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ISOLATION, PURIFICATION AND CHARACTERIZATION OF THE NATTOKINASE FROM *BACILLUS* SPECIES.

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ABSTRACT

Nattokinase is an enzyme that belongs to the class of subtilis. This is a serine protease containing about 275 residues of amino acid. This is found to be85% similar to Factor VII of the haemoglobin factor and got fibrin-like operation. Nattokinase finds its uses in pharmaceuticals, health welfare etc. as a thrombolytic enzyme, which is useful for treating blood associated illnesses and cardiovascular diseases. Crude nattokinase was partly purified by ammonium salt precipitation (40%). Protein assessment was conducted using the Lowry's method after dialysis and the enzyme assay. The overall protein content of the pure enzyme is 0.138 mg/ml and the typical activity was determined to be 0.53 U/ml/min. In order to affirm the purity of nattokinase, the HPLC showed 99.17 per-cent purity and we got their peals at RT 5.4 min.

Key words: Nattokinase, serine protease, thrombolytic enzyme, HPLC, Cardiovascular diseases.

INTRODUCTION

Nattokinase is an enzyme whose origin dates back to the later part of the Edo period (1600-1868) in Japan. Natto is the product of normal soya bean fermentation by Bacillus in the straw in which the soya beans were wrapped. The botanical source for nattokinase is glycine and it appears as a fine yellow white powder **[1].**

The bacterium of the bacillus includes a fermentation cycle that gives the beans their famously sour scent, nutty flavour and slippery texture. Bacillus natto is rod shaped bacteria with a relatively high heat resistance. Compared to other bacteria with maximum tolerance of 20°C-30°C, Bacillus subtilis natto has a heat tolerance of 40-50°C **[2,3].**

Nattokinase, an active ingredient isolated from natto, is a naturally occurring enzyme with 20,000 ± 500 Daltons molecular weight **[4]**. This is a heat stable at 60°C with a pH solution spectrum of 6-12. Although the human body has several types of thrombi-creating enzymes, it uses only one plasmin to decompose and dissolve thrombi **[5,6]**.

The characteristics of nattokinase closely mimic those of plasmin. Nattokinase, isolated from natto, is a fibrinolytic enzyme that essentially breaks down fibrin strands and keeps together the thrombi of this fibrin. Nattokinase has a protein structure composed of a long polypeptide chain composed of 275 residues of Ala at the N-terminal **[7-9]**.

This hydrolyses fibrin vey powerfully. Nattokinase is the most active fibrinolytic enzyme present in approximately 200 foods testes for oral fibrinolytic therapy **[10]**. Nattokinase has a number of nutritional features, which are often referred to as mega safe food **[11]**. It is known to have a high fibrinolytic function that aides in cardiovascular diseases and few more **[12,13]**. Medically, it has the ability to combat muscle spasms, chronic fatigue syndrome, cardiac disease, heart attack, angina (chest pain) elevated blood pressure arteriosclerosis (hardening of the arteries), deep vein thrombosis, haemorrhoids etc. **[14-16].** Nattokinase has a high homology to subtilisin enzymes and DNA sequencing reveals 99.5 and 99.3 per-cent homology to subtilisin E and amylosacchariticus, respectively **[17-20].**

MATERIALS AND METHODS

Collection of samples:

Samples have been obtained from various sites. Soil collected near the poultry field from Vibhuti khand, Gomti Nagar, soup stick made up of soya flour collected from shatabdi express and soybean pulses& nuggets taken from home **[21].**

Isolation of nattokinase producing bacteria from samples:

Originally, the samples obtained were serially diluted in 0.85 per-cent NaCl solution and then scattered over nutrient agar plates to achieve a variety of bacterial colonies **[22]**. Through streaking, these colonies were turned into pure cultures **[23]**. Colonies have been chosen on the basis of numerous morphological studies. The pure cultures were tested for nattokinase activity by conducting casienolytic and haemolytic activity **[24]**.

Biochemical characterization of culture:

For strain identification, Gram Staining and few other biochemical tests were performed defined by Bergeys manual **[25]**.

Selection and optimization of of production media:

Four separate production media have been chosen and screened on the basis of best bacterial growth. Further the selected media was optimized using one factor at a time **[26]**.

Table 1: showing composition of all production media

S. No.	Constituent	Quantity
Production media 1		
1.	Glucose	10 g/l
2.	Yeast extract	10 g/l
3.	K_2HPO_4	01 g/l
4.	$MgSO_4$. $7H_2O$	0.5 g/l
Production media 2		
1.	Glucose	1%
2.	Peptone	5.5%
3.	$CaCl_2$	0.5%
4.	$MgSO_4$	0.2%
Production media 3		
1.	Maltose	20 g/l
2.	Peptone	10 g/l
3.	K_2HPO_4	2.0 g/l
4.	$MgSO_4$	1 g/l
5.	Yeast extract	10 g/l
6.	Glucose	2.0 g/l

Production media 4		
1.	Beef	3.5 g/l
2.	Tryptone	5.5 g/l
3.	NaH_2PO_4	3 g/l
4.	$MgSO_4$	0.2 g/l
5.	$ZnSO_4$	0.1 g/l
6.	NaCl	5 g/l

Fermentation and purification of nattokinase:

The fermentation medium was designed for the processing of nattokinase. The shake flask **[27]** process was used for the processing of nattokinase with additional salt precipitation (40per-cent ammonium sulphate) and dialysis was executed for purification purpose **[28]**.

Estimation of the protein concentration in nattokinase:

The concentration of the protein was estimated by performing the Lowry's method **[29]**.

Enzyme assay for nattokinase:

The casienolytic assay is used to quantify the enzyme. Once the substrate added to enzyme, then incubated at 37C for 15 min, additional TCA (trichloroacetic acid) was added to avoid the reaction and then alkaline copper sulfate was added then OD was taken at 680 nm in spectrophotometer after adding folins reagent **[30**.

HPLC chromatogram analysis of nattokinase:

The samples were firstly dissolved in solvents, and then allowed to float under high pressure into the chromatographic tube. In the column, the sample is separated into its components **[31]**.

RESULTS AND DISCUSSIONS

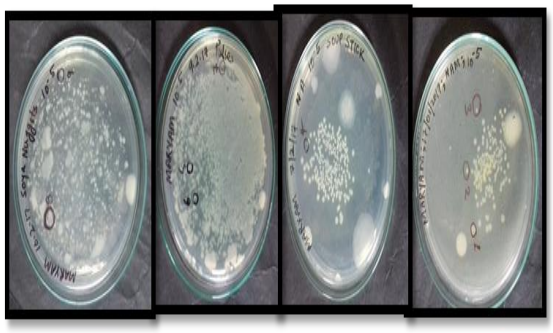
Sample collection:

Samples were collected from different places which have the possibility of having nattokinase activity.

Figure 1: Collected samples.

Isolation of the microbes by serial dilution and agar plating method:

Microbes from different samples were isolated by serial dilution and spread plating technique. Mixed cultures were obtained after spreading as shown in fig. below.



a) poultry soil sample b) soup stick sample c) soybean pulses sample d) soya nugget sample

Figure 2: Mixed culture plates bacterial colonies isolated from a) poultry soil sample b) soup stick sample c) soybean pulses sample d) soya nugget sample

Colony morphology

The cultures obtained from different samples were differentiated on the basis of their morphology. The physical characteristics of the colonies such as shape, margin, elevation, pigmentation, texture and opacity of each colony obtained from the mixed cultures were studied.

Table 2: The morphology of all the selected colonies

Culture	Shape	Margin	Elevation	Texture
C-1	Irregular	Entire	Flat	Hard
C-2	Circular	Entire	Raised	Soft
C-3	Circular	Entire	Raised	Soft
C-4	Irregular	Discrete	Flat	Soft
C-5	Circular	Entire	Raised	Soft
C-6	Circular	Entire	Flat	Soft
C-7	circular	Entire	Flat	Soft
C-8	Irregular	Entire	Raised	Soft
C-9	Irregular	Discrete	Flat	soft

Culture Name	Surface	Pigmentation	Opacity
C-1	Rough	Off- white	Opaque
C-2	Smooth	Light orangish	Translucent
C-3	Smooth	Orangish	Translucent
C-4	Smooth	Off- white	Opaque
C-5	Smooth	Off- white	Translucent
C-6	Smooth	White	Translucent
C-7	Smooth	Off- white	Opaque
C-8	Smooth	Off- white	Opaque
C-9	Smooth	Off- white	Opaque

Pure culture:

Pure cultures were obtained by discontinuous and continuous streaking of the selected colonies on the nutrient agar plates as shown below.

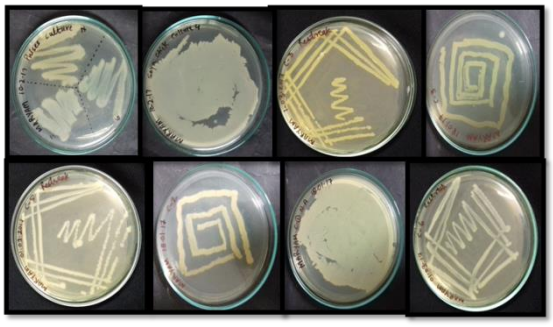


Figure 3: Pure culture plates

Screening of pure culture:

Pure cultures obtained were screened for the nattokinase activity. For this, the pure cultures were streaked in sterilized casein agar media plate. And after the incubation at 37°C for 48 hours the potential activity of the cultures by examining the core zone of hydrolysis. Culture-5 shows best result after 2° screening in which good zone of hydrolysis appears. Hence, it is selected for further optimization.

Table 3: shows the remark of the cultures after primary and secondary screening. Secondary cultures showed that Culture 5 has maximum core zone of hydrolysis.

Culture No.	1° Screening	2° Screening
C-1	-	-
C-2	+	-
C-3	++	++
C-4	+	-
C-5	+++	+++
C-6	++++	++
C-7	+++	-
C-8	++	-
C-9	+	-

Identification of bacterial culture:

From the screening result it became clear that culture-5 has maximum nattokinase activity. Further identification was done by different techniques in accordance with Bergey's manual as mentioned below:

Table 4: Biochemical characterization for the identification of culture -5.

S. No.	Test	Result
1.	Gram Staining	**+**
2.	Endospore Test	**+**
3.	Mannitol test	**-**
4.	VP-Test	**+**
5.	MR test	**-**
6.	Catalase test	**+**

From the results above, and in accordance with Bergey's Manual, it can be concluded that the microbe may be ***Bacillus subtilis.***

Selection of production media (72 hrs.):

On the basis of the bacterial growth production media 3 was selected for the optimization and fermentation purpose.

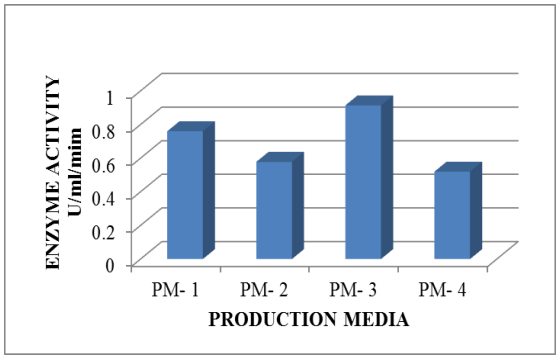


Figure 4: graph showing effect of production media on enzyme activity

Optimization of production media:

The production media used is optimized according to the need of the culture so as to get the best result for the growth and enzyme activity. Different physical and chemical factors affecting production were changed such as nitrogen source, carbon source, pH etc. After incubating the culture in optimized media for 48 hrs, O.D was taken and compared with standard graph. As a result, best nitrogen and carbon sources were discovered along with best working pH and temperature.

(A) Effect of different nitrogen sources:

In this, production media was varied by testing the nitrogen sources and beef extract came out to be the best nitrogen source for the bacterial culture. **Figure 5** shows the O.D measured and activity of the bacterial culture.

Table: Effect of different Nitrogen sources Varied in the Production Media

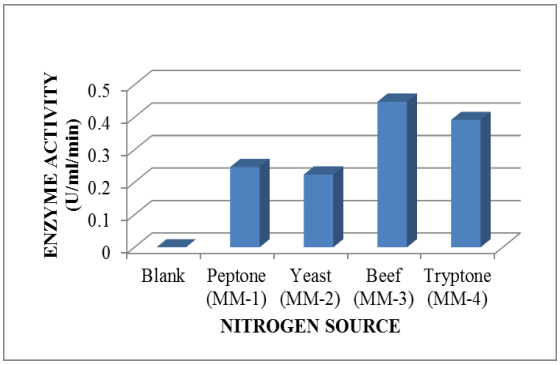


Figure 5: Graphical representation of effect of nitrogen source on enzyme activity

(B) Effect of percentage of beef as nitrogen sources:

Different % of beef extract was tested and O.D was measured. 2.5% of beef extract gave good results with fibrin as substrate for bacterial culture.

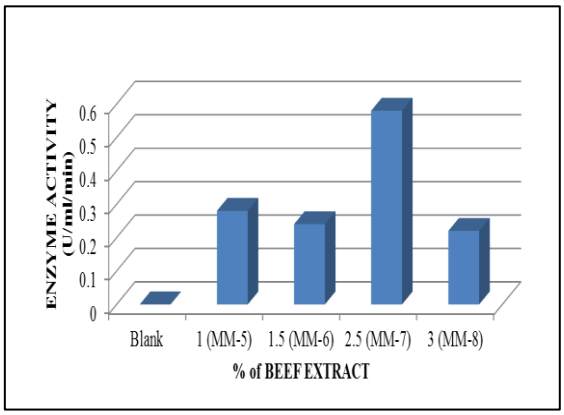


Figure 6: Graph showing effect of % of beef extract on enzyme activity

(C) Effect of different concentration of carbon sources:

In this, production media was varied by testing the carbon sources and sucrose came out to be the best carbon source for the bacterial culture.

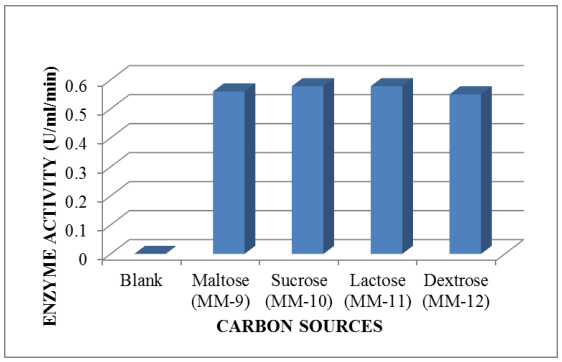


Figure 7: Comparative analysis of carbon source on enzyme activity

(D) Effect of % of carbon sources:

Different % of lactose was tested and O.D was measured and original concentration i.e. 22g/l came out to be best with fibrin as substrate for bacterial culture.

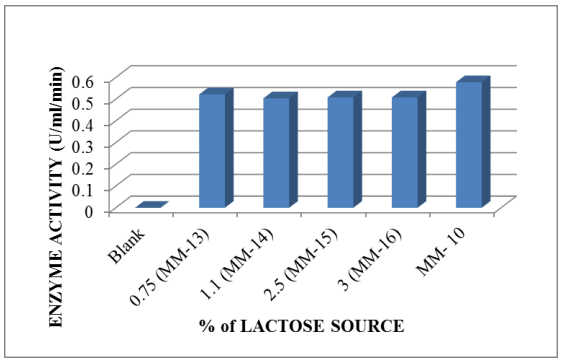


Figure 8: Graph showing effect of % of lactose on enzyme activity

(E) Effect of different types of metal ions:

On varying the metal ions, original concentration of Mg^{2+} was found to be best for the bacterial culture.

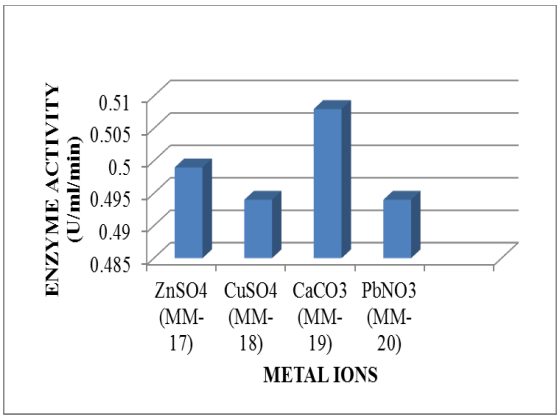


Figure 9: Graphical analysis of metal ions on enzyme activity

(F) EFFECT OF DIFFERENT pH ON THE PRODUCTION MEDIA

Inoculate the culture in the flask for above optimised media at pH= 5,7,9 and 11. Measure O.D at 680 nm. pH 7.08 gave the best results which was the original pH.

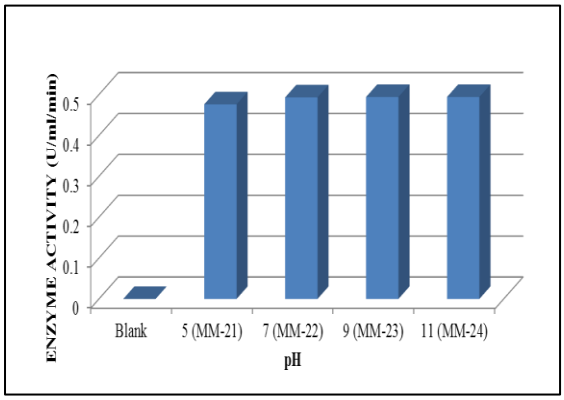


Figure 10: Graph expressing effect of pH on enzyme activity

Fermentation and downstream processing:

Fermentation is done using shake flask method in the optimised media under optimum conditions. After fermentation, centrifuge the crude enzyme and collect the supernatant and store it at 4°C. Total supernatant measured was 34 ml. The crude enzyme obtained after precipitation was salt precipitated. Once salt precipitation is completed, centrifuge the enzyme and dissolve the pellet in Tris buffer of pH= 7. 6ml of enzyme in Tris buffer was obtained. Dialysis is done in order to remove the salt present in the enzyme.

Estimation of protein content by Lowry's method

Once standard graph is plotted, Lowry's assay for salt precipitated crude enzyme and dialysis protein is performed. The reading is compared with the Lowry's standard graph and total protein concentration is determined. Figure 11 below shows the protein content of the precipitated and crude enzyme.

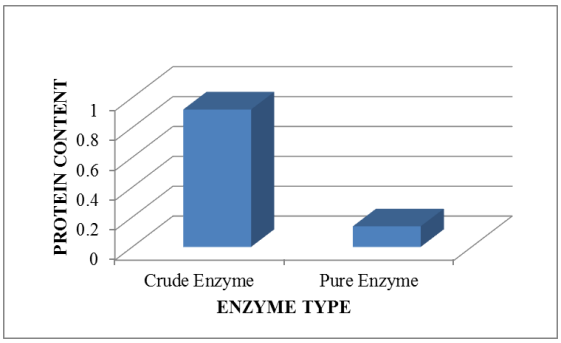


Figure 11: Graph comparing the protein content of crude and pure enzyme

Assay of Nattokinase enzyme

Assay is done in order to find out the activity of different form of enzyme like Crude salt precipitate and pure enzyme. The reading is then compared with the standard graph and enzyme activity is calculated.

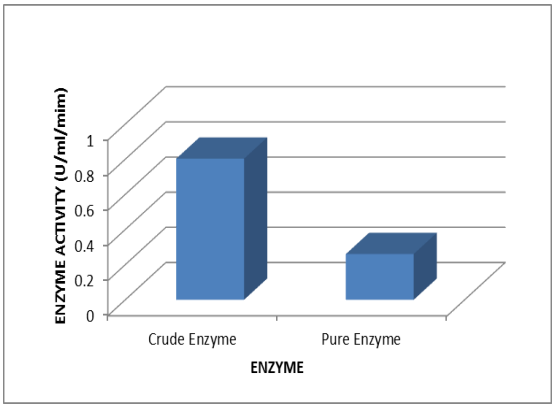


Figure 12: Graph associating the result of pure and crude enzyme

Characterization of Nattokinase:

(A) Effect of different temperature on the enzyme activity

To check the activity at different temperature, the enzyme with substrate was incubated at different temperature like 37°C, 22°C, 55°C and RT for 15 min.

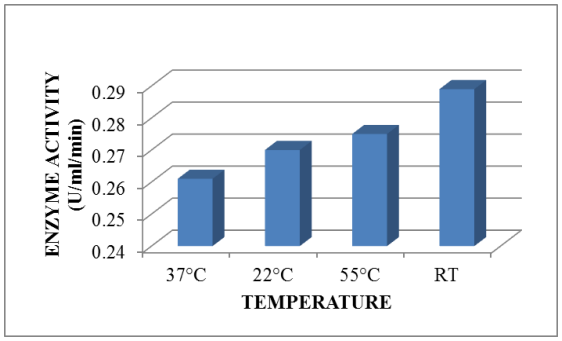


Figure 13: Graph showing effect of temperature on enzyme activity

(B) Effect of pH on enzyme activity

To check the activity at different temperature, the enzyme with substrate was incubated at different temperature like 5,7,9 and 11

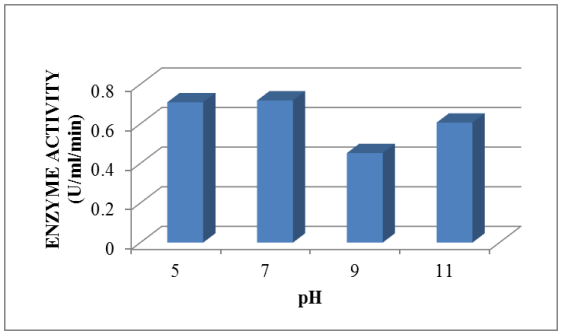


Figure 14: Graph showing effect of pH on enzyme activity

(C) Effect of activators on the enzyme activity

Activators are known to increase the efficiency of enzyme. $MgSO_4$ and $CaSO_4$ are used as activators to check the activity of enzyme. Incubate the enzyme with substrate for 15 min at 37°C.

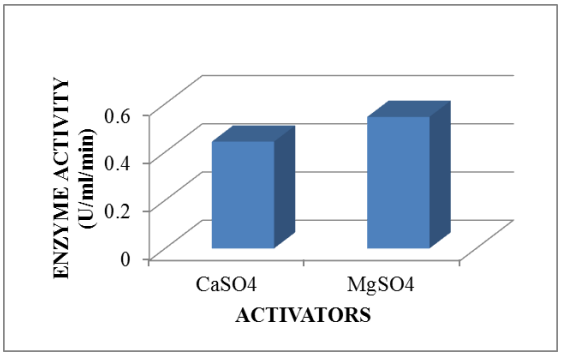


Figure 15: Graph presenting effect of activators on enzyme activity

(C) Effect of inhibitors on the enzyme activity

Inhibitors are known to decrease the efficiency of enzyme. EDTA and SDS are used as inhibitors to check the activity of enzyme. Incubate the enzyme with substrate for 15 min at 37°C.

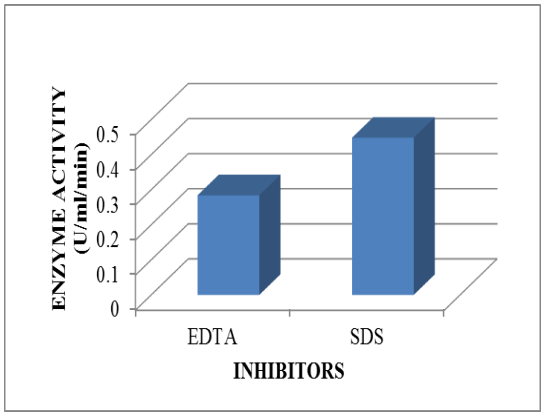


Figure 16: Graph showing effect of inhibitors on enzyme activity

Percentage purity of Nattokinase by HPLC after Dialysis

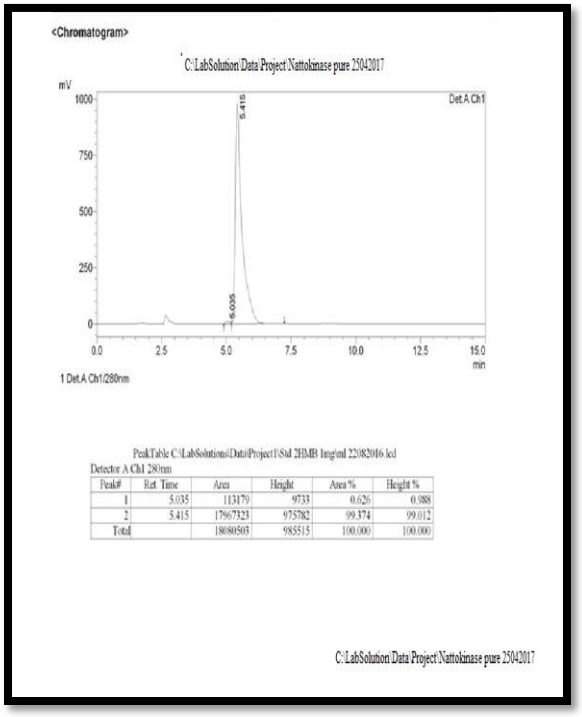


Figure 17: Above graph represent the HPLC of Pure Nattokinase in which show purity of Nattokinase 99.174% at RT 5.415 min.

After Dialysis of enzyme we check purity of enzyme by HPLC then we found that percentage purity of our enzyme is 99.17%, which confirm by the peak of our sample was came at RT 5.4515. We need Standard sample for compare our result but we compare our result with different research paper thorough this confirm that peak of Nattokinase was came at RT 5.415.

CONCLUSION

According to our current work includes further enhancing the activity of our enzyme after purification because during characterization of enzyme we found that enzyme would be loses their activity. In future we also find out the exact species of bacteria by help of 16S RNA sequencing and also evaluate their in-vivo application with the help of pure enzyme.

The present study entitled as In vitro studies on media optimization, purification and characterization of fibrinolytic activity of nattokinase from *Bacillus* species.

The project work begins with the isolation of Nattokinase producing bacteria from soil sample and from soyabeans by serial dilution method and agar plate method .The isolate was screened for nattokinase activity , study of growth curve parameters of +ve isolate , selection of different media for the optimum production of the nattokinse , physiological conditions were optimized so as to favour the proper growth, nattokinase production in Submerged Fermentation, ammonium salt precipitation & dialysis followed by the characterization of the enzyme on basis of pH and temperature. On the basis of the results obtained of the following conclusions can be drawn which are as follows.

Among all four isolates, the isolate **C05** was identified as the best isolate for the nattokinase production. The growth of the isolate was found to be best at pH 7 and at the temperature of 37^0C.

Crude enzyme was partially purified by salt precipitation and followed by the dialysis. The effect of activators and inhibitors can also be studied. After this also check the purity of our enzyme by Chromatography technique i.e. HPLC.

REFERENCES

[1] Ku, T. W., Tsai, R. L., & Pan, T. M. (2009). A simple and cost-saving approach to optimize the production of subtilisin NAT by submerged cultivation of Bacillus subtilis natto. *Journal of agricultural and food chemistry*, *57*(1), 292-296.

[2] Dabbagh, F., Negahdaripour, M., Berenjian, A., Behfar, A., Mohammadi, F., Zamani, M., ... & Ghasemi, Y. (2014). Nattokinase: production and application. *Applied microbiology and biotechnology*, *98*(22), 9199-9206.

[3] Wei, X., Luo, M., Xu, L., Zhang, Y., Lin, X., Kong, P., & Liu, H. (2011). Production of fibrinolytic enzyme from Bacillus amyloliquefaciens by fermentation of chickpeas, with the evaluation of the anticoagulant and antioxidant properties of chickpeas. *Journal of agricultural and food chemistry*, *59*(8), 3957-3963.

[4] Kotb, E. (2012). Fibrinolytic bacterial enzymes with thrombolytic activity. In *Fibrinolytic Bacterial Enzymes with Thrombolytic Activity* (pp. 1-74). Springer, Berlin, Heidelberg.

[5] Lee, D. G., Jeon, J. H., Jang, M. K., Kim, N. Y., Lee, J. H., Lee, J. H., ... & Lee, S. H. (2007). Screening and characterization of a novel fibrinolytic metalloprotease from a metagenomic library. *Biotechnology letters*, *29*(3), 465.

[6] Kim, O. V., Xu, Z., Rosen, E. D., & Alber, M. S. (2013). Fibrin networks regulate protein transport during thrombus development. *PLoS Comput Biol*, *9*(6), e1003095.

[7] Kotb, E. (2012). Fibrinolytic bacterial enzymes with thrombolytic activity. In *Fibrinolytic Bacterial Enzymes with Thrombolytic Activity* (pp. 1-74). Springer, Berlin, Heidelberg.

[8] Grunwald, P. (Ed.). (2019). *Pharmaceutical Biocatalysis: Fundamentals, Enzyme Inhibitors, and Enzymes in Health and Diseases*. CRC Press.

[9] Sinha, S., & Verma, L. (2014). *Intravitreal Injections*. JP Medical Ltd.

[10] Faran, G. E. (2015). *Mutagenesis of Indigenous Streptococcus equisimilis isolates for Enhanced Production of Streptokinase* (Doctoral dissertation, UNIVERSITY OF AGRICULTURE, FAISALABAD).

[11] He, F. J., & Chen, J. Q. (2013). Consumption of soybean, soy foods, soy isoflavones and breast cancer incidence: differences between Chinese women and women in Western countries and possible mechanisms. *Food Science and Human Wellness*, *2*(3-4), 146-161.

[12] Kamada, M., Hase, S., Fujii, K., Miyake, M., Sato, K., Kimura, K., & Sakakibara, Y. (2015). Whole-genome sequencing and comparative genome analysis of Bacillus subtilis strains isolated from non-salted fermented soybean foods. *PLoS One*, *10*(10), e0141369.

[13] Wei, X., Luo, M., Xu, L., Zhang, Y., Lin, X., Kong, P., & Liu, H. (2011). Production of fibrinolytic enzyme from Bacillus amyloliquefaciens by fermentation of chickpeas, with the evaluation of the anticoagulant and antioxidant properties of chickpeas. *Journal of agricultural and food chemistry*, *59*(8), 3957-3963.

[14] Sharif, U. H., Kamarudin, A. S., & Huda, N. (2019, July). Proximate and amino acid composition of quail meat treated with mega floral booster addition. In *IOP Conference Series: Earth and Environmental Science* (Vol. 287, No. 1, p. 012031). IOP Publishing.

[15] Bhadekar, R., & Parhi, P. (2016). Non-dairy functional foods: Potential of probiotics. In *Microbes in Food and Health* (pp. 1-27). Springer, Cham.

[16] Ju, S., Cao, Z., Wong, C., Liu, Y., Foda, M. F., Zhang, Z., & Li, J. (2019). Isolation and Optimal Fermentation Condition of the Bacillus subtilis Subsp. natto Strain WTC016 for Nattokinase Production. *Fermentation*, *5*(4), 92.

[17] Xin, X. (2016). Investigations of the industrial compatible aqueous purification techniques and intrinsic stabilizing factors for nattokinase.

[18] Harris, S. R., Morrow, K., Titgemeier, B., & Goldberg, D. (2017). Dietary Supplement Use in Older Adults. *Current Nutrition Reports*, *6*(2), 122-133.

[19] Harris, S. R., Morrow, K., Titgemeier, B., & Goldberg, D. (2017). Dietary Supplement Use in Older Adults. *Current Nutrition Reports*, *6*(2), 122-133.

[20] Yarnell, E. (2017). Herbs for atrial fibrillation. *Alternative and Complementary Therapies*, *23*(3), 102-111.

[21] Gu, S. B., Zhao, L. N., Wu, Y., Li, S. C., Sun, J. R., Huang, J. F., & Li, D. D. (2015). Potential probiotic attributes of a new strain of Bacillus coagulans CGMCC 9951 isolated from healthy piglet feces. *World Journal of Microbiology and Biotechnology*, *31*(6), 851-863.

[22] Dubey, R., Kumar, J., Agrawala, D., Char, T., & Pusp, P. (2011). Isolation, production, purification, assay and characterization of fibrinolytic enzymes (Nattokinase, Streptokinase and Urokinase) from bacterial sources. *African Journal of Biotechnology*, *10*(8), 1408-1420.

[23] Borah, D. E. B. A. J. I. T., Yadav, R. N. S., Sangra, A. N. K. U. S. H., Shahin, L. U. B. A. N. A., & Chaubey, A. K. (2012). Production, purification and characterization of nattokinase from Bacillus subtilis, isolated from tea garden soil samples of Dibrugarh, Assam. *Asian J Pharm Clin Res*, *5*(3), 124-125.

[24] Obeid, A. E. F. E., Alawad, A. M., & Ibrahim, H. M. (2015). Isolation and characterization of bacillus subtillus with potential production of nattokinase. *Int. J. Adv. Res*, *3*(3), 94-101.

[25] Wang, S. L., Wu, Y. Y., & Liang, T. W. (2011). Purification and biochemical characterization of a nattokinase by conversion of shrimp shell with Bacillus subtilis TKU007. *New biotechnology*, *28*(2), 196-202.

[26] Ju, S., Cao, Z., Wong, C., Liu, Y., Foda, M. F., Zhang, Z., & Li, J. (2019). Isolation and Optimal Fermentation Condition of the Bacillus subtilis Subsp. natto Strain WTC016 for Nattokinase Production. *Fermentation*, *5*(4), 92.

[27] Siraj, S. G. (2011). *Comparative studies on production of nattokinase from bacillus subtilis by changing the nitrogen source* (Doctoral dissertation, RGUHS).

[28] Eldeen, K. I., Ibrahim, H. M., Elkhidir, E. E., & Elamin, H. B. (2015). Optimization of culture conditions to enhance nattokinase production using RSM. *American Journal of Microbiological Research*, *3*(5), 165-170.

[29] Hmood, S. A., & Aziz, G. M. (2016). Optimum conditions for fibrinolytic enzyme (Nattokinase) production by Bacillus sp. B24 using solid state fermentation. *Iraqi Journal of Science*, *57*(2C), 1391-1401.

[30] Devi, C. S., Mohanasrinivasan, V., Sharma, P., Das, D., Vaishnavi, B., & Naine, S. J. (2016). Production, purification and stability studies on nattokinase: a therapeutic protein extracted from mutant Pseudomonas aeruginosa CMSS isolated from bovine milk. *International Journal of Peptide Research and Therapeutics*, *22*(2), 263-269.

[31] Ibrahim, H. M., Bashir, K. I., Elkhidir, E. E., Alnour, M. I., & Elyas, O. (2015). Statistical Optimization of Culture Conditions for Nattokinase Production by Batch Fermentation. *Int. J. Curr. Microbiol. App. Sci*, *4*(11), 143-153.