

OPTIMAZATION OF PROTEASE PRODUCED FROM CRONOBACTER SAKAZAKII R4

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Article Received on
08 April 2017,

Revised on 29 April 2017,
Accepted on 19 May 2017

DOI: 10.20959/wjpr20176-7596

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ABSTRACT

Cronobacter sakazakii was supplied from biotechnology department/college of science primarily identified according to their cultural and morphological tests and biochemical characteristics. The ability of these isolates in protease production was examined. Results showed that all five isolates of *C.sakazakii* were protease producers with variable degrees. Among them, the isolate symbold *C.sakazakii* R4 was the most efficient in protease production. The specific activity of protease produced in crude filtrate was 35.8 U/mg protein. Upon that, this isolate was selected to determine optimum conditions for

protease production. Results showed that maximum protease production was achieved after supplementation of the production medium (pH7) with 0.5% glucose, 1.5% tryptone and 0.1% KH₂PO₄ and inoculation with 10⁴ CFU /ml of fresh bacterial culture and incubated at 30 °C in shaker incubator (150 rpm) for 24h. Under these conditions, the specific activity of protease produced in culture medium was sharply increased to 80.4U/mg protein.

KEYWORDS: *Cronobacter sakazakii* *C.sakazakii* symbol 80.4U/mg protein.

INTRODUCTION

Cronobacter sakazakii cells are Gram negative, facultative anaerobic, non spore forming bacterium belongs to the family *Enterobacteriaceae*. The earliest recorded use of the name was *C. sakazakii* who derived the name to honour the Japanese microbiologist Riichi Sakazaki.^[1] Prior to this, five other names were used, including the “Urmenyi and Franklin bacillus”, “yellow coliform”, “yellow *cronobacter*”, “pigmented *cloacae* A organism” and most notably “yellow-pigmented *cronobacter cloacae*”.^[1]

C. sakazakii is an enteric pathogen that can be found in PIF and associated with neonates in neonatal intensive care units (NICU) infections. It can cause NEC, bacteraemia, and meningitis resulting in a 40-80% mortality rate among infected infants and 20% of the survivors also develop serious neurological disorders.^[2,3]

C. sakazakii is an opportunistic pathogen, which is associated with a number of clinical presentations in neonates, especially with those who have underlying conditions such as prematurity and low weight at birth. Moreover, the infection is not limited to neonates, it can occur in several age groups with less severity.^[4]

Many species of the genus *Cronobacter* produced a variety of extracellular protease, which are critical to the maintenance of cellular function.^[5] Proteases are the hydrolase enzymes that catalyze the hydrolysis of the peptide bonds in the primary structure of proteins and peptides. Proteases are present in a wide variety of living organisms and they also show different physiological, physicochemical, biological, chemical functions on the earth. Proteases constitute one of the most important groups of industrial enzymes and accounting for about 60% of total worldwide enzyme market because of their various application in many industries such as detergent, food, leather, silk, dairy and pharmaceutical industry.^[6,7] Many industrial protease take place at high temperature and therefore, the thermostable proteases are suitable for these industries, for example, the detergent industry is performed at 20-60°C at a pH 7.5-10.5, proteases are particularly important for this application because they are both stable and active under high temperature and in the presence of surfactants.^[8]

MATERIALS AND METHODS

Chemicals

All chemicals (99% purity) used in this study were purchased from Hi-Media Laboratories, Merck (Mumbai, India) and Sigma(U.S.A).

Culture and Growth Condition

C.sakazakii was obtained from Biotechnology Department, College of Science AL-Nahrain University, Iraq. The strain was maintained on nutrient agar slants having pH 7.0 at 37°C slants and subcultured for every 15 days.

Production of enzyme

Protease enzyme production was carried out using standard media glucose, 0.5% (w/v); tryptone 1.5% (w/v); KH₂PO₄ 0.1% (w/v); before inoculated with a 10⁴ CFU of fresh bacterial culture and incubated at 30 °C in shaker incubator (150 rpm) for 24h. culture medium was harvested and was subjected to centrifugation at 6000 rpm for 20 min to obtain crude extract, which was used as enzyme source.

Screening ability of *Cronobacter sakazakii* isolates for protease production

Semi quantitative screening

Each isolate of *C. sakazakii* streaked on nutrient agar medium and incubated at 37 °C for 24h. A single colony was then taken and placed on the skim milk agar medium plate. The plate was incubated at 37 °C for 24h. Ability of *C. sakazakii* in protease production was measured based on presence of clear halo zone around each colony.

Quantitative screening of protease

Production was achieved by determining the enzyme activity and specific activity according to the following steps.

Assay of protease enzyme Activity

Protease activity was assayed according to Manachini *et al.*, (1989), by incubating 0.8 ml of the 1% casein solution with 2.0 ml of enzymatic solution for 30 minutes at 37 °C. Reaction was stopped by adding 1 ml of the 5 % TCA and chilled in an ice bath for 10-15 min before, the solution was centrifuged at 6000 rpm for 15 minutes. Control test was prepared by adding 1 ml of 5 % TCA to 0.8 ml of 1 % casein solution and then adds 0.2 ml enzymatic solution. The absorbency for the supernatant was measured at 280 nm using UV-VIS spectrophotometer. The enzymatic activity was estimated depended on the degradation of casein protein to small peptides and soluble amino acids, The absorbance was measured at 280 nm since one unit (U) of enzyme activity was defined as the amount of enzyme required to produce an increase in absorbance at 280 nm equal to 0.01 in one minute under experimental conditions according to the following equation.

$$\text{Enzyme activity} = \frac{\text{Absorbance at 280 nm}}{0.01 * 30 * 0.2}$$

0.01: Constant

30: Reaction time (min)

0.2: Enzyme volume (ml)

$$\text{Specific activity (unit/mg)} = \frac{\text{Enzyme activity}}{\text{protein concentration}}$$

Protein estimation

Protein estimation was determined according to (Bradford, 1976).

Optimization of media

The media used for production of protease was subjected to optimization with respect to different carbon, nitrogen and phosphate sources along with physical parameters like pH, temperature, inoculum size, incubation period and agitation

Effect of different carbon sources

Standard media was supplemented with various carbon (Sucrose, Lactose, Glucose, Fructose and starch and maltose).

Optimum carbon source concentration

To determine the effect of optimum carbon concentration on protease production by the isolate of *C.sakazakii*, an optimum carbon source from previous step was added.

Effect of different nitrogen sources

Standard media was also optimized with different inorganic nitrogen sources such as (ammonium nitrate, Yeast extract, Casein, Tryptone and Peptone).

Optimum nitrogen source concentration

To determine the effect of optimum nitrogen concentration for protease production by isolate of *C.sakazakii*, an optimum nitrogen source was added to the production medium at concentrations of 0.5%, 1%, 1.5%, 2%, 2.5% and 3%.

Effect of different phosphate source

Various phosphate sources (K_2HPO_4 , KH_2PO_4 , Na_2HPO_4 , and NaH_2PO_4) were used to determine the optimum for protease production.

Optimum phosphate source concentration

To determine optimum phosphate source concentration for protease production, an optimum phosphate source was added to the production medium at concentrations 0.05, 0.1, 0.15, 0.2 and 0.25%.

Optimum inoculum size

Effect of different inoculum size of the selected isolate of the over producer isolate on protease production was studied. This was achieved by inoculating the production medium, individually, with an inoculum size ranging between (1×10^2 - 1×10^8) CFU/ml.

Optimum pH

To determine the effect of medium pH on protease production by the isolate *C.sakazakii*, pH of the production medium was optimal adjusted individually to different pH value (4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5 and 9). Then the optimum pH was used to supplement the production medium in the next step of optimization.

Optimum incubation temperature

In order to determine the optimum temperature for protease production by isolate of *C.sakazakii*, the production medium was incubated at different incubation temperatures (25, 30, 35, 40, 45 and 50°C), respectively, and then the optimum incubation temperature was set at the next steps of optimization.

Optimum incubation period

Effect of the incubation period on protease production by isolate of *C.sakazakii* was studied by incubating the production medium at different periods of time (12, 24, 36, 48, 60, and 72 hrs) to determine the optimum period for enzyme production. Then the optimum incubation period was set at the next steps of optimization.

Optimum agitation speed

Optimum shaking speed for protease production by the selected isolate was examined by incubating the production medium at different shaking speeds (100, 140, 150, 200 and 250 rpm) to determine the optimum agitation speed for protease production.

RESULTS AND DISCUSSION**Identification of *Cronobacter sakazakii* isolates**

The isolates of *Cronobacter sakazaki* was supplied from biotechnology department were primarily identified according to their cultural and morphological characteristics. Results showed that colonies of these isolates were yellow in color with mucoid and smooth produce yellow pigment undistrupted and easy to remove from the agar with a loop when grown on TSA agar medium, but when grown on MacConky agar, they were purple in color and lactose

fermentation after incubation at 37°C for 24-48hrs. bacterial cells were gram negative, rod in shape, non-spore forming and occur singly when examined under light microscope. These isolates were subjected to API 20 E systems and biochemical analysis for final-identification. Results mentioned in table (1).

Table (1): Biochemical tests for identification of *C. sakazakii* isolates.

Test	Result
Oxidase	+
Indole	-
Citrate utilization	+
Urease	-
Motility	+
Catalase	+

(-): negative result, (+): Positive result.

Screening ability of *C. sakazakii* in protease production

Two methods used for screening the ability of the *C. sakazakii* in protease production. The first was semi-quantitative screening which depends on the formation of halo of hydrolysis on skim milk agar medium, and the second was the quantitative screening which depends on the determination of specific activity of protease produced by *C. sakazakii*.

Semi- quantitative screening

Semi- quantitative screenings for protease production by *C.sakazakii* was achieved by detecting the ability to produce protease by the formation of halo of hydrolysis around the colony when grown on skim milk agar medium. Results in Figure (1) showed that these isolates were able to hydrolyze skim milk agar medium and forming halos of hydrolysis.

Quantitative screening

Isolates of *C. sakazakii* were screened quantitatively to examine their ability in protease production by growing in the production broth medium for 24 h at 37°C, then were centrifuged, and specific activity of protease in the crude filtrates was determined. Results showed that the isolates all were protease producers. Specific activity of protease in culture filtrates was ranged between 28.2 and 35.8 U/mg protein and it was found that *C.sakazakii* R4 was the most efficient, as shown in table (2), and it was selected to determine the optimum conditions for protease production.

Table (2): Specific activity of protease produced by local isolates of *C.sakazakii* after 24hrs. of incubation at 37°C in production broth medium (pH7.0) in shaker incubator at 100 rpm.

Isolates	Specific activity (U/mg)
<i>C.sakazakii</i> R1	28.2
<i>C.sakazakii</i> R2	31.2
<i>C.sakazakii</i> R3	28.7
<i>C.sakazakii</i> R4	35.8
<i>C.sakazakii</i> R5	33.8

Optimum conditions for protease production

Optimum Carbon Source

Six carbon sources (glucose, starch, sucrose, fructose, lactose, and maltose) were used as a sole source for carbon and energy to determine the optimum for production of protease by *C.sakazakii* R4. Each of carbon sources was added to the production medium in a concentration of 1% w/v. Results mentioned in figure (2) showed that the maximum production of protease by *C.sakazakii* R4 was achieved when glucose was used as a sole source for carbon and energy with specific activity of 40.8 U/mg in the culture filtrate. Results indicated that among the different sugars supplemented in the liquid media, easily metabolizable sugar was glucose and it give highest yield of protease with specific activity of 40.8 U/mg.

This may be because cells can readily transport glucose from the medium inside the cells^[9,10] found the best carbon source for protease production from *Bacillus subtilis* was glucose.



Figure (1): Proteolytic activity of *C.sakazakii* R4 on 10% skim milk agar after incubation at 37°C for 24hrs.

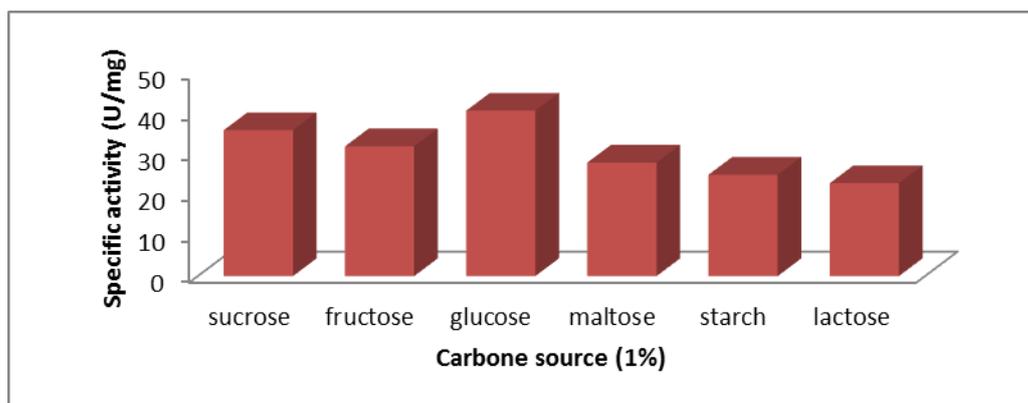


Figure (2): effect of carbon source on protease produce by *C. sakazakii*R4 after incubation at 37°C for 24 hrs in shaker incubator at 100 rpm

Optimum carbon source concentration

In order to determine the optimum carbon source concentration for protease production by *C.sakazakii* R4, six concentrations of glucose (0.5, 1, 1.5, 2, 2.5, and 3%) were used separately to determine the optimum for protease production. Results illustrated in figure (3) showed that production of protease by *C. sakazakii*R4 varied according to the concentration of glucose. Maximum production of protease was achieved when the production medium was supplemented with 0.5 % glucose, and specific activity reached 46.3 U/mg at this concentration of carbon source. This indicates that this concentration of carbon source is the best for providing the microorganism with the needed energy for growth found the best carbon source for protease production from *Bacillus subtilis* was glucose 0.5%.^[10]

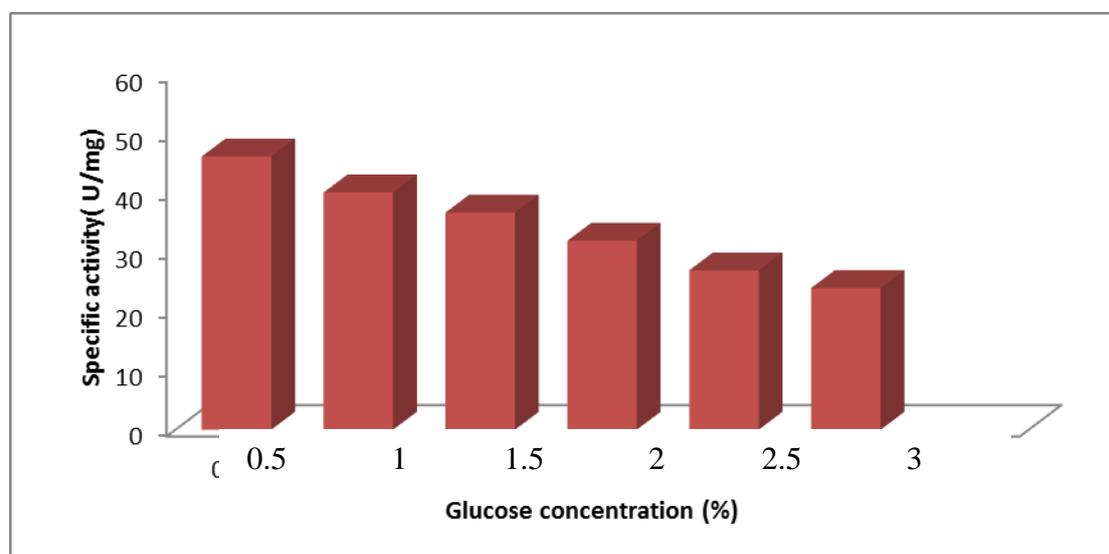


Figure (3): Effect of glucose concentration on protease production by *C.sakazakii*R4 after incubation at 37°C for 24 hr in shaker incubator at 100rpm.

Optimum nitrogen source

Five nitrogen sources were used to determine the optimum for protease production from *C.sakazakii*R4. These nitrogen sources include casein, peptone, yeast extract, ammonium nitrate, and tryptone which were added to the production medium in a concentration of 1.5 %. Results illustrated in figure (4) showed that production of protease by *C.sakazakii* R4 was varied according to the type of nitrogen source, and it was found that maximum production of protease was obtained when tryptone was used to supplement the production medium in a concentration of 1.5%. Specific activity of protease in culture filtrate of *C.sakazakii* 4 was 46.3 U/mg.^[11], found that the best nitrogen source for protease production from *Aeromonas hydrophila* was tryptone.

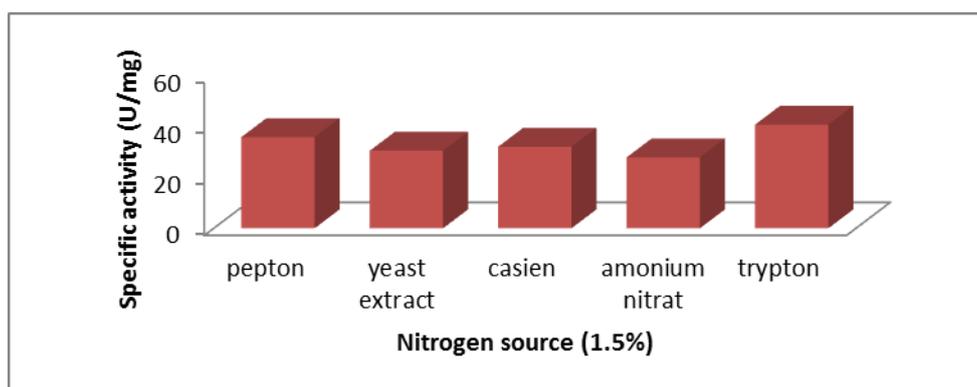


Figure (4): effect of different nitrogen sources on protease production by *C. sakazakii* R4 after incubation at 37°C for 24 hrs. in shaker incubator at 100 rpm

Optimum nitrogen source concentration

In order to determine the optimum nitrogen source concentration for protease production by isolate *C.sakazakii* R4 various concentrations (0.5, 1, 1.5, 2, 2.5 and 3%) of the most efficient source of nitrogen (tryptone) were used.

Results in figure (5) showed that maximum production of protease was obtained when tryptone was used to supplement the production medium in a concentration of 1.5%. Specific activity of protease in culture filtrate was 46.4 U/mg after using tryptone as a nitrogen source in a concentration of 1.5%. Al-tae, (2005) institute that the best concentration of nitrogen source for protease production from *Aeromonas hydrophila* was 1.5%.

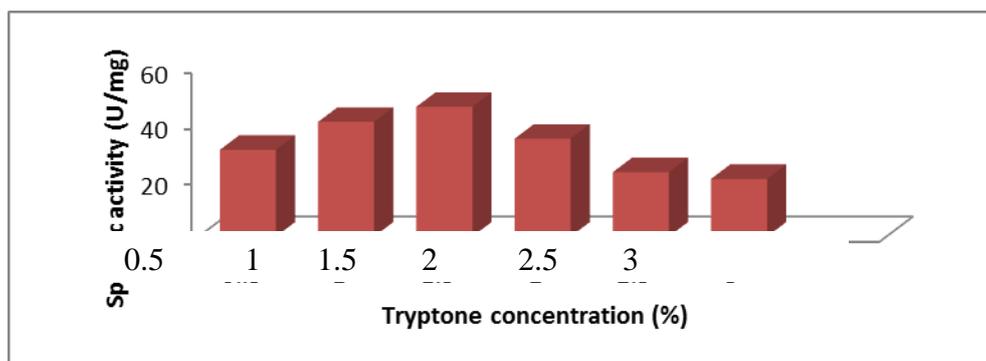


Figure (5): Effect of tryptone concentration on protease production by the *C.sakazakii* R4 after incubation at 37°C for 24 hrs. in shaker incubator at 100 rpm

Effect of phosphate source

In order to determine the optimum phosphate source for Protease production by *C.sakazakii* R4, four phosphate sources were used. These phosphate sources included potassium dihydrogen phosphate (KH_2PO_4), dipotassium hydrogen phosphate (K_2HPO_4), disodium hydrogen phosphate (Na_2HPO_4) and Sodium dihydrogen phosphate (NaH_2PO_4). They were added to the production medium in a concentration 0.005%. Results illustrated in figure (6) exhibited that the production of protease by *C.sakazakii* R4 varied according to the type of phosphate source. Maximum production of protease was achieved when the production medium was supplemented with KH_2PO_4 . Enzyme specific activity reached 52.3 U/mg, when the above phosphate source was used.

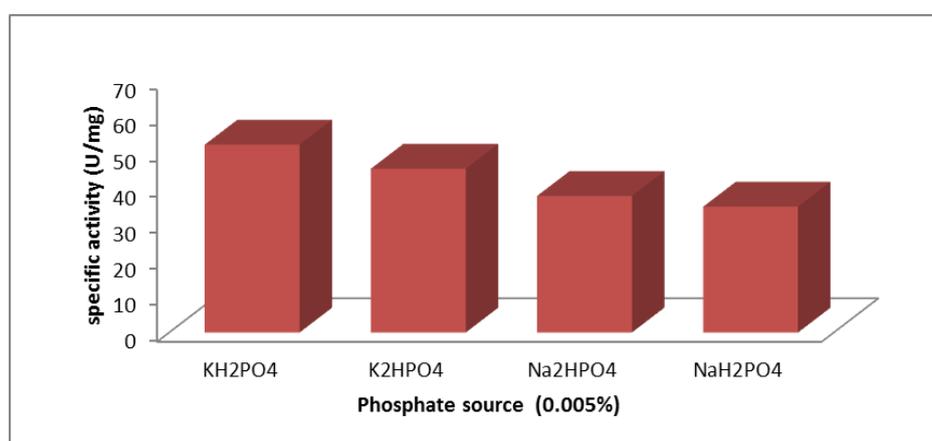


Figure (6): Effect of phosphate source on protease production by the *C. sakazakii* R4 incubation at 37°C for 24 hrs. in shaker incubator at 100 rpm.

The composition of nutritional substances and growth factors in the medium, represents the suitable environmental conditions for the microorganism. It controlled the rate of growth and

the final harvest.^[12] noticed that the best phosphate source for protease production from *Bacillus subtilis* was KH_2PO_4 .

Effect of phosphate source concentration

Optimum concentration of KH_2PO_4 for protease production by isolate *C.sakazakii* R4 was examined using various concentrations (0,005, 0.05, 0.1,0.15, 0.2, and 0.25%) of KH_2PO_4 for protease production. Results indicated in figure (7) show that maximum production of protease was obtained when KH_2PO_4 was used to supplement the.

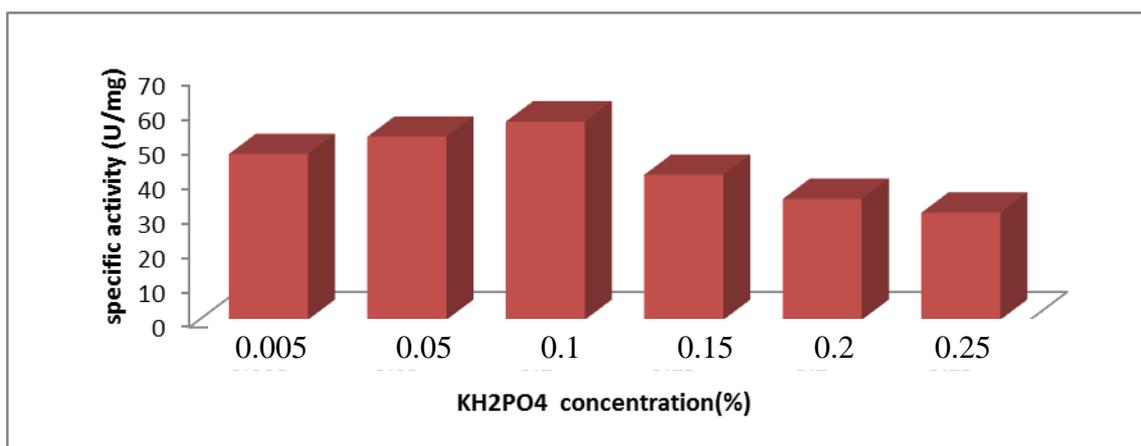


Figure (7): Effect of phosphate concentration on protease production by the *C. sakazakii* R4 after incubation at 37°C for 24 hrs. in shaker incubator at 100 rpm

production medium at a concentration of 0.1%.protease specific activity in culture filtrate reached 57.4U/mg at the above concentration. This suggests that scaling up of protease production by this organism could be done with 0.1% of KH_2PO_4 .

3.3.7 Optimum inoculum size

Six inoculum sizes were used to determine the optimum for protease production by the *C.sakazakii* R4 . Inoculum sizes of (10^2 , 10^3 , 10^4 , 10^5 , 10^6 , 10^7 , and 10^8 CFU) were used to inoculate the production medium. Results illustrated in figure (8) showed that production of protease by *C.sakazakii*R4 was affected by the inoculum size of microorganism. It was found that protease production was increased slightly with the increase of the inoculum size to 10^4 CFU, then it was decreased with the increase of the inoculum size. However, maximum protease production with 10^4 CFU and specific activity reached to 64.6 U/mg.

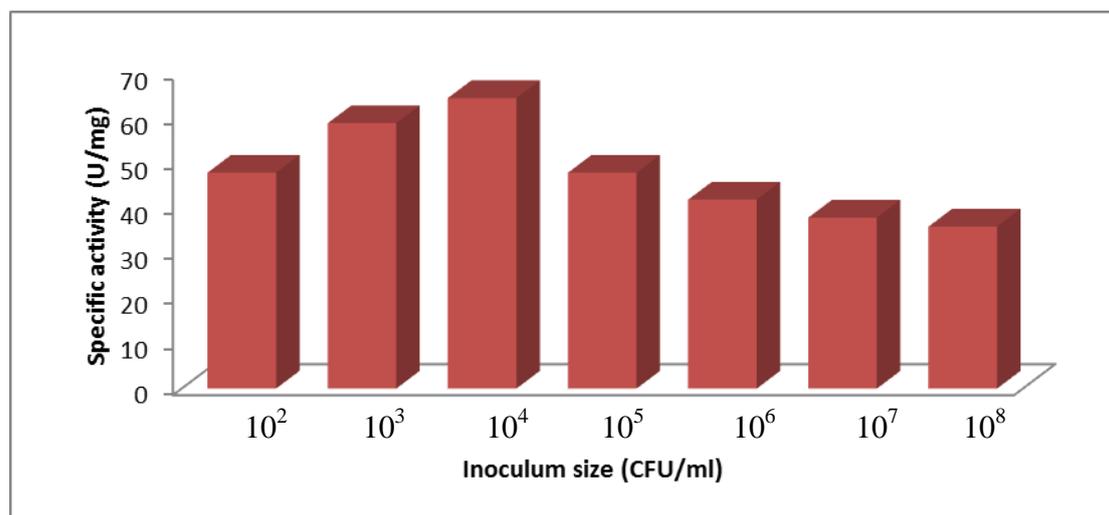


Figure (8): Effect of inoculum size on protease production by *C. sakazakii* R4 after incubation at 37°C for 24 hrs. in shaker incubator at 100 rpm.

Decreasing protease specific activity at higher inoculum size (more than 10^4 CFU) may be attributed to the crowding of cells, competitions on nutrients and increasing toxic secondary metabolites which inhibit growth cells, leading to a decrease in production of enzyme.^[13]

Optimum pH

In order to investigate the effect of the initial medium pH on protease production by the *C.sakazakii* R4, the production medium was adjusted to different pH values ranged between pH 4 and pH 9. Results mentioned in figure (9) showed that maximum protease production was obtained when the pH value of the production medium was adjusted to 7, at this value the enzyme specific activity in culture filtrate was 64.7 U/mg.

A decrease or increase in hydrogen ions concentration causes pH changes in the culture medium which may lead to drastic changes in the three-dimensional structure of proteins because H^+ and/or OH^- compete with hydrogen bonds and ionic bonds in an enzyme, resulting in enzymes denaturation.^[14] On the other hand, the effect of pH on enzyme production resulted from its role in the solubility of the nutritional substances and its effect on the ionization of the substrate and its availability to the microorganism, in addition to its effect on the stability of the produced enzyme, others mentioned that protease production depends on the initial and final pH of the culture medium.^[15]

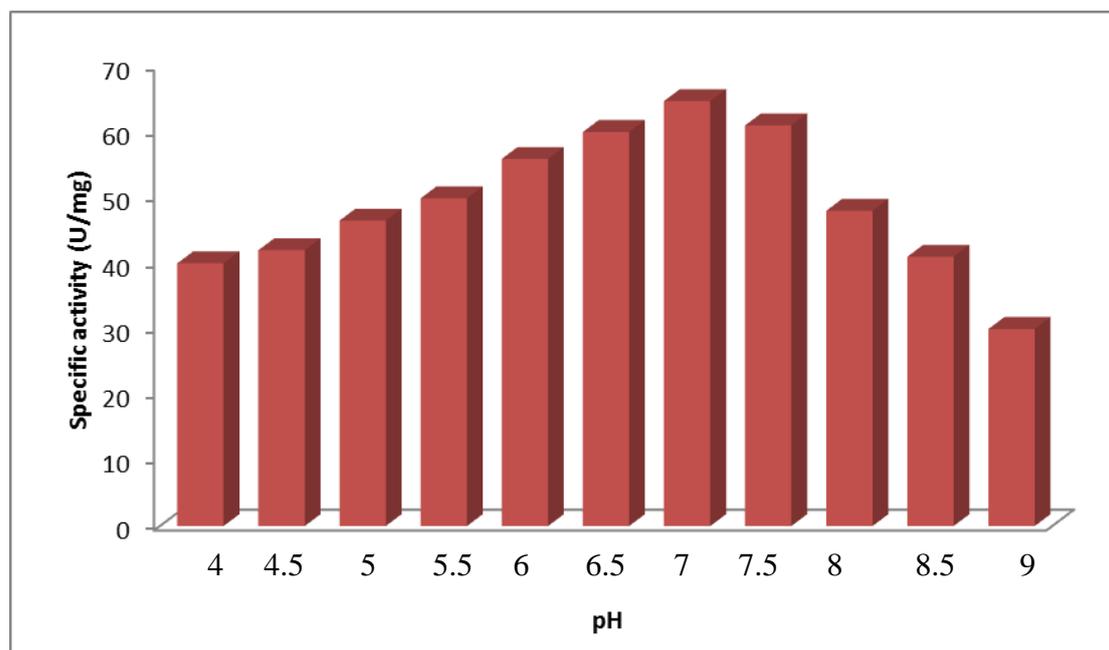


Figure (9): Effect of medium pH on protease production by the *C. sakazakii* R4 after incubation at 37°C for 24 hrs. in shaker incubator at 100 rpm

The optimum pH of culture medium was 6.9 and 7 for the production of protease by *Aeromonase hydrophilia*, while pH 8.0 appropriate for growth and biomass production.^[16,17] found that the appropriate range of pH used in the production of protease from bacteria *Yersinia. ruckeri* was 6.1 to 9.5, while the optimum pH for the production of the enzyme is 8.1. In a other study submitted by^[18] mentioned that the optimum pH for the protease production by *Aeromonas. sobria* reached 7.5.

Optimum incubation temperature

In this study different incubation temperatures (25, 30, 35, 40, 45 and 50°C) were used to determine the optimum temperature for protease production by the *C. sakazakii* R4. Figure (10) shows that the maximum production of protease was occurred when the microorganism was grown in the production medium and incubated at 30°C. Under these conditions, protease specific activity reached 71.4 U/mg.

For any enzymatic reaction, temperature below or above the optimal temperature will drastically reduce the rate of reaction. This may be due to the enzyme denaturation, or to losing its characteristics of three-dimensional structure. Denaturation of a protein involves the breakage of hydrogen bonds and other non-covalent bonds.^[14] Also, temperature affects

microorganism growth by oxygen solubility in the medium, kinetic energy of molecules, or the reaction velocity of molecules. So, all these factors may affect protease production.^[15,19]

Temperature has a significant effect on enzyme structure and enzyme production. High temperature affects metabolism of the living cells and may destroy many sensitive enzymes used in protease production. Furthermore, it may change enzyme structure and reduces its activity or by influencing extracellular enzyme secretion, possibly by changing the physical properties of the cell membrane.^[20]

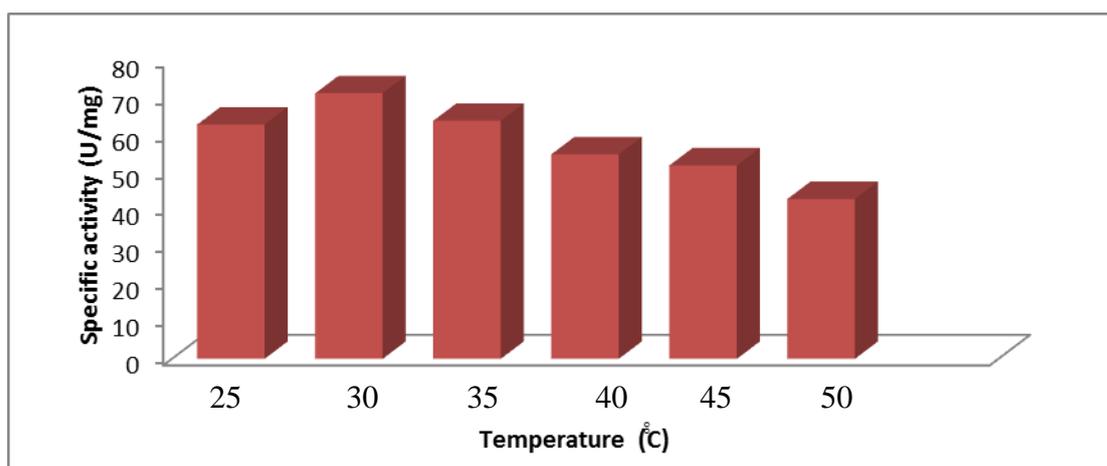


Figure (10): Effect of incubation temperature on protease production by the *C. sakazakii* R4 for 24hrs. in shaker incubator at 100 rpm

Effect of incubation period

Optimum protease production by *C.sakazakii* R4 was determined after different incubation periods (12, 24, 36, 48, 60, and 72hours). Results presented in figure (11) showed that enzyme production was initiated after the first 12 hrs of incubation with gradual increase in productivity with the increase in the incubation period. Enzyme specific activity reached 71.5U/mg after 24 hours of incubation, then it began to decline reaching 60.0U/mg after 42 hours of incubation. The decline after 42 hours of incubation might be referred to the adverse effect of the metabolism products in culture medium which were produced continuously throughout growth of bacteria.^[21] The researches found that optimum incubation period for extracellular protease production by *Bacillus* sp.^[22], was at 24 h. , and by *Bacillus clausii*^[23] showed that cell density increased with time and protease activity reached its maximum after 24hrs. of incubation.

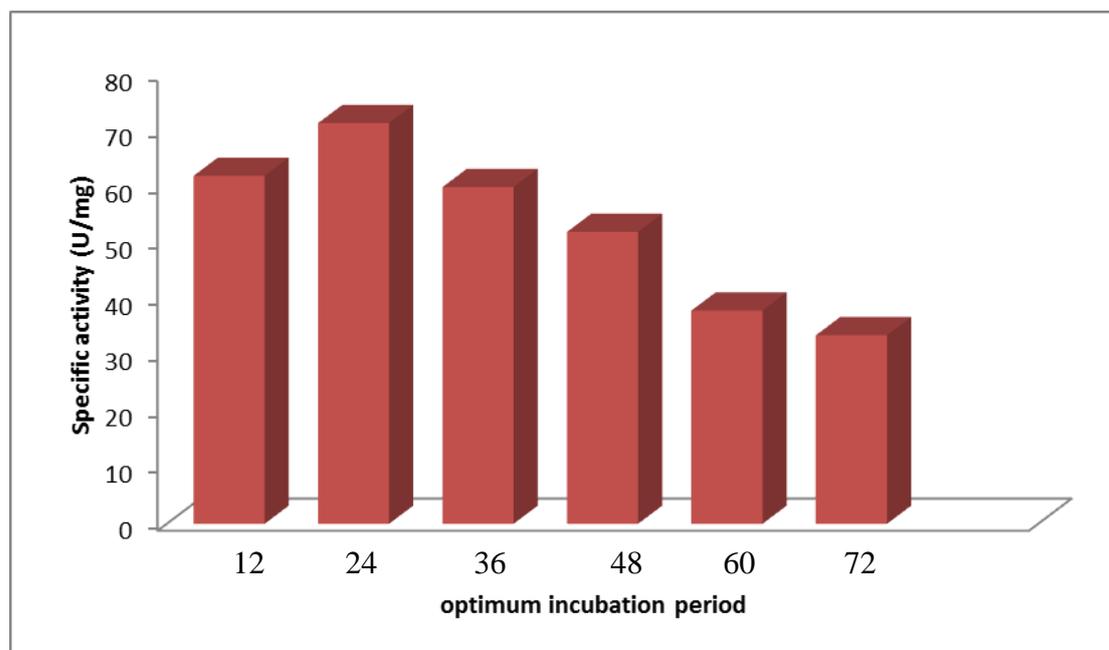


Figure (11): Effect of incubation period on protease production by the *C.sakazakii* R4 after incubation at 30°C in shaker incubator at 100 rpm.

Effect of agitation speed

Effect of agitation speed on protease production by *C.sakazakii* R4 was examined throughout incubation at different speeds of agitation (100, 140, 150, 200, and 250 rpm) in a shaker incubator. Results illustrated in figure (12) show an increase in the protease production and enzyme specific activity reached 80.4 U/mg when the culture medium was incubated in a shaker incubation at 150 rpm, then it began to decrease with the increase in the agitation speed. This may due to the moderate agitation speed that supplies growth medium with sufficient amount of oxygen for growth and production of different metabolites. This result is approaching other study which reported an increase in bacterial growth under shaking conditions compared to static. Maximum enzyme production observed when agitation speed at ranged between 100 to 200 rpm.^[24] achieved maximum enzyme product at 100-200 rpm.^[25] found that vigorous agitation suppressed enzymes production causing mechanical inactivation of the enzymes. Vigorous agitation seems to affect the catalytic activity of the enzymes

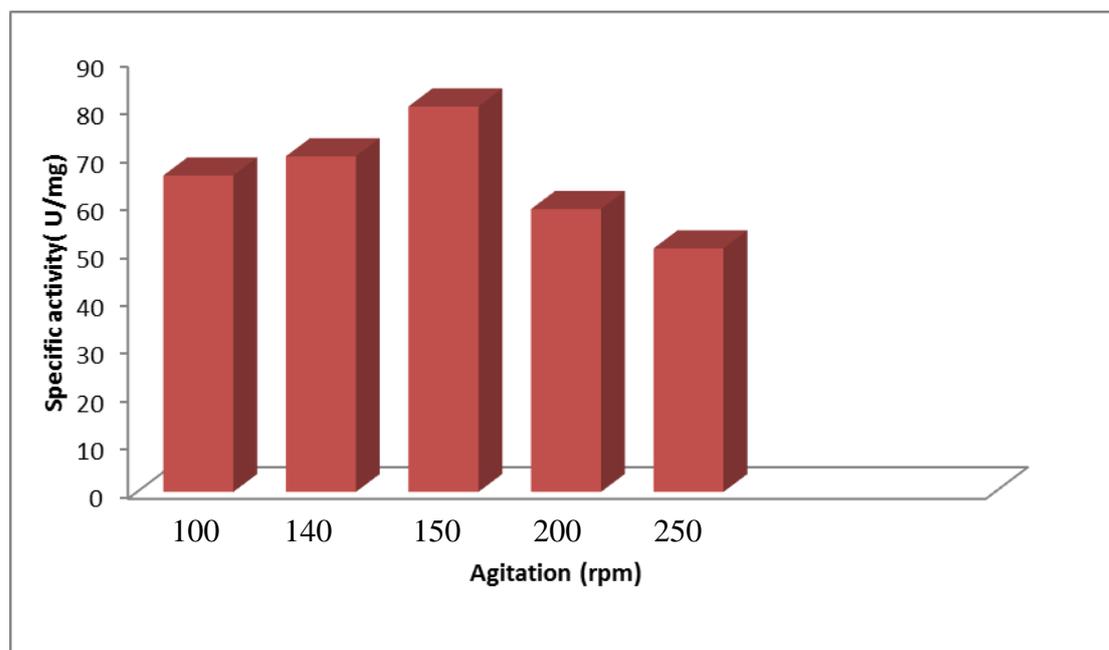


Figure (12): Effect of agitation on protease production by the *C. sakazakii* after incubation at 30°C for 24 hrs.

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