ISSN: 0067-2904

Optimum conditions for fibrinolytic enzyme (Nattokinase) production by *Bacillus* sp. B24 using solid state fermentation

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Abstract

The present study was aimed to screen the ability of local isolates of *Bacillus spp*. (56 isolates) for nattokinase production using solid state fermentation, then optimize the nutritional conditions for enzyme production. The isolates were subjected to the primary and secondary screening process to select the *Bacillus* isolate which give the highest production of enzyme. It was found that *Bacillus* sp. B24 had the highest productivity of the enzyme (25.58U/mg protein). The optimum conditions for nattokinase production were performed by the solid state fermentation and found that the wheat bran was the best medium at initial moisture ratio 1.0:1.0 (w/v) using distilled water as moisturizing solution with initial pH of 7.0 after inoculation with 5×10^7 cell/ml for 72 hrs, the enzyme specific activity was 38.65U/mg protein.

Keywords: *Bacillus* B24, Nattokinase, enzyme screening, optimization.

تحديد الظروف المثلى لانتاج الانزيم المحلل لليفين (الناتوكاينيز) باستعمال *Bacillus* sp. B24 بوساطة تخمرات الحالة الصلبة

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الخلاصة

هدفت هذه الدراسة الى غربلة العزلات المحلية لبكتريا *Bacillus* spp (56 عزلة) المنتجة لانزيم الناتوكاينيز بوساطة تخمرات الحالة الصلبة فضلا عن تحديد الظروف المثلى لانتاج الانزيم. خضعت هذه العزلات الى الغربلة الاولية والثانوية لاختيار العزلة الاغزر انتاجا للانزيم. وجد ان العزلة *Bacillus* sp. B24 امتلكت اعلى انتاجية للانزيم حيث بلغت الفعالية النوعية للانزيم25.58 وحدة/ملغم بروتين. كانت الظروف المثلى لانتاج الانزيم من العزلة المنتجة بوساطة تخمرات الحالة الصلبة باستعمال وسط نخالة الحنطة المرطبة بالماء المقطر بنسبة 1:1 (وزن/حجم) رقم هيدروجيني ابتدائي 7.0 وحجم لقاح 5×10^7 خلية/مل بعد فترة حضن 72 ساعة وتم الحصول على فعالية نوعية للانزيم 38.65 وحدة/ملغم بروتين.

Introduction

Nattokinase is a potent fibrinolytic enzyme, which primarily extracted from a Japanese fermented food named "natto" [1]. Fibrinolytic enzymes were produced from different types of microorganisms, and the most important among them was the genus *Bacillus* isolated from conventional fermented foods [2]. Fibrinolytic enzyme (nattokinase) has plasmin-like bio-characteristic that degrades fibrin in various pathways [3], it was studied by many researchers and reveals to have a high fibrinolytic activity than natural thrombolytic protease in the blood "plasmin" [4]. It has been used for more than 20 years in Japan [5], and it was demonstrated to possess effective fibrinolytic activity, which could be increased and prolonged in the blood plasma when taken orally [6]. Its catalytic center has 275 amino

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acids, and contains three conserved residues, Asp-32, His-64, and Ser-221, with molecular weight and isoelectric point were 27700 Dalton and 8.7, respectively. The enzyme gene sequence is analogous to those of other subtilisin family members (99.5% homology with subtilisin E, 86% with subtilisin BPN0, and 72% with subtilisin Carlsberg) [2,7,8]. Despite its name, NK is not a kinase enzyme, but a serine protease of the subtilisin family. In 1993, it was classified as a substilin-like serine protease, depend on its high sensitivity to protein substrate Suc-Ala-Ala-Pro-Phe-pNA for substilin. It shows a strong fibrinolytic activity thus comes under fibrinolytic enzymes also [8]. It has diverse advantages over the clinically used fibrinolytic agents like suitable oral administration, preventive and prolonged effect and stability in the gastrointestinal tract. Furthermore, NK can be absorbed from the intestinal tract and subsequently stimulate fibrinolysis [6], make it to be a potential clot-hydrolyzing agent for the treatment of a cardiovascular diseases. In addition to the main nattokinase works to support healthy blood circulation, it has many medical uses. Other clinically fibrinolytic agents, streptokinase and urokinase, are expensively, unstable in the intestinal tract as well as causing haemorrhage [9,10]. The aim of this study was to obtain an efficient local isolate of nattokinase producer *Bacillus* that produces an enzyme with high productivity, and then successful optimization of the enzyme production medium using solid state fermentation technique.

Materials and Methods

Source of bacterial isolates

In the present study, fifty six local isolates of *Bacillus* were used. These isolates were obtained ready from department of Biotechnology-College of science - University of Baghdad.

Maintenance of bacterial isolates

Bacterial isolates were cultured on a nutrient agar slants, then incubated for 24 hrs at 37°C and kept in the refrigerator at 4°C until use. The selected isolate was preserved by sub-culturing every four weeks.

Preparation of bacterial inoculum suspension

Isolates from nutrient agar stocks were inoculated into brain heart infusion broth and incubated for 24 hrs at 37°C before using for tests. After the incubation period, cells in the suspension were counted by heamocytometer and the cells suspension was diluted using the same broth until obtaining 2.5×10^7 cell/ml.

Screening the *Bacillus* isolates for nattokinase production

Primary screening (Semi-quantitative screening)

Primary screening using casein agar medium

All *Bacillus* isolates were screened for the production of nattokinase by plate assay using casein agar medium. Casein agar medium was prepared by dissolving casein (2.0 gm), peptone (0.5 gm) and agar (1.5 gm) in 90 ml distilled water, pH was adjusted to 7.0. The final volume was completed to 100 ml with distilled water; then sterilized by autoclaving at 121°C for 5 min. Then, the medium (20 ml) was distributed into a petri dish, and a well (8 mm in diameter) on a solidified plate was punched for sample application [11]. Fifty microliter of bacterial inoculum containing 2.5×10^7 cell/ml were inoculated on casein agar plate and then incubated at 37°C for 24 hrs. Colorless zone of casein hydrolysis around the well was an indication of nattokinase secretion.

Primary screening using fibrin plate medium

This assay was carried out according to Chang *et al*. with some modification [12]. The fibrin plate medium was prepared by dissolving 1.5 gm of fibrin in 40 ml of 0.2 N NaOH; pH was adjusted to 7.0 with 6 N HCl and the volume was then completed to 50 ml with distilled water which then sterilized by autoclaving. One gm of agarose was dissolved in 50 ml of 0.2 N NaOH (agarose was dissolved with heating). Fibrin solution and agarose solution were mixed and the medium (15 ml) was dispensed into sterilized petri dish, solidified, and the hole of 7 mm in diameter was made for sample application. Twelve isolates with high productivity based on casein hydrolysis were cultivated on fibrin plate. Twenty microliter of isolates inoculum consisting of 2.5×10^7 cell/ml were transferred into fibrin plate hole and incubated at 37°C for 24 hrs. Colorless zone was observed directly and the radius of a zone was measured.

Secondary screening (Quantitative screening) using solid state fermentation medium

Isolates with maximum productivity based on casein and fibrin hydrolysis were selected and cultivated on fermentation medium. Solid state fermentation medium composed of 5.0 gm of wheat bran and 5.0 gm of soybean meal contained in a 250 ml Erlenmeyer conical flask were humidified with 10 ml

of distilled water. After pH was adjusted to 7.0, the flasks materials mixed, cotton-plugged and autoclaved at 121◦C for 15 min. [13]. The fermentation medium was inoculated with 1.0 ml of 24 hrs old culture isolate with bacterial number of 2.5×10^7cell/ml and incubated at 37◦C for 48 hrs. After incubation, enzyme produced in each flask was extracted by addition of 100 ml of distilled water, and the mixture was placed in the rotary shaking incubator (190 rpm) for 1 hr. The crude extracts were centrifuged at 10,000 rpm for 30 min., and then the supernatant was filtered through Whatman filter paper No.1. The clear supernatant was considered as a crude enzyme and it was assayed for nattokinase activity by casein digestion method.

Determination of nattokinase activity

Method described by Senior (1999) was used to measure the activity of the nattokinase enzyme [14]. Casein solution (1.8 ml) was incubated in a water bath at 37◦C for 5 min., then 0.2 ml of enzyme solution was added to the substrate and incubated for 20 min. The reaction was stopped by adding 3 ml of ice cold TCA reagent and centrifuged at 10,000 rpm for 10 min. A blank was prepared using the same steps except the addition of TCA reagent into casein solution before the addition of enzyme solution. The amount of TCA-soluble products formed can be measured by reading the absorbency of the supernatant at 275 nm. using UV-VIS spectrophotometer.

Unit of enzyme activity was defined as the amount of enzyme that caused an increase of 0.01 in the absorbance at 275 nm. within one min. under the experimental conditions. Enzyme activity was calculated according to the following equation:

Enzyme activity (U/ml) = Absorbance at 275 nm. /0.01 × 20 × 0.2

Where:

0.01: Constant

20 : Reaction time (min.)

0.2 : Enzyme volume (ml)

Determination of protein concentration

Bradford method was used to measure the concentration of protein [15].

Determination of specific activity [16]

The specific activity of the enzyme was calculated as follow:

Specific activity (U/mg protein) = Enzyme activity (U/ml)/ Protein concentration (mg/ml)

Optimum conditions for nattokinase production

Optimum solid substrate

To investigate the effect of solid substrate on nattokinase production, four types of substrates were tested. These substrates included soybean meal, rice husk, wheat bran and soybean meal and wheat bran combination with ratio 1:1 (w/w). Ten grams of each agricultural wastes were prepared, and then inoculated with 1.0 ml of the selected bacterial inoculum containing 2.5×10^7cell/ml and incubated at 37 ºC for 48 hrs. After filtration, the volume of crude extract (clear supernatant) of each flask was measured and it was applied for nattokinase productivity according to the following equation:-

$$\text{Enzyme productivity (U/gm substrate)} = \frac{\text{Enzyme activity(U/ml)}\times\text{Volume of crude extract(ml)}}{\text{Amount of substrate (gm)}}$$

Optimum moisture ratio

Distilled water was added to the selected medium at different ratio 1:0.5, 1:1 and 1:2 (w/v). Media were autoclaved, inoculated with 1.0 ml of selected bacterial inoculum containing 2.5×10^7 cell/ml and incubated at 37 ºC for 48 hrs.

Optimum moisturizing solution

Distilled water and different mineral salt solutions (MS) included (%): MS-1 (0.5 NH_4NO_3, 0.2 KH_2PO_4, 0.2 $MgSO_4$ and 0.1 NaCl), MS-2 (0.1 Fe_3SO_4, 0.1 $MgCl_4$ and 0.1$MgSO_4$) and MS-3 (0.2 KH_2PO_4, 0.04 $CaCl_2$, 0.5 NH_4NO_3 and 0.02 $MgSO_4$) were added separately to the selected medium in moisturizing ratio 1:1 (w/v). Then autoclaved and inoculated with 1.0 ml of selected bacterial inoculum consisting of 2.5×10^7cell/ml and incubated at 37 ºC for 48 hrs.

Initial pH

To determine the optimum pH for nattokinase production, selected production medium was moistened separately with distilled water at pH values 5, 6, 7, 8 and 9 and phosphate buffer at pH

values 6, 7 and 7.5. Inoculum of 2.5×10^{7} cell/ml was added to each flask which then incubated at 37 ºC for 48 hrs.

Optimum inoculum size

The selected medium was inoculated with 1.0 ml of 24 hrs old culture isolate with different bacterial number (1×10^{6}, 1×10^{7}, 2.5×10^{7}, 5×10^{7} and 1×10^{8}cell/ml) and it was incubated at 37 ºC for 48 hrs.

Optimum incubation period

Different incubation times were examined to determine the suitable incubation period for nattokinase production. The selected medium was humidified with distilled water at optimum pH, inoculated with 1.0 ml of 24 hrs old culture isolate with bacterial number of 5×10^{7}cell/ml and incubated at 37 ºC for 24, 48, 72, 96, 120 and 144 hrs.

Results and discussion

Screening the *Bacillus* isolates for nattokinase production

Primary screening of nattokinase producing isolates

In this study fifty six bacterial isolates were obtained for the semi-quantitative method and inhibition zone assay. A simple method of screening was followed to determine NK producing bacteria on casein agar medium. The efficiency of bacterial isolates was compared by estimating the ratio between the colorless zone diameter around bacterial cultures (Z) and the growth culture diameter (G).

All *Bacillus* isolates were screened, fifty three isolates were nattokinase producer when clear zone of inhibition around the casein agar plate well was formed Figure-1A. The diameter of clear zone were different from one isolates to another, twelve *Bacillus* isolates were gave the highest clearance zone ratios (2.5-3.4), while the others were ranged between (1.1-2.4).

Another screening method was applied to detect nattokinase producing bacteria using fibrin plate medium. Twelve bacterial isolates with high productivity, selected from previous experiment, were cultivated on fibrin plate. Results showed that all twelve isolates were NK producer through the formation of clear zone in the medium Figure-1B.

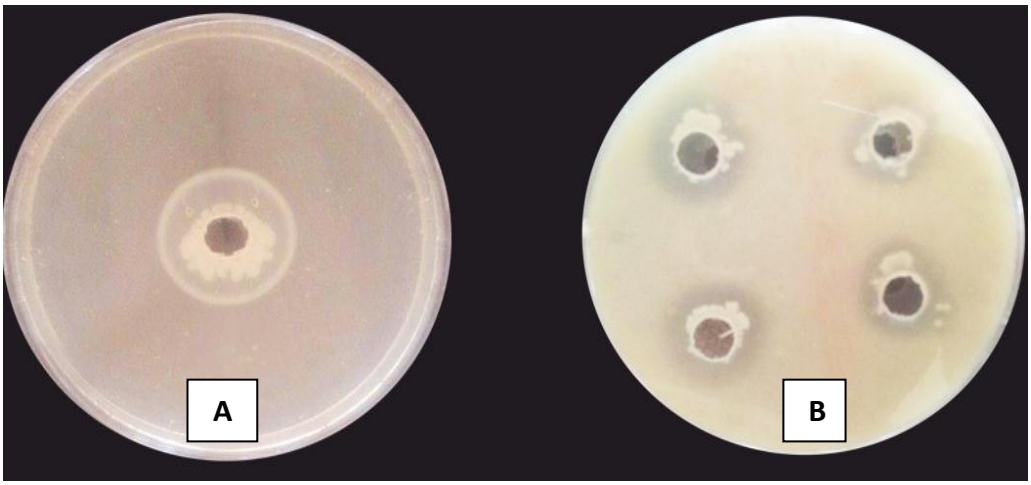

Figure 1- Proteolytic activity of *Bacillus* spp A: Casein hydrolysis B: Fibrin digestion

Padmapriya and Williams, (2012) screened forty bacterial isolates using casein agar plate medium, positive results for protease activity appeared only with twelve *Bacilllus* isolates. Of the twelve isolates, *Bacillus subtilis* was considered to be the best and high protease enzyme producing strain, it showed zone size of 2.98cm and standard culture zone size of 3.0 cm [17]. Raju and Divakar, (2013) screened five isolates of bacteria which were isolated from soil samples. These bacterial isolates were screened on fibrin plate medium, and all five isolates produced clear zone around the growth of bacteria [18].

Upon semi-quantitative screening for the highest NK producing isolates on casein agar medium and fibrin plate medium, twelve isolates were chosen for further screening (Quantitative screening) and named (B9, B17, B19, B24, B30, B33, B34, B36, B42, B45, B53 and B54) in order to choose an efficient *Bacillus* sp. isolate for NK production.

Secondary screening (Quantitative screening) of NK producing isolate

For more detection and meticulous selection of efficient bacterial isolate to produce NK, the twelve isolates of *Bacillus* spp. with maximum zone of hydrolysis in primary screening were screened again for their enzymatic activity using casein digestion method. Among twelve isolates, *Bacillus* sp. B24 gave the highest enzymatic activity, NK specific activity in crude supernatant was 25.58U/mg protein, whereas the NK specific activity for the other isolates were ranged between 2.63 and 24.32U/mg protein. Based on these results, the isolate *Bacillus* sp. B24 was selected to be used for further studies.

According to the results of primary screening and secondary screening it was exhibited that there were a clear differences in the efficiencies of the nattokinase production from various sources of microbial isolates were observed, these differences refer to the isolates nature and its genetic background [19].

Optimum conditions for nattokinase production

Optimum solid substrate

Different types of solid substrate were examined for their efficiency in nattokinase production, these substrates included soybean meal, rice husk, wheat bran and soybean meal and wheat bran combination with ratio 1:1 (w/w). Of all substrates, wheat bran was the best substrate for production of NK from *Bacillus* sp. B24. The specific activity of NK using wheat bran was 33.33U/mg protein with productivity 190.9U/gm substrate, while the specific activity of NK using soybean meal, rice husk and soybean meal together with wheat bran were 5.27, 15.94 and 26.14U/mg protein with productivity 22.15, 16.23 and 174.68U/gm substrate, respectively as shown in Figure-2.

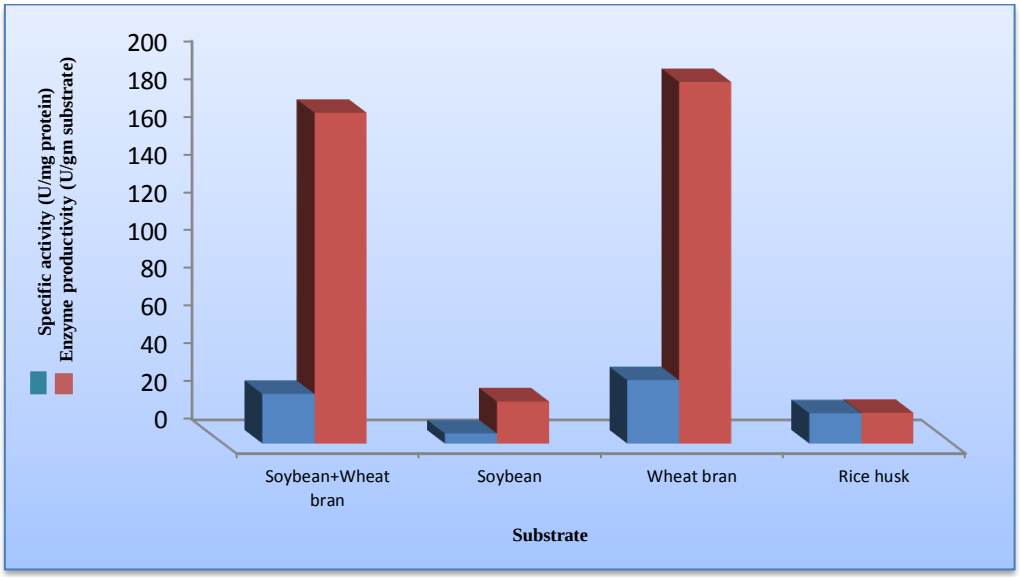

Figure 2- Effect of different types of substrate on nattokinase production from *Bacillus* sp. B24 using solid state culture after incubation at 37˚C for 48 hrs.

According to results, the maximum enzyme production was gained by using wheat bran and was therefore selected to be used for improving NK production as solid substrate. This substrate is rich in different nutrients such as proteins, vitamins, functional compounds and lipids [20], it also supplied compounds, which can stimulate the production of required biomolecules [21]. Beside being wheat bran a substrate and a simple, it composed of co-product of agricultural origin unpolished that contain all the nutrients needed for the microorganism growth [22].

Akcan and Uyar (2011) found that lentil husk was the best substrate for extracellular alkaline protease production among different agro-industrial residues (rice husk, lentil husk, wheat bran, cotton stalk, millet cereal, crushed maize) using *Bacillus subtilis* RSKK96 in SSF with specific activity 3937U/mg [23].

3.2.2 Optimum moisture ratio

Impact of moisture ratio used for moistening of enzyme production media was appraised and the results were documented in Figure-3. The production medium was moistened with different volumes of distilled water (1:0.5, 1:1 and 1:2 w/v), it was found that moistening of media with moisture ratio of 1:1 (w/v) supported maximal NK specific activity 34.92U/mg protein. When moisture ratio of 1:0.5

was used, only 10.92U/mg protein of NK could be obtained. Whereas, a specific activity of 29.52U/mg protein could be obtained when 1:2 (w/v) moisture ratio was used.

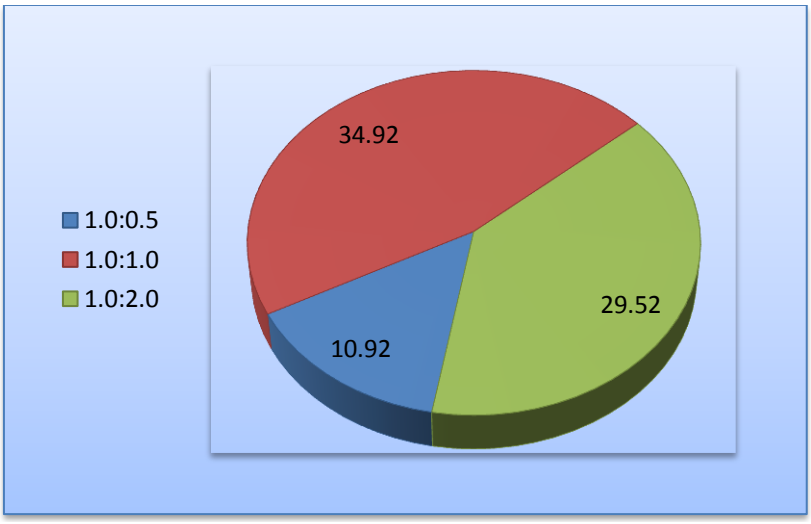

Figure 3- Effect of different moistening ratio on nattokinase production from *Bacillus* sp. B24 using solid state culture after incubation at 37˚C for 48 hrs.

In solid state fermentation, the moisture level has a great effect on the physical properties of the substrate. Most of solid substrates used in solid state fermentation are indissoluble in water hence, water will have to be absorbed onto the substrate particles, which can be utilized by the microorganisms for its growth and metabolic activity. Thus, the hydration degree of the substrate plays a crucial role on the bacterial growth and consequently the enzyme production [24]. Moreover, water causes the swelling of the solid substrate and promotes good consumption of substrates by the microbial cells. Increasing moisture ratio is thought to have reduced the substrate porosity, consequently limiting the O_2 transfer into the substrate [25, 26]. Otherwise, a decreasing moisture level leads to lower degree of swelling, solubility reduction of the nutrients of the substrate and a higher water tension [27].

The optimum moisture ratio for fibrinolytic enzyme production from different *Bacillus* species had been determined such as, a moisture ratio 1:2 w/v which was used to produce the alkaline protease from thermoalkalophili *Bacillus* SP.JB-99 in which enzyme specific activity 4200U/gm (Shivasharana and Naik, 2012) [28]. Furthermore, Akcan and Uyar (2011) found out that the optimum initial moisture level for extracellular alkaline protease production using *Bacillus subtilis* RSKK96 was 30% in which enzyme activity was more than 3500U/mg [23].

Optimum moisturizing solution

The type of moisturizing solution was also examined to investigate the best moistening solution for NK production. Distilled water and a variety of mineral salt solutions were tested. Distilled water gave the highest specific activity 35U/mg protein, whereas MS-1, MS-2 and MS-3 gave 16.93, 23.28 and 22.79U/mg protein, respectively Figure-4.

The reason that the production increases when using distilled water may be due to the substrate (wheat bran) in the production medium which contains a lot of nutrients that are sufficient for the production of the enzyme without needing for its addition or increase its concentration in the moisturizing solution, compared with distilled water. In addition Pandey *et al*. (1999) mentioned that, the water has an impact on physico-chemical substrate properties, which in turn affect enzyme production [29].

During alkaline protease production with thermoalkalophilic *Bacillus* SP.JB-99, different moistening solutions were used (chemically defined medium, tap water, different mineral salt solutions (MS) and distilled water). A mineral salt solution MS-1 (0.5 NH_4NO_3, 0.2 KH_2PO_4, 0.2 $MgSO_4$ and 0.1 NaCl) produced maximum alkaline protease activity (5200U/gm) (Shivasharana and Naik, 2012) [28].

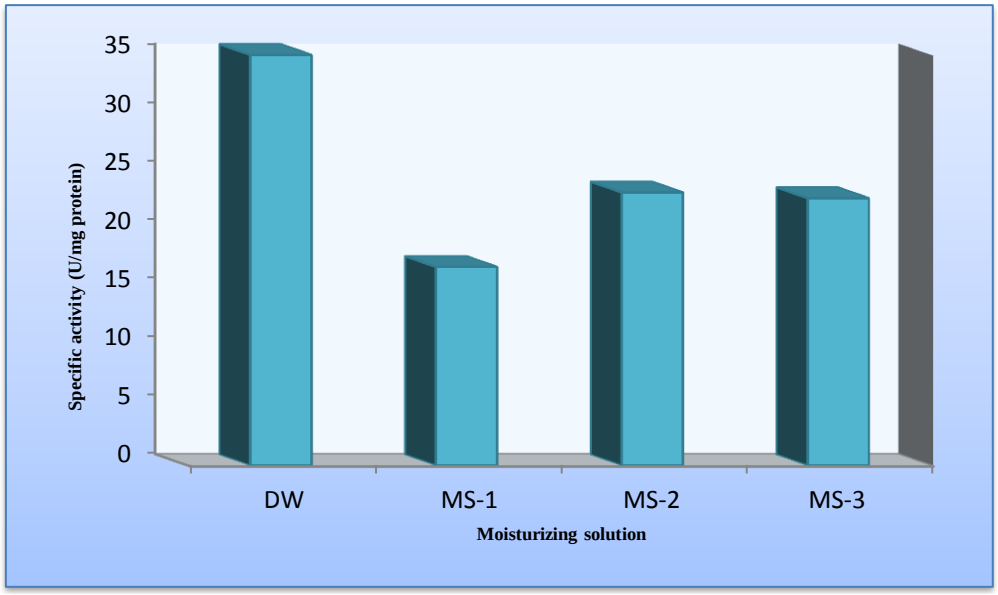

Figure 4- Effect of different moisturizing solution on nattokinase production from *Bacillus* sp. B24 using solid state culture after incubation at 37˚C for 48 hrs.

Initial pH of the medium

To study the effect of the initial pH on NK production, *Bacillus* sp. B24 was grown on production medium with different pH values. pH of medium was adjusted to 5, 6, 7, 7.5, 8 and 9. The optimum pH for NK production was observed when medium moistened with distilled water at pH 7.0, moistening of medium with distilled water gave higher rate of NK productivity (35.3U/mg protein) than phosphate buffer (11.07U/mg protein) in pH 7. Furthermore, it was observed that decrease in NK production supported by initial pH 6, 7.5 and 8. Sharp decrease happened in acidic pH (5) and alkaline pH (9) Figure-5.

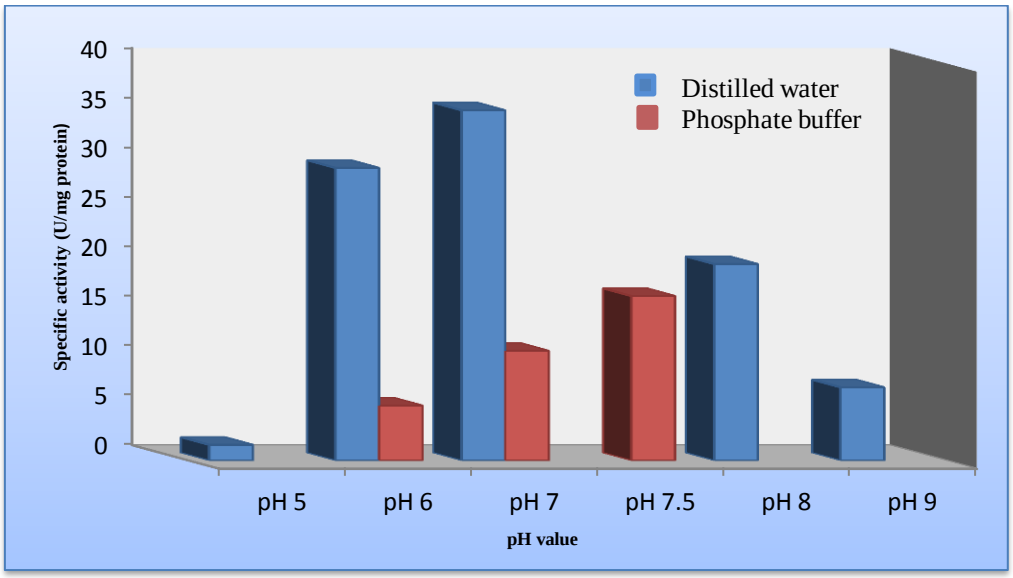

Figure 5- Effect of pH on nattokinase production from *Bacillus* sp. B24 using solid state culture after incubation at 37˚C for 48 hrs.

Generally, the effect of pH in enzyme production is attributed to its role in the solubility of the medium nutritional substances, its influence on the substrate ionization and its availability for the microorganism, in addition to its influence on the enzyme stability [30]. On the other hand, pH of the culture media often influence the fermentation course and enzyme production rate, since pH of the medium drastically affects the conformation of the plasma membrane, and subsequently affects the membrane bound ribosomes involved in protein synthesis [31].

The result of this study is consistent with the result of Siraj (2011), who found that highest production of nattokinase from the *Bacillus subtilis* was 7.0 [32]. Kumar *et al.* (2015) also found that optimum pH for fibrinolytic protease production from *Bacillus circulans* was 7.0 in which activity was 1.74U/ml after 36 hrs [33].

Optimum inoculum size

Optimal inoculum level required for maximum NK production by *Bacillus* sp. B24 was detected by inoculating the production medium with different levels of inocula and assaying enzyme productivity. From the results presented in Figure-6, it was apparent that 5×10^7 cell/ml inoculum level was optimal since maximal specific activity (38.65U/mg protein) was obtained when compared to other levels tested. Levels below and above 5×10^7 cell/ml showed a slightly decline in the NK production.

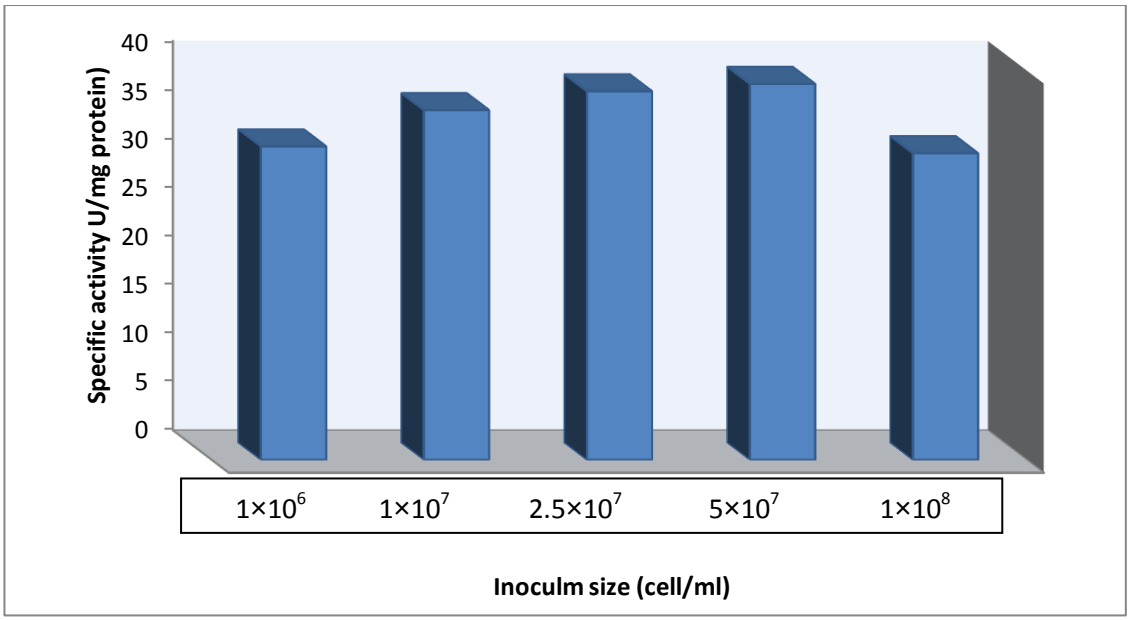

Figure 6-Effect of inoculum size on nattokinase production from *Bacillus* sp. B24 using solid state culture after incubation at 37˚C for 48 hrs.

The exception of the optimum inoculum level, the enzyme productivity descended away either sides this may be due to high inoculum size and rapid growth, resulting in a higher rate of oxygen and substrate consumption, a phenomenon that was named as "fast metabolism". Hence, higher inoculum size lead to a decrease in enzyme production. Whereas at low inoculum size, the cells needs longer time to multiply to adequate number in order to utilize the substrate and produce enzyme [39]. On the other hand, an increase in the size of inoculum would ensure a rapid propagation and biomass synthesis. In spite of that, after a certain limit, enzyme production could decline because of exhaustion of nutrients due to the increased biomass, which would result in a decline in activity of the enzyme [40]. Hence, in the present study optimum requirements for inoculum levels were determined.

In other studies, various suitable inoculum size were used to produce fibrinolytic enzyme from different *Bacillus* species such as, the inoculum size 1×10^5cell/g wet weight was the optimum size for production of fibrinolytic enzyme from *Bacillus lichniformis* B4 with an activity of 19.185U/ml [41]. Whereas inoculum size 2.0% was the optimum size for the production of fibrinolytic protease from *Bacillus cereus* GD55 on which the activity was 46.24 ± 0.98U/gm [42].

Optimum incubation period

The optimum incubation period was studied in order to detect the bacterial growth phase in which the NK was produced, therefore different incubation times (24, 48, 72, 96, 120 and 144)hrs were used. From the results Figure-7, it was observed that NK production commenced during the first 24 hrs and the specific activity was enhanced after 48hrs and reach to maximal specific activity after 72 hrs of incubation, which was 38.68U/mg protein. Whereas after 96 hrs of incubation the specific activity was decreased with increasing the incubation, this may be due to the change in the conditions of culture along this periods such as diminishing of oxygen, nutrients and accumulating of toxic metabolites which inhibit the bacterial growth. Some studies have demonstrated that the production of the enzyme starts in the early stages of microbial growth [34, 35].

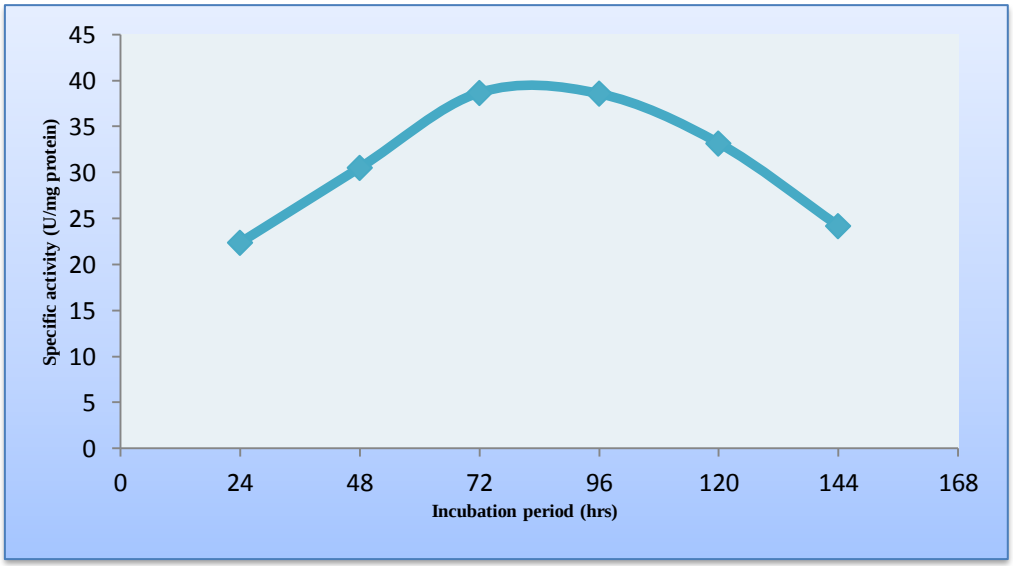

Figure 7- Effect of incubation period on nattokinase production from *Bacillus* sp. B24 using solid state culture after incubation at 37˚C.

Similar result was obtained by Raju and Goli, (2014), who found that highest level of fibrinolytic protease production from *Bacillus cereus* GD55 was occurred at 3 days of fermentation [36], while optimum nattokinase production by *Pseudomonas* spp. TKU015 was observed during 60 hrs (Wang *et al.*, 2009) [37]. Pouryafar *et al.* (2015) reported that optimum alkaline protease production by *Bacillus licheniformis* ATCC 21424 was observed by 48 hrs of incubation [38].

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