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PRODUCTION, OPTIMIZATION AND PARTIAL PURIFICATION OF NATTOKINASE FROM *BACILLUS CEREUS;* A STRAIN ISOLATED FROM GRAPE WINE

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ABSTRACT

In the current study a potent fibrinolytic protease producing *Bacillus cereus* VMI2 was isolated from Grape wine (*Vitis vinifera*) and studied the protease activity and fibrinolytic activity. The organism was identified by morphological, biochemical and molecular analysis. The potent *Bacillus cereus* VMI2 showed significant clot lysis activity in 2h of incubation. In order to enhance the fibrinolytic activity, the medium was optimized. Inoculum size, pH, temperature, nitrogen and carbon were selected for the optimization. Compared with base medium the optimized medium with fructose, beef extract and 1000 µL inoculum volume were incubated at 35°C and showed significant increase in fibrinolytic activity. The potent strain also showed a maximum of enzyme production and enzyme activity at 6th h which showed the optimum time for production of nattokinase. The FTIR-HPLC results confirmed the presence of nattokinase. The molecular weight of the enzyme was analysed by SDS PAGE. It was found to be a 29kDa protein, confirmed the presence of nattokinase.

Keywords: Nattokinase. *Bacillus cereus*. Cardiovascular diseases. Fibrinolytic enzymes. Clot busters

INTRODUCTION

CVD or cardiovascular diseases have become one of the significant reasons for increase in mortality rate across the globe. Moreover, thrombosis is regarded as the severe blood clotting condition which results in thrombophlebitis, phlebitis, and venous thrombosis **(Kotb et al., 2012) (Souza et al., 2010).** Despite the possibility of a cerebral hemorrhage as a result, different anticoagulants, including heparin, are currently used to treat thrombosis disorders. It has been suggested that plasminogen activators used in thrombolytic therapy are a superior form of treatment to other anticoagulants **(Malke et al., 1984) (Pajovic et al., 2018)**. Different microorganisms act as a source for various enzymes including *Bacillus* which is commonly found in traditional fermented foods **(Uyar et al.,2011).** Nattokinase (NK) is a sticky enzyme present in Japanese food natto which is a cheese like food made from fermented soybeans. The fermentation of soybeans with *Bacillus subtilis*, a Gram-positive bacteria produces the fibrinolytic protease nattokinase. **(Dabbah et al., 2014).** Grape wines are made mainly from species *Vitis vinifera* **(Li et al., 2017).** Grape wines are rich in phenolic compounds that have potent anti-oxidant activity. Red grapes when compared with white grapes have more total polyphenol content (300–5000 mg/L) which is about ten times more than that of white wines which has 60–200 mg/L content **(Li et al., 2017).** Matured soybeans have been used in isolation of *Bacillus* and have proven the ability to produce nattokinase. **(Li et al.,2022).** *B. subtilis* in particular is known to cause ropiness in wines. This helps to improve the quality of wine due to the production of long-chain polysaccharides. The species of the genus *Bacillus* are known to be great producers of industrial enzymes. These bacteria are known to produce about 50% of industrially important enzymes **(Schallmey et al., 2004).** *Bacillus cereus* has a great role in wine production as well as is a strong producer of fibrinolytic enzyme nattokinase. Therefore, having a potent producer for the production of nattokinase is an industrial need. It opens the vast opportunities for mass production of nattokinase which is a highly demanded fibrinolytic protease. The current study focussed on the production, partial purification, and media optimization for enhanced production of nattokinase from *Bacillus cereus* VMI2 isolated from grape wine. This work is the report on the nattokinase producing bacterial strain isolated from household wine.

MATERIALS AND METHODS

Isolation of *Bacillus* sp. from food samples

Four fermented home-made food samples like curd, batter, grape wine and nutmeg wine were collected from different parts of Vellore, Tamil Nadu, India. The fermented food samples were serially diluted in 0.85% saline upto 10^{-7} and spread plate was performed using 100µL from 10^{-3},10^{-5}, and 10^{-7} dilutions on nutrient agar media (Hi-Media, India). The culture plates were kept at 37°C for 24h of incubation. Based on the morphology of the colonies, they were selected and subjected to Gram's staining. Isolated strains were then screened for protease activity on skim milk agar medium **(Merlyn et al., 2021) (Das et al., 2010).** Plates were incubated for 24h at 37°C.

Screening for protease activity

Primary and secondary screening

Selected strains were screened for protease activity by plating them on skim milk agar plates (10%) and incubated for 24h at 37°C. The plates were then observed for zone of clearance **(Merlyn et al., 2021).** Based on the zone of hydrolysis the selected strains were selected for secondary screening. The selected strains were incubated in nutrient broth at 37°C for 24h. The culture broth was centrifuged at 4°C, 10,000 rpm for 10 min and the cell free suspension was collected. The cell free supernatant of 100µl was then added into the wells (1mm diameter) cut on skim milk agar plates and incubated. Further the plates were incubated at 37°C for 24h after incubation the diameter of zone of hydrolysis was measured.

Soft agar overlay method

The selected strains were screened for the detection of fibrinolytic protease production by soft agar medium method using casein and human plasminogen in soft agar **(Schallmey et al., 2004) (Wang et al., 2009).** The selected strains were streaked on overlay soft agar medium. The medium was prepared using 10 mL of 0.8% agarose, 10% skim milk, 150 mM NaCl, 200 µl of fresh human plasma, and 50 mM Tris-HCl at pH 8.0. The plates were incubated overnight, at 37°C. A clear zone around the colonies indicated fibrinolytic protease.

Modified Holmstorm method

Clot lysis activity of the crude supernatant (100µL,200µL,300µl,500µl) extracted from the selected strains were analysed by modified Holmstorm method. **(Holmstorm 1965) (Mohanasrinivasan et al., 2013) (Pajovic et al., 2018).** The positive control used was streptokinase from hemolytic *Streptococci*(sigma), 10,000 KU.

Selection of strain and identification

Morphological and biochemical characterization of the selected strain isolated from grape wine was done. The results were interpreted using Bergey's manual of

bacteriology **(Brown 1939).** Pure isolates of the strain were maintained on nutrient agar slants.

Molecular characterization of the strain VMI2

The selected potent strain was identified by 16S rDNA sequencing. DNA was isolated from the strain VMI2 and 5 µl of sample was added to 25 µL of PCR reaction solution (5 µL of deionized water,1.5 µL of forward primer (5' AGAGTTTGATCTGGCTCAG 3') and 12 µL of Taq Master Mix and reverse primer (5' TACGGTACCTTGTTACGACTT 3') were used for PCR. The amplified 16s rDNA sequences were run in BLAST using National Center for Biotechnology Information (NCBI) database **(Altschul et al. 1990).** The alignment was evaluated using the program MUSCLE 3.7 **(Edgar et al. 2004).** MEGA 11 software with bootstrap values were used for the analysis of phylogeny **(Kumar et al., 2016)**. The 16s rDNA sequence was submitted to Genbank and accession number was generated.

Production medium optimization

Production medium for nattokinase was supplemented with dried shrimp shell powder and inoculum size of 1% was inoculated into it. It was then incubated at 37°C in the shaker for 48h **(Mohanasrinivasan et al., 2013).** Media optimization with carbon sources like (1% of glucose, fructose, glycerol, sucrose) nitrogen sources (2% of yeast extract, beef extract, soy-bean meal (commercially available meal maker) inoculum size (50µL, 100 µL, 250 µL, 500 µL and 1000 µL), pH (pH 4, pH 6, pH 7, pH 10, pH 14) and temperature (25℃, 35℃, 40℃, and 55℃) enriched with shrimp shell powder (1%) **(Dabbah et al., 2014) (Ku et al., 2009).**

Growth kinetics of *Bacillus cereus* VMI2

Growth rate of the organism *Bacillus cereus* VMI2 was analysed in the optimized production medium. The strain VMI2 was inoculated into the production medium and incubated for 12h at 37 °C. 1% of log phase culture was inoculated into 100mL of production medium and incubated at 37 ℃ for 24h. **(Babu and Subathra 2015).** The growth kinetics, protein content and enzyme activity were checked at regular intervals of 2h, it was observed from 0^{th} to 12^{th} h. The protein content and enzyme activity were determined by Lowrys method and casein hydrolysis assay respectively. **(Lowry 1951) (Cupp-enyard 2008).**

Partial purification of the enzyme

The cell free suspension was used for the ammonium sulphate precipitation at different levels of saturations (20-100% w/v). The precipitated samples were collected and spined at an rpm of 12,000 at 4 °C for 20 min. After dissolving the pellet in Tris-HCl (20 mM) of pH 7, the sample was dialyzed using tris HCl buffer. **(Rajaselvam et al 2021)**. The partially purified enzyme of 80% saturation was then checked for clot lysis activity by modified Holmstorm method. **(Holmstorm 1965)**

Enzyme characterization

HPLC AND FTIR

Partially purified sample (60-80%) was subjected to HPLC with mobile phase of NaH_2PO_4 and CH_3OH in the ratio 95:5. The mobile phase was filtered using syringe filter (0.45µm) and analysed **(Mohanasrinivasan et al.,2013).**
FTIR spectra of partially purified enzyme (60% and 80%) partially purified sample was measured using a Perkin Elmer FTIR spectrometer.

SDS-PAGE

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was done to identify the protein weight and purity of the enzyme produced by *Bacillus cereus* VMI2 **(Vaithilingam et al., 2016).**

RESULTS

Isolation and screening

Screening for protease activity

Among all the selected ten strains only three strains showed zone of hydrolysis on skim milk agar (Figure 1B). Zone of hydrolysis was compared among the three strains and further examined for secondary screening under same conditions. Among the three strains *Bacillus cereus* VMI2(Figure 1A) isolated from grape wine showed a zone of hydrolysis with 20mm (Figure 1C) of diameter and the protease activity was confirmed.

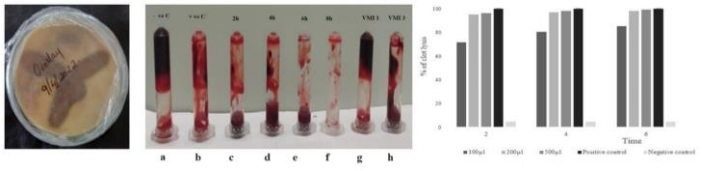
A) **B)** **C)**

Figure 1 a) Pure culture of *Bacillus cereus* VMI2 on nutrient agar b) primary screening on skim milk agar medium c) secondary screening on skim milk agar medium.

Screening for fibrinolytic protease activity

The soft agar over lay method and clot lysis

The fibrinolytic protease activity of the selected strain was confirmed by soft agar overlay method (Figure 2A). Among the three protease positive strains VMI2 isolated from grape wine showed maximum zone of hydrolysis. Compared with the three protease positive strains, significant clot lysis activity was observed for the strain VMI2 crude sample. The crude samples showed less clot lysis activity in stipulated time. After 2h of incubation 200µl of crude sample extracted from VMI2 showed 95.3% of clot lysis activity (Figure 3B). After 4^{th} h the clot lysis was gradually increased, and a complete lysis was observed in the 6^{th} h. The crude supernatant from the strain VMI2 exhibited a maximum of 97% of clot lysis. (Figure 2C)



A) **B)** **C)**

Figure 2 Enzyme activity (A) soft agar overlay method (B) clot lysis (a) negative control, (b) positive control, (c) clot lysis activity of VMI2 after 2h (d) after 4h (e) after 6h (f) after 8h (g) clot lysis activity of VMI1 after 8h (h) clot lysis of VMI3 after 8h of crude supernatant extracted from selected three bacterial strain (C) percentage of clot lysis activity of VMI2 after 2h,4h and 6h

Biochemical and molecular characterization

The strain *Bacillus cereus* VMI2 was found to be Gram positive, non-motile, indole (-), MR (-), VP (+), citrate (+), TSI (-), nitrate (-). Based on this the bacterial genus was identified as *Bacillus* sp. The 16S rDNA sequence was deposited Genbank database and the sequence alignment was checked. The results showed maximum similarity with *Bacillus cereus*. Genbank accession number of the selected strain was ON680844. The phylogenetic tree (Figure 4) showed direct similarity with *Bacillus cereus*. Phylogenetic tree based on maximum comparison with bootstrap values more than 60%.

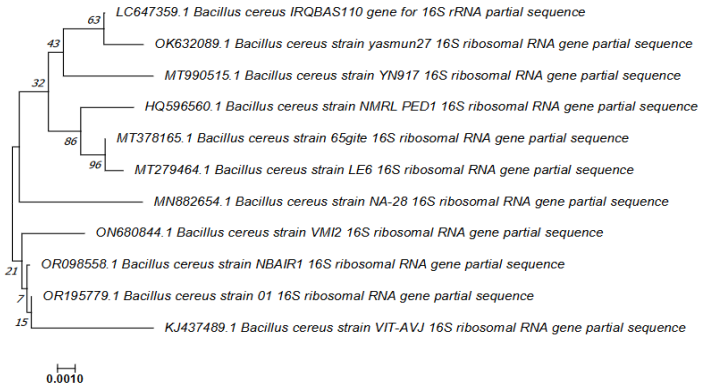
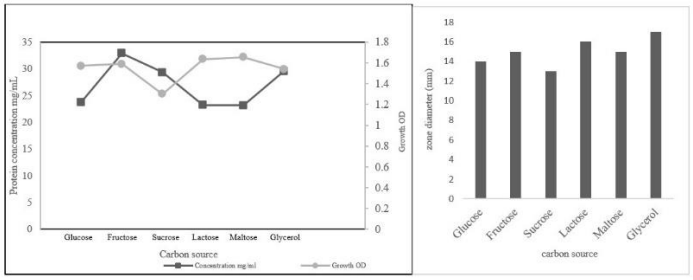


Figure 4 Phylogenetic tree of *Bacillus cereus* VMI2

Medium optimization for enzyme production

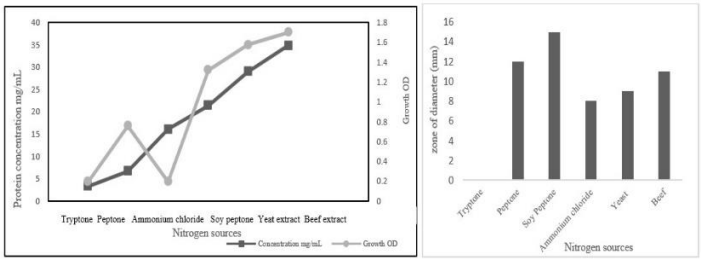
The production of nattokinase from *Bacillus cereus* VM2 isolated from grape wine showed maximum enzyme activity of 32. 88 mg/mL in the medium containing 1% of fructose (Figure 5A). The least protein concentration was for the medium with maltose (23.15 mg/mL). The zone of hydrolysis was 16mm for glycerol while sucrose showed 12mm of zone diameter (Figure 5B). The selected carbon source fructose had 15.6 mm of zone diameter (Figure 5B). For the optimization of

nitrogen source beef extract (34.7 mg/mL) gave more protein concentration and ammonium chloride showed less protein (3.25 mg/mL) content. The growth rate was maximum for beef extract and least in tryptone. (Figure 6A). While the zone of hydrolysis was maximum for soy peptone and completely absent in peptone. The maximum of active protein was found in beef extract which showed a zone diameter of 11.5mm (Figure 6B). At pH 11 the strain *Bacillus cereus* VMI2 isolated from grape wine produced 9.20 mg/mL of protein. The enzyme production was reduced to 3.63mg/mL at pH 5(Figure 7A). From pH7 the protein content was gradually increased and reached a maximum at pH 11. This indicates the strain VMI2 isolated from grape wine can grow well in the alkaline condition. Zone of hydrolysis at neutral and alkaline pH were considerably the same. The zone size was maximum at neutral pH 7 (13mm) while pH 11 has 10mm of zone diameter. While in pH 3 the zone was completely absent (Figure 7B). Similarly for temperature optimization the suitable temperature for maximum enzyme production and activity was 35℃. There was a considerable increase in enzyme activity and protein concentration at 35℃ and had no activity at other temperatures (Figure 8A). Zone of hydrolysis on skim milk agar showed maximum diameter at 35℃ (10mm) and a zone size of 8mm in 40℃ (Figure 8B). The suitable inoculum size optimization showed a log phase bacterial culture of 1000 µL gave maximum of enzyme production. The growth rate was maximum at 1000µL of inoculum size (Figure 9A). The skim milk agar zones were similar for all inoculum size of 50 µL, 100 µL, 250 µL and 500 µL (10mm) while the maximum was for 1000 µL of inoculum size (11.5mm) (Figure 9B).



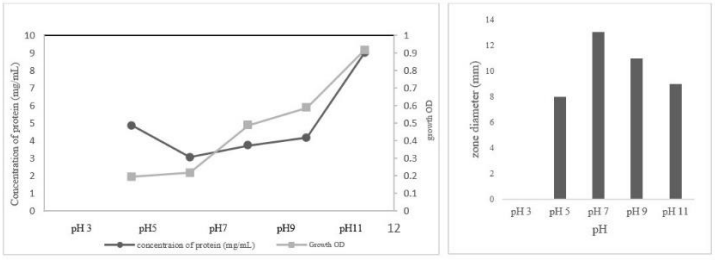
A) B)

Figure 5 Optimization of carbon source a) Growth rate and protein content b) enzyme activity



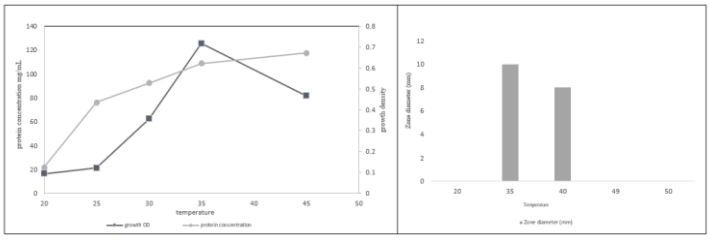
A) B)

Figure 6 Optimization of nitrogen source a) Growth rate and protein content b) enzyme activity



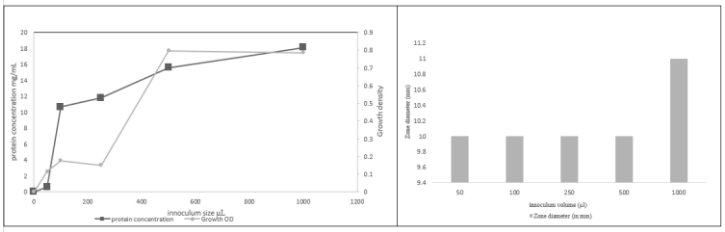
A) B)

Figure 7 Optimization of pH a) Growth rate and protein content b) enzyme activity



A) B)

Figure 8 Optimization of temperature source a) Growth rate and protein content b) enzyme activity



A) B)

Figure 9 Optimization of innoculum size a) Growth rate and protein content b) enzyme activity

Growth kinetics of *Bacillus cereus* VMI2

The growth kinetics of *Bacillus cereus* VMI2 showed no significant increase in first 2h. The growth rate gradually increased with respect to time. In the 4th h the rate increased and reached a maximum at 6th h (Figure 10). From there the decline phase started and the rate declined slowly at 8th h and 10th h.

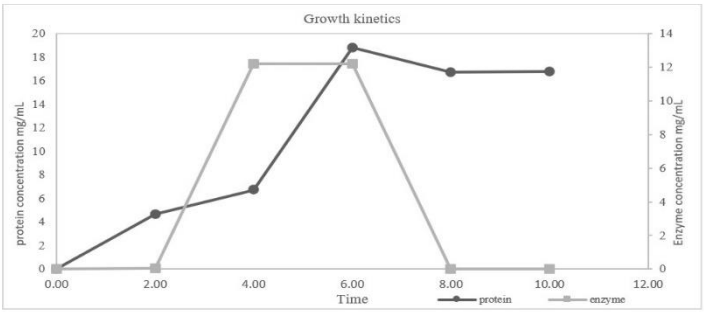


Figure 10 Growth kinetics of *Bacillus cereus* VMI2 showing maximum enzyme concentration at 6th h (12.21 mg/mL) and maximum protein at 6th hour (18.81mg/mL).

Partial purification of nattokinase

The clot lysis activity of the partially purified enzyme samples (20%,40%,60% and 80%) were observed after 1h of incubation at 37℃. Complete lysis of clot was observed after 1h incubation in 80% of saturated partially purified enzyme (Figure 11)

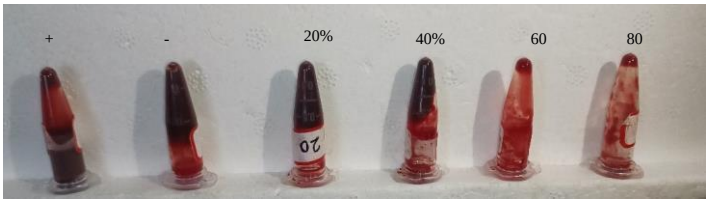


Figure 11 Clot lysis of partially purified *Bacillus cereus* VMI2

SDS-PAGE

The results of the sample protein bands were compared with the protein marker (lower range, GeNei 311017500A) and it was found to be 27kDa (Figure 12) confirmed the production of nattokinase by *Bacillus cereus* VMI2.

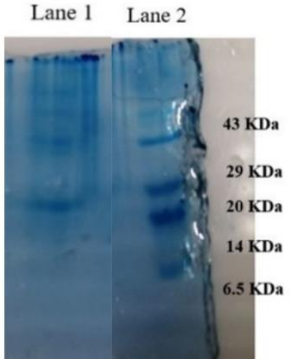
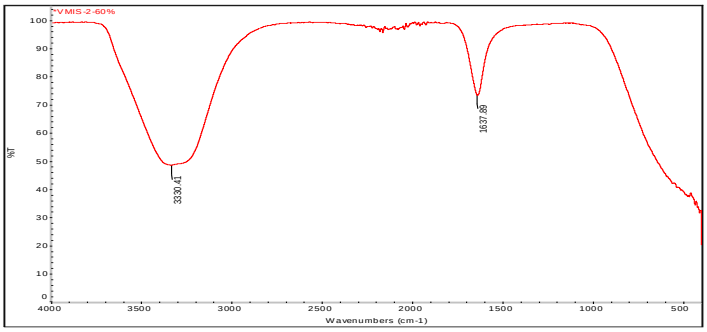


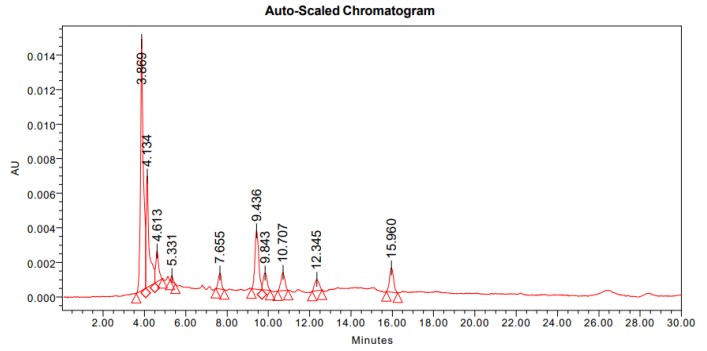
Figure 12 SDS-PAGE of partially purified *Bacillus cereus* VMI2 (lane 1) with protein marker (lane 2)

FTIR AND HPLC

The spectral analysis by Fourier transform- infra red revealed the presence of amine group in the partially purified fibrinolytic enzyme produced by *Bacillus cereus* VMI2, having a strong stretching at 3330.41 cm^{-1}. And strong stretching at 1637.89 cm^{-1} showing the presence of anhydride C=O. this confirms the enzyme as serine protease (Figure 13 A).
The partially purified sample showed peak at 10.7 min, the results were compared with the previous reports **(Mohanasrinivasan et al.,2013)** the retention time for the standard nattokinase was reported as 10.6 min (Figure 13B).



A)



B)

Figure 13 a) FTIR profile of partially purified *Bacillus cereus* VMI2 b) HPLC chromatogram of partially purified VMI2 showed a peak at retention time of 10.7 min.

DISCUSSION

This research study emphasizes on the production and partial purification of nattokinase from a bacterial strain isolated from home-made grape wine. The potential of the fibrinolytic activity was confirmed by clot lysis assay. The wine samples were chosen because of the presence of *Bacillus* in wine which helps for the ropiness of wine and improves the quality of it **(Yu et al., 2021).** Among the ten isolates *Bacillus cereus* VMI2 showed potent fibrinolytic activity. Nattokinase producing *Bacillus* species were isolated from different fermented food samples (**Merlyn et al., 2021)**. Soy based fermented foods were identified as one of the important sources for nattokinase producers. According to **Yao et al.,2022** reported a bacterial strain isolated from soy milk as an efficient producer of nattokinase. Most of the strains isolated from food sources showed protease activity, anti-hypertensive, anti-thrombolysis, anti-inflammatory and anti-viral activity **(Yao et al., 2022**). In the present study, the strain isolated from home-made wine showed complete lysis after 6h. In a previous study, *Bacillus amyloliquefaciens* KJ10 **(Rajaselvam et al.,2021)** isolated from soybean paste showed a maximum clot lysis activity of 28% in 1h. When compared to the previous study, the strain *Bacillus cereus* VMI2 exhibited a maximum of 95.3% cloy lysis in 2h. Thus, the strain *Bacillus cereus* VMI2 is identified as one of the potent fibrinolytic protease producers. Fermented foods are one of the good sources for nattokinase production. Fermentation of hemp seed meal by solid state fermentation improved the rate and yield of enzyme production **(Zhang et al., 2023)**. The optimized medium has significant role in enzyme production and its yield. The optimization of medium was performed with suitable carbon and nitrogen source, pH, temperature and inoculum size. Defatted soybean and glucose were identified as the optimum nutrient for enhanced nattokinase production **(Ku et al., 2009)** (**Xiao et al., 2022**). In our study, the medium supplemented with fructose and beef extract showed a maximum enzyme production. The optimum pH and temperature were found to be 11 and 35°C respectively. The addition of yeast extract, glycerol, and soy peptone in the production medium enhanced the production of nattokinase. **(Berenjian et al., 2014)**. For the commercial production of nattokinase, low-cost substrates have to be used in the production medium **(Wang et al., 2009).** In the current study maximum growth rate and protein content was observed at 6^{th} h in *Bacillus cereus* VMI2. In a previous research report, *Bacillus subtilis* WTC016 strain produced maximum nattokinase in the stationary phase **(Ju et al., 2019).** When compared to *Bacillus subtilis* WTC016, the strain *Bacillus cereus* VMI2 isolated from grape wine showed maximum enzyme activity and growth rate in the logarithmic phase (after 6h). Nattokinase producing *Bacillus* species were also reported from different sources like rust **(Mohanasrinivasan et al 2013)**. The molecular size of nattokinase ranges from 28Kda -30Kda **(Weng et al.,2017) (Ngan et al., 2022).**

CONCLUSION

In the present study, nattokinase was produced and partially purified from potent strain *Bacillus cereus* VMI2 isolated from home-made fermented wine sample. The fibrinolytic property of *Bacillus cereus* VMI2 was confirmed by modified Holmstorm and soft agar overlay method. This study also focused on the growth kinetics and media optimization for enhanced nattokinase. Further purification and strain improvement will be done to enhance the production of nattokinase. Thus, our research data indicated the presence of Natto producing *Bacillus* sp. in homemade grape wine

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Conflict of interest: There is no actual or potential conflict of interest.

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