *K. pneumoniae K3*

Purification step	Size (ml)	Protein concentration (mg/ml)	Inulinase activity (U/ml)	Specific activity (U/mg)
Crud extract	150	112.6	165.8	1.47
(NH4) SO4 at 70%	70	34.3	240.7	7.01

Table 2: MICs (µg/ml) of ceftazidime antibiotic for *S. aureus* and *E. coli* strains in tubes and plates with or without inulinase

Antibiotic	Bacteria	MIC for antibiotic alone (µg/ml)	Diameter of inhibition zone(mm)	MIC for antibiotic and inulinase (µg/ml)	Diameter of inhibition zone(mm)
Ceftazidime	*E. coli*	1024	28	64	22
	S. aureus	256	26	16	19

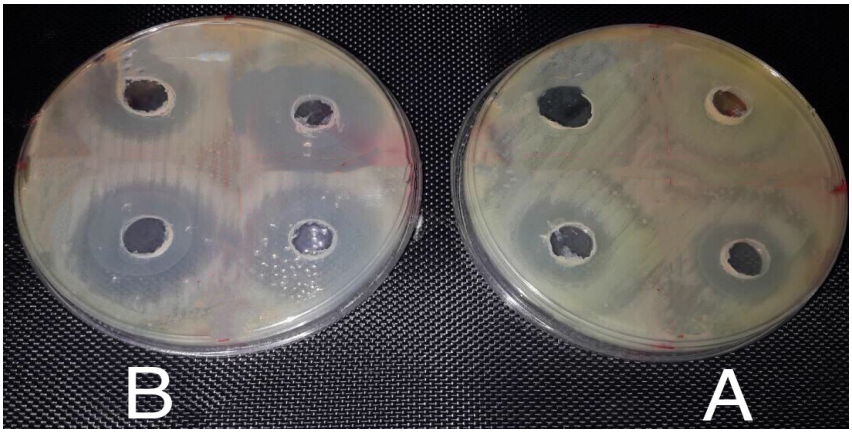

Figure 3: (A) Diameter of inhibition zones of ceftazidime against *E. coli*
(B) Diameter of inhibition zones of ceftazidime and inulinase combination against *E. coli*

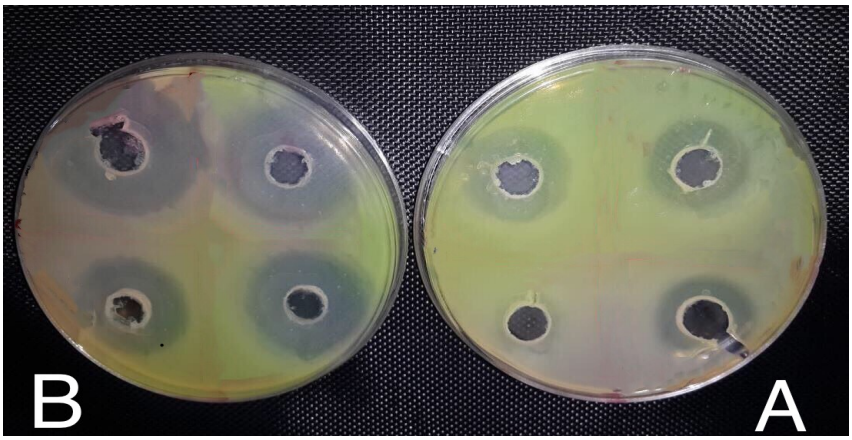

Figure 4: (A) Diameter of inhibition zones of ceftazidime against *S. aureus*
(B) Diameter of inhibition zones of ceftazidime and inulinase combination against *S. aureus*

Discussion

In this research work, the inuilnase extraction and purification from the bacterium *K. pneumoniae* is discussed. For the first time *Klebsiella sp.* shows excellent results in the production of inuilnase. Inuilnase enzymes must be manufactured either when the substrate is existent with the surrounding medium or in the presence of structurally related compounds to this enzyme (Peter & Robinson, 2015). The activity of Inulinase are various depending on the fermentation conditions (Neagu et al.,2012). The results show that the highest inulinase activity is 240.7(U/ml) by precipitate inulinase with ammonium sulfate at saturation of 70% this suggests that the difference in inulinase production may be related to the microbial strain, composition of fermentative media and parameters of the fermentation process. Purification of inuilnase from this specious of bacteria are so simple by using the crude extract or precipitation method with using ammonium sulfate. Many generations of bacteria were developed a virulence resisting against antibiotics include ceftazidime. So, purified microbial inulinase from *K. pneumoniae* can be a new biotic medicine (Andrea Du Toit 2018) especially when it is combined with classical antibiotic.

Conclusion:

Inulinase is considered as a very important industrial enzyme. Therefore the need to produce and purify inulinase from new microbial isolates is required. In this study, we have successfully purified inulinase from *Klebsiella pneumoniae*. The purified enzyme shows a broad spectrum bioactivity against microbial pathogens. Ceftazidime antimicrobial activity against bacteria, is increased when a combination between inulinase and ceftazidime had done.

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