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PHYSICAL AND CHEMICAL CHARACTERIZATION OF ALKALINE PROTEASE FROM *BACILLUS SUBTILIS* VBC7 USING AGRO WASTE AS SUBSTRATE

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ABSTRACT

Alkaline proteases are the most important group of industrial enzymes that hydrolyse the peptide bond of proteins into small peptides. The industrial demand of alkaline protease predominantly from the microbial origin has been recently increased and enhances the research for alkaline protease with high stability at extreme industrial conditions. Thus, this study is aimed to characterize the alkaline protease from bacterial isolate, *Bacillus subtilis* VBC7 screened from dairy waste dumped soil. Extracellular alkaline protease production was carried out in alkaline broth by submerged fermentation. The production medium with 10% ground nut extract at pH 10 and 40 °C enhanced the alkaline protease activity (712 U/mL) than other wastes such as coconut pulp extract and sesame seed extract. This optimized media increased the bacterial growth rate and activity of alkaline protease compared to the unsupplemented basal medium. Further, alkaline protease was partially purified and assessed their molecular weight (~30 k Da) in 12 % SDS -PAGE. The enzyme activity was observed at pH 10 and 40 °C and stable over a wide range of pH (5-12), and temperatures (10-70 °C). In addition, enzyme activity was stimulated in the presence of Mg^{2+}, Ca^{2+} and Mn^{2+} and was unpretentious after treating with surfactants (SDS, Triton X-100, Tween 80), organic solvents (ethanol, methanol, chloroform, acetone and hexane) and protease inhibitors (EDTA and β-mercaptoethanol). These compatible features could lead the enzyme as a potential candidate among the industrial sectors.

Keywords: Alkaline protease; agro waste; submerged fermentation; enzyme activity; metal ions; surfactants

INTRODUCTION

India is a resource hub for engendering agro wastes as our country is an agro based. Nearly, 62 million tonnes of agro wastes are generated in India per year with 4% of annual growth rate (PIB, 2016). This agricultural waste should be properly managed as they have good nutritional values and thus, they have value for their economics. Further, agricultural waste management play major role in ecological cycle and thus interdependent relationship is sustained in the ecosystem. Such agro wastes could be utilized to produce commercially valuable products in order to improve their economical values and to improve waste remediation. Organic wastes have been utilized for producing garbage enzymes which are used as antimicrobial agents to treat domestic, municipal and industrial drainage system and to clean air and remove the bad odor **(Arun and Sivashanmugam. 2015).** To enhance the cost effective production of various industrial important enzymes, the massive agro waste have been used as an alternative and sustainable substrate. One such most industrial important enzyme, proteolytic enzymes can be produced by solid –state and submerged fermentation process using various agricultural waste such as soybean meal, wheat bran, cotton seed meal, mustard oil cake, ground nut oil cake, coconut oil cake, banana peel, orange peel, etc. **(Ramachandran *et al*., 2007; De Castro and Sato. 2013; Sukumprasertsri *et al*., 2013**).
Proteases are the largest group of enzymes with invincible role in industrial sectors including detergents, food, pharmaceutical, leather, peptide synthesis, soy processing, extraction of silver from recycled X ray film (**Sharma *et al*., 2019**), etc. Alkaline proteases comprised 60% of the global enzyme market and considered as most precious among other commercial enzymes **(Annamalai *et al*., 2014)**. Although proteases are widespread in nature, microbial sources are most preferred one and account for around two-thirds of commercial production worldwide **(Selvam *et al*., 2016**). A variety of bacteria such as *Pseudomonas aeruginosa* **(Andrejko *et al*., 2019)**, *Flavobacterium psychrophilum* **(Secades *et al*., 2001)**, *Achromobacter lyticus* **(Norioka and Sakiyama. 1993)**, *Thermoactinomyces vulgaris* **(Ding *et al*., 2020)**, *Serratia marcescens* **(Bach *et al*., 2012)**, *Streptomyces* sp. **(Sarkar and Suthindhiran. 2020)**, *Bacillus subtilis* **(Auther *et al*., 2012)** have been previously reported for alkaline protease. Amongst these, *Bacillus* sp. is the most prominent source of soluble extracellular enzymes especially neutral and alkaline proteases with potential to secrete more than 20g/L **(Masi *et al*., 2021; Rehman *et al*., 2017; Anandharaj *et al*., 2016)**. Further, *Bacillus* sp. is widely distributed in soil and recycles the available nutrients by secreting macromolecule degrading hydrolases like amylase, cellulase, phosphatase, protease, etc. During industrial processing *Bacillus* strains tolerated the unfavourable conditions, nutrient depletion, abiotic stress etc. **(Contesini *et al*., 2017).**
The increasing industrial demand of alkaline protease has provoked the research to hunt for new microbes and that can be a source of these valuable enzymes. With this, the study is mainly focused to find out the effect of various agro wastes (coconut pulp extract, ground nut extract, sesame seed extract) design the cost effective media and to increase the yield of enzyme. Also this study aimed to determine the various physiochemical properties of alkaline protease produced by *Bacillus subtilis* VBC7.

MATERIAL AND METHODS

Protease producing bacterial isolates

Alkaline protease producing bacterial isolate VBC7 was isolated from soil collected from area where dairy waste dumped in Salem, India. The diluted soil sample (in 0.1% peptone water) was spread plated on nutrient agar medium (pH 7.0) and incubated at 37 °C for 24 h. The isolated colonies were subcultured 3-4 times in order to get pure culture of each isolate. All the isolates were screened for alkaline protease production by plating on alkaline agar medium (pH 10.0) consisting of (g/L) glucose (10.0), yeast extract (5.0), peptone (5.0), K_2HPO_4 (1.0), $Mg_2SO_4.7H_2O$ (0.2), Na_2CO_3 (10.0), and agar (15.0), with skim milk (10 % w/v) (Hi media, India). The potential isolates those produced clear halo zone around their growth were further identified *via* standard morphological and biochemical tests (Senthilkumar *et al*., 2017) and confirmed by 16S rRNA gene sequencing using the universal primers 27F (5'-AGAGTTTGATGMTGGCTCAG-3'), and 1492R **(**5'-GGTTACCTTGTTACGACTT-3'). The obtained 16S rRNA gene sequences were analysed for their homology using CLUSTAL W software.

Alkaline protease assay

The isolate showing positive proteolytic activity in alkaline agar medium supplemented with skimmed milk (clear halo zone) was subjected for assaying alkaline protease activity. About 2 % broth culture of the isolate (2.5×10^8 CFU/mL) was inoculated into the 250 mL conical flask containing 100 mL of liquid protease production medium as the alkaline agar medium, except that of agar and skimmed milk and incubated at 37 °C for 48 h. The cell growth was monitored by recording OD_{595} of the broth culture for every 4 h. Alkaline protease activity was determined

using cell free extract (CFS) as described by **Ibrahim *et al*. (2015)**. Briefly, the CFS was collected by centrifuging the broth culture at 4 °C, 10000 rpm for 10 min. About 1 mL of reaction mixture was prepared by adding 0.5 mL of 50mM glycine-NaOH (pH 10.0) containing casein (1%) and 10 mM $CaCl_2$ was pre-incubated at 50 °C for 5 min and was added with equal amount of CFS. This reaction mixture was incubated at 60 °C for 45 min. The enzymatic reaction was stopped by adding 2 mL of tricholoroacetic acid solution (5 % v/v). The mixture was centrifuged (10,000 ×g for 20 min) at 4 °C and the supernatant containing soluble peptides was neutralized using 1N NaOH. About 500 µL of 1 N folin-phenol reagent was added to the neutralized supernatant and the developed colouration was measured at 660 nm. Concurrently, 1mM tyrosine as reference was used to prepare standard curve. One unit of protease activity was defined as the quantity of enzyme which releases 1 µM of tyrosine per min.

Optimization of production media

Effect of agro waste as substrate in protease production

The agro waste used in this study, coconut pulp extract, groundnut extract and sesame seed extract were collected from their respective milling site. These wastes were assessed for their effect on bacterial growth and alkaline protease production was investigated. The protease production liquid medium was supplemented individually with different concentration of (0, 5, 10, 15 %) agro wastes and inoculated with 2 % of broth culture containing 2×10^8 CFU/mL. It was incubated at 37 °C and 150 rpm. Protease activity was measured for every 4 h up to 64 h of fermentation. The nutrition contents such as carbohydrate, protein, lipid, ash and moisture content of the agro waste were determined by following association of official analytical chemists protocol **(AOAC, 2010)**.

Effect of pH and temperature

The bacterial isolate was grown in protease production media adjusted with pH ranging from 4-12 (at the interval of 1 unit) and incubated at 37 °C. The enzyme activity was assayed by the above mentioned after 48 h incubation. Similarly, the protease production was carried out at different temperature by incubating the culture broth at various temperatures (20, 30, 40, and 50 °C) and the enzyme activity was measured after 48h incubation.

Cell growth and protease production kinetics

The optimized production media was inoculated (pH 9) inoculated with 2 % of 24 h old VBC7 broth culture (2×10^8 CFU/mL) and incubated for 48 h at 40 °C. Cell growth was measured recording the OD_{595} value of broth culture for every 4 h. Similarly, the enzyme activity was checked using CFS aliquots collected aseptically at every 4 h (**Ibrahim *et al*., 2015**)

Alkaline protease extraction and partial purification

The fermentation medium (pH 9.0) was centrifuged at 9000 ×g, 4 °C for 10 min and the CFS containing crude protease was collected. The crude protease was precipitated by adding ammonium sulphate up to 60 % saturation and incubated overnight at 4 °C. The precipitate was collected by centrifuging the suspension at 7000 ×g for 10 min and dissolved in minimal amount of 10 mM phosphate buffer (pH 7.0). The suspension was dialyzed against the same buffer at 4 °C with subsequent changes of the buffer. Then the concentration of protein was measured using Pierce BCA protein assay kit (Thermo Fisher Scientific, USA) and enzyme activity was also determined.

Characterization of alkaline protease

SDS-PAGE analysis

The molecular weight of partially purified alkaline protease was determined by SDS-PAGE with 12 % gel. The gel was stained with Coomassie Brilliant Blue R-250 and the enzyme molecular mass was compared with standard protein ladder of 11-250 k Da. (New England Biolabs, USA).

Enzyme activity at various conditions

To investigate the effect of temperature and pH, metal ions, surfactants and inhibitors, the partially purified enzyme solution was incubated at different temperature (10-100 °C at the interval of 10 units) for 30min and pH (4-12, at the interval of 1 unit) for 1 h. Then the enzyme activity was assayed.
Similarly, the enzyme solution was separately treated with various metal ions such as magnesium, calcium, ferrous, manganese, copper, zinc and mercury (1 and 5 mM), surfactants such as SDS, Triton X-100, Tween 80 (1, 5 and 10 %), organic solvents such as ethanol, methanol, chloroform, acetone, hexane (10 and 20 %) and inhibitors such as ethylenediaminetetraacetic acid (EDTA), phenylmethylsulfonylfluoride (PMSF) and β–mercaptoethanol (1 and 5 mM) for 1 h and then their residual activity was assessed.

RESULTS AND DISCUSSION

In India, agriculture is one of the major sectors producing huge quantity of solid waste that may be global health threat if they are allowed to accumulate extensively for long time. Thus, such waste must be recycled to the valuable product to minimize the cost of the product production and to enable the waste remediation. Agro wastes have been used to produce the industrial important enzymes such as L-asparaginase **(Shakambari *et al*., 2018)**, alpha amylase **(Rajagopalan and Krishnan. 2008)**, xyalanase **(Knob *et al*., 2013)**, inulinase **(Dilipkumar *et al*., 2014)**, protease **(Elumalai *et al*., 2020)**, etc. This study is mainly focused to utilize the agro waste products like extracts of coconut pulp, ground nut and sesame seeds as the substrate for alkaline protease enzyme. The protease producing isolate, VBC7 was screened from the dairy waste dumped soil as this isolate produced clear halo zone around its growth (Figure 1). The phenotypic including morphological and biochemical features shown that the isolate VBC7 is a Gram positive, endospore forming non motile rods with catalase producing ability but not oxidase. Other biochemical features and ability to utilize various carbon sources are listed in Table 1 and 2 The sequencing of 16S rRNA gene and BLAST analysis revealed 100% homology with *Bacillus subtilis* and confirmed the isolate VBC7 as *B. subtilis* VBC7. The 16S rRNA gene sequences were submitted to GenBank with accession No. MZ148584. According to industrial point of view, *Bacillus* sp. is highly anticipated and most recognized genus as they are currently employed in various sectors, including food, beverage, pharmaceutical, medical, leather and detergent industries **(Schallmey *et al*., 2004)**. This special industrial interest is not only due to their generally recognized as safe (GRAS) status and also to their fastest growth and their potential for secreting extracellular proteins especially versatile enzymes.

Figure 1 Screening of VBC7 from dairy waste dumped soil isolates using alkaline agar medium (pH 10.0) supplemented with skimmed milk. The clear zone indicated the protease prducing ability of VBC7.

Table 1 Biochemical characterization of *B. subtilis* VBC7

Characteristics	Results
Indole test	-
Methyl red test	-
Voges- Proskeur test	+
Citrate utilization test	+
Urease test	-
Oxidase test	-
Catalase test	+
Nitrate reduction	-
Starch hydrolysis	+

Table 2 Carbohydrate fermentation profile of *B. subtilis* VBC7

Characteristics	Results
Lactose	+
Mannitol	+
Glucose	+
Maltose	+
Fructose	+
Sucrose	-
Galactose	+
Xylose	+
Rhamnose	+
Sorbitol	+
Cellulose	-

+: Positive, -: Negative

Alkaline protease activity of B. *subtilis* VBC7

The ability of *B. subtilis* VBC7 to produce the alkaline protease under submerged fermentation condition was assessed using protease producing basal medium at alkaline pH 10.0. The figure 2a depicted that after 4 h incubation the growth was continuously increased up to 28 h and followed by statute line. Nevertheless, the enzyme production was increased after 8 h only and continuously increased along with the growth pattern. The growth pattern of *Bacillus* sp. NPST-AK15 was slightly varied from the current strain as NPST-AK15 strain as it reached stationary

phase after 26 h only (Ibrahim *et al*., 2015) indicating growth pattern may be varied from each strain due to their source, growth conditions and other parameters. The maximum enzyme production was at 38 h with highest activity (246 U/mL) then the production rate was nearly constant up to 42 h and started to decline thereafter. The enzyme secretion pattern of the current strain is quite similar to *Bacillus* sp. NPST-AK15 (Ibrahim *et al*., 2015), *Bacillus* sp. Po2 (Patel *et al*., 2006) *Bacillus* sp. B001 (Deng *et al*., 2010) as in all these cases, maximum enzyme production was observed at the late of the stationary phase. All these findings clearly revealed the key role of extracellular protease in metabolism and organism's survival (Patel *et al*., 2006).

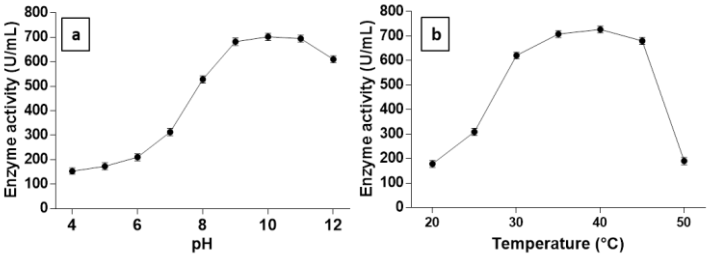

Figure 2 Time course study of cell growth and alkaline protease production (measured by assessing activity) from *B. subtilis* VBC7. For kinetics study, VBC7 was grown in (a) basal media and (b) optimized media and incubated at 40 °C for 48 h. Each data point indicates mean±SD, N= 3

Optimization of alkaline protease production media under submerged fermentation

The figure 3a, b & c depicted that protease production was greatest in submerged fermentation with agro waste as substrate. Among the three substrates (coconut pulp extract, ground nut extract and sesame seed extract), 10 % ground nut extract supplemented fermentation media showed highest enzyme activity (712 U/mL) at 48 h incubation at 37 °C (Figure 3b). Further incubation decreased the enzyme production because the substrate became limiting to the bacterial growth. This is followed by sesame seed extract with 698 U/mL of enzyme activity at 48 h incubation (Figure 3c). The current result is agreed with **Elumalai *et al*. (2020)** who reported ground nut oil cake added medium increased the growth and protease production up to 334 U/mL at 72 h from *B. subtilis* B22 when compared with other agro wastes such as coconut oil cake, soybean meal, cotton seed and wheat bran.
The nutritional composition of three waste products, coconut pulp extract, ground nut extract and sesame seed extract were assessed and showed in figure 4. High amount of carbohydrate and protein was present in ground nut extract and followed by sesame seed extract and these two waste products only produced protease with high enzyme activity indicating nutritional composition especially carbohydrate and protein content majorly influencing the protease production during submerged fermentation. This result is synchronized with the statement of **De Castro and Sato**. **(2013)** who articulated that substrate must be rich in carbon and nitrogen content for enhanced bacterial growth and improved fermentation process.

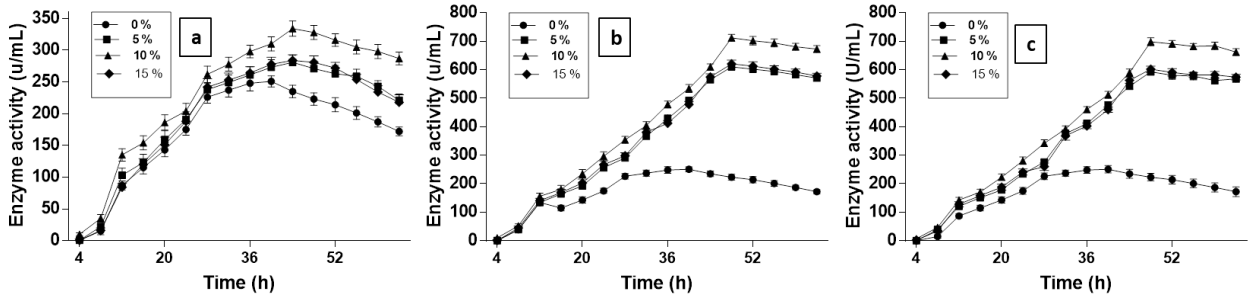

Figure 3 Alkaline protease production by *B. subtilis* VBC7 in submerged fermentation to optimize the production media using agro waste (a) Coconut pulp extract, (b) Ground nut extract, (c) Sesame seed extract as substrate

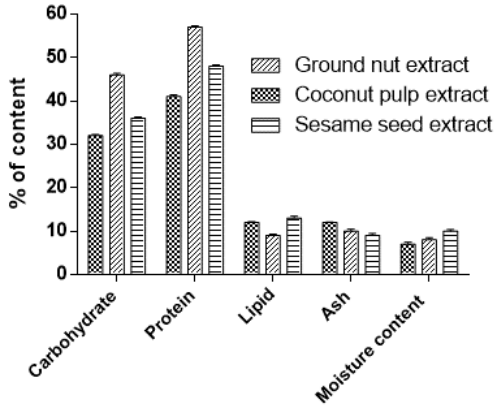

Figure 4 Nutritional composition of agricultural waste used to produce alkaline protease from *B. subtilis* VBC7

Effect of pH and temperature on protease production

Bacillus sp. is commonly preferred for industrial applications due to their acidophilic, alkalophilic and thermophilic potential and this will be varied from each strain based on their source and environmental conditions. Further, the growth temperature and media pH are the most critical factors influencing the enzyme production. Thus, in this study, the current VBC7 strain was assessed for efficiency to produce the extracellular alkaline protease under various pH an temperatures in broth supplemented with ground nut extract (10 %). The VBC7 strain could produce alkaline protease at wide range of pH from 4-12 and maximum production (712 U/mL) was observed at pH 10 (Figure 5a). At pH 4-6, the lesser enzyme production was recorded indicating the importance of growth pH in metabolic reactions of bacteria especially protein secretion. **Horikoshi *et al*. (2011)** clearly stated that a bacterial strain growing under alkaline condition require the similar condition for their better metabolic process. With respect to temperature, the isolate VBC7 could show enzyme production at the temperature ranging from 25-45 and maximum production (717 U/mL) was observed at 40 °C after 48 h incubation time. There was a sudden decrease in enzyme activity was recorded at 50 °C. Similarly, minimal enzyme production (178 U/mL) was recorded at 20 °C (Figure 5b).

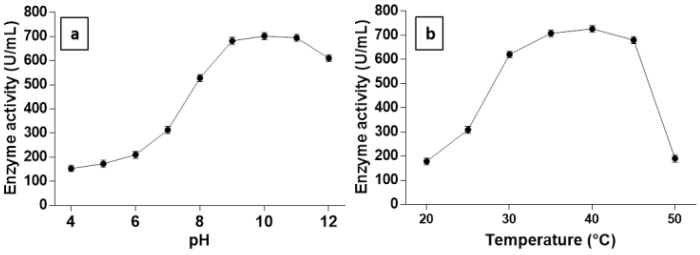

Figure 5 Effect of (a) pH and (b) temperatures in alkaline protease production (measured as activity) carried out in medium supplemented with 10 % ground nut extract (optimized substrate). The enzyme activity was determined at 48 h.

Kinetics of cell growth and alkaline protease production

Under optimized condition (pH 10, temp 40 °C) the fermentation was carried out in enzyme production media supplemented with 10% of ground nut extract (based on the optimization study). The figure 2b showed that after 4h the growth was exponentially increased up to 30 h and then reached stationary phase. Alkaline protease secretion was synchronized with the growth pattern but the secretion was started at the initial stage of logarithmic phase. The maximum biomass (OD_{595}: 2.96) and protease activity (768 U/mL) was attained at 22 and 36 h respectively. After that enzyme production was stayed nearly constant up to 48 h. Wang *et al*. (2008) reported that maximum production of protease (573 U/mL) was at 48h incubation. The present results clearly indicate that *B. subtilis* VBC7 could be completely utilized the substrate and other nutrients within lesser duration (22 h). Thus the present strain, VBC7 could be efficient to utilize the agro waste as cheaper substrate and to convert them into valuable protease enzyme.
The alkaline protease produced from submerged fermentation was pelleted by ammonium sulphate precipitation and purified further by dialysed against sodium phosphate buffer. The dialysate was further checked for their protein concentration

and enzyme activity. The protein concentration and specific activity of the alkaline protease obtained from basal broth, optimized broth, ammonium sulphate precipitated sample and dialysate were listed in Table 3 The enzyme activity was comparatively higher than that of the previously reported alkaline protease, 473U/mL from *B. firmus* (**Annamalai *et al*., 2014**), 432 U/mL from *B. subtilis* B22 **(Cui *et al*., 2015)**, 380 U/mL from *Bacillus* sp. **(Khan *et al*., 2011)**.

Table 3 Summary of protein concentration and specific activity of alkaline protease from *B. subtilis* VBC7 at different duration of purification

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mL)	Purification fold
CFS collected from basal media	956.23	2,35,462.07	246.24	1
CFS collected from optimized production media	1786.41	13,72,195.11	668.13	1
Ammonium sulphate precipitate obtained from optimized medium	87.34	76,691.63	916.34	1.4
Dialysate sample	28.71	30,654.4	1267.71	1.9

The molecular mass of partially purified of alkaline protease was analysed by SDS-PAGE. The existence of more than 2 bands revealed the necessity of further purification process for their industrial application and the molecular weight was about ~30 kDa (Figure 6).

Annamalai *et al*. (2014) reported a similar kind of result (21 kDa) for alkaline protease from *B. firmus*. But current result is nearly similar to most of the previously reported proteases from *Bacillus* strains such as *B. cereus* TKU006 with 33 kDa **(Wang *et al*., 2008)**, *Bacillus* sp. B001 with 28 kDa **(Deng *et al*., 2010)**, *B. firmus* Tap5 with 34 kDa **(Joshi *et al*., 2010)** and different from *B. subtilis* with 45 kDa **(Cui *et al*., 2015)**.

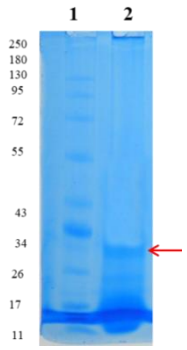

Figure 6 SDS-PAGE analysis of alkaline protease partially purifed from *B. subtilis* VBC7. Lane 1: molecualr markers ranging from 11-250 kDa, Lane 2: dialysate sample containing alkaline protease (30 kDa).

Effect of pH, temperature, metal ions, surfactants, organic solvents and inhibitors on enzyme activity

The alkaline protease activity was checked after treating at various physical and chemical conditions to assess their optimum reactive condition and stability. The partially purified alkaline protease of *B. subtilis* VBC7 was active over a wide range of pH from 5 to 12 and maximum activity (100 %) was observed at pH 10 and minimal activity (45 %) was observed at pH 4. Further, the alkaline protease was stable over low pH 5 and retained 50 % of its activity whereas at pH 12, 70 % activity was retained (Figure 7a). The results indicate the broader pH stability of alkaline protease from *B. subtilis* VBC7 and this feature makes the enzyme suitability for various industrial sectors including food, detergent, tanning, etc. **Hadder *et al*.** (**2009)** reported that pH 9-12 is optimum for anticipated industrial applications and thus the current alkaline protease is found to be more appropriate for industrial applications. Similarly, the alkaline protease from *B. subtilis* VBC7 was active over a wide range of temperature ranging from 10 to 70 °C revealing their thermostability nature. The maximum enzyme activity (100 %) was recorded at 40 °C and the enzyme retained its activity up to 80 °C with 30 % (Figure 7b). Thus the optimal temperature for protease activity was 40 °C which is concurrent with previously reported from *B. subtilis* B22 **(Uttatree and Charoenpanich, 2016)** and *B. firmus* CAS 7 **(Annamalai *et al*., 2014)**.

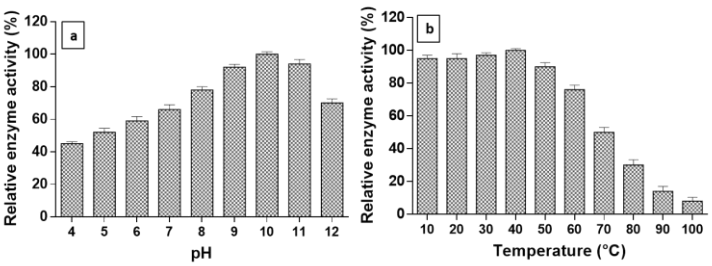

Figure 7 Paritally purified alkaline protease activity after treating at different (a) pH, (b) temperatures for one hour.

Table 4 and 5 depicted the activity of alkaline protease in the presence of various metal ions, surfactants, organic solvents and inhibitors. The enzyme activity was stimulated when treated with Mg^{2+}, Ca^{2+} and Mn^{2+} and significantly reduced by Cu^{2+}, Fe^{2+}, Zn^{2+} and Hg^{2+} treatment. A similar kind of stimulatory effect of Mg^{2+}, Ca^{2+}, Mn^{2+} was reported by **Annamalai *et al*. (2014)** and **Hadder *et al*. (2009).** These metals (Mg^{2+}, Ca^{2+}, Mn^{2+}) may prevent the unfolding of the protease by which they preserve the native form and activity of the enzyme **(Sinha *et al*., 2013)**. In contrast, metal ions, Fe^{2+} and Zn^{2+} affect the catalytic site of the enzyme and leads to reduction in enzyme activity **(Farhadian *et al*., 2015)**. Similarly, Hg^{2+} moderately reduced the activity to 75 % at 1 mM and completely minimized the activity to 23 % at 5 mM concentration and their inhibition is reported by **Farhadian *et al.* (2015)**, **Annamalai *et al*. (2014)** and **Jaouadi *et al.* (2008)**.

Table 4 Effect of different concentration of the metal ions and surfactants on alkaline protease activity. The values are expressed as mean±SD of three independent experiments.

Compounds	Conc.	% of residual activity
Metal ions		
Mg^{2+}	1 mM	112±2.6
	5 mM	135±2.9
Ca^{2+}	1 mM	120±2.4
	5 mM	150±3.5
Fe^{2+}	1 mM	88±4.0
	5 mM	63±2.6
Mn^{2+}	1 mM	106±3.5
	5 mM	136±2.9
Cu^{2+}	1 mM	91±4.0
	5 mM	78±3.5
Zn^{2+}	1 mM	93±2.0
	5 mM	75±2.9
Hg^{2+}	1 mM	75±2.6
	5 mM	23±3.5
Surfactants		
SDS	1 mM	93±4.0
	5 mM	68±2.0
Triton X-100	1 mM	100±0
	5 mM	88±2.6
Tween 80	1 mM	100±0
	5 mM	83±4.5

Table 5 Effect of different concentration of organic solvents, and inhibitors on alkaline protease activity. The values are expressed as mean±SD of three independent experiments.

Compounds	Conc.	% of residual activity
Organic solvents		
Ethanol	10 %	95±2.6
	20 %	89±4.5
Methanol	10 %	92±2.9
	20 %	82±2.0
Chloroform	10 %	100±4.0
	20 %	95±3.5
Acetone	10 %	94±2.6
	20 %	85±2.9
Hexane	10 %	100±0
	20 %	92±3.5
Inhibitors		
EDTA	1 mM	100±0
	5 mM	94±2.9
PMSF	1 mM	60±4.5
	5 mM	0±0
β-mercaptoethanol	1 mM	97±3.5
	5 mM	85±2.6

With the respect of surfactants treatment, the enzyme showed 100 % activity at 1mM of non-ionic surfactants (Triton X-100 and Tween 80) and the activity were decreased to 88 and 83 % after treated with 5mM of Triton X-100 and Tween 80 respectively. But the anionic detergent like SDS reduced the protease activity from 100 % to 93 and 68 % at 1 and 5mM concentration respectively. The alkaline protease exhibited >90 % of activity after treated with 20 % chloroform and hexane and 10 % of these solvents not interfered with enzyme activity. More than 80 % of activity was observed with 20 % ethanol, methanol and acetone treated protease sample. Generally solvents are toxic in nature and can disturb the structural and hydrophobic interactions leads to lose of enzyme of activity **(Jain *et al*., 2012)**. Nevertheless, the protease from *B. subtilis* VBC7 was active after treated with solvents indicating their solvent tolerant potential of alkaline protease. Similar solvent tolerant proteases were reported from *B. subtilis* DR8806 **(Farhadian *et al*., 2015)**, *B. subtilis* B22 (**Elumalai *et al*., 2020)**, *B. cereus* **(Shah *et al*., 2010)**, *B. licheniformis* K7A **(Hadjidj *et al*., 2018)**. The tolerance of alkaline protease against the solvents is due to the presence of large amount of acidic (negative charged) amino acids than basic (positively charged) amino acids **(Jain *et al*., 2012)** and the negative charges makes more stable by forming a hydrated ion network with cations **(Elumalai *et al*., 2020)**.

Similarly, the protease inhibitors like EDTA and β-mercaptoetahnol was not displayed any significant effect on enzyme activity at their 1 mM concentration and 10 % of activity was reduced at their 5mM concentration. But the least concentration of PMSF (1 mM) minimized the activity to 60 % and the high concentration (5 mM) completely lost the enzyme activity. The protease was not affected by EDTA revealing protease as metalloprotease class of enzymes as EDTA does not affect active site of protease. In case of PMSF, it completely inhibited the enzyme activity. **Uttatree and Charoenpanich**. **(2016)** stated that PMSF reserved the enzyme activity by blocking the serine at the active site of protease. This result is consistent with previous reports on *B. licheniformis* **(Hadjidj *et al*., 2018)** and *B. subtilis* B22 **(Elumalai *et al*., 2020)**.

CONCLUSION

Production of microbial alkaline proteases by exploiting agro wastes not only unravels the burden of environmental pollution and also increases the economic value of these wastes. In addition these wastes could also improve the yield of enzyme at cost effectively. In this present study, a potential alkaline protease producing bacterial isolate, *B. subtilis* VBC7 was screened from dairy waste dumped soil. The protease production was significantly improved (712 U/mL) in optimized media containing 10 % ground nut extract at pH 10 and 40 °C. The catalytic activity of enzyme was stimulated by metal ions such as Mg^{2+}, Ca^{2+} and Mn^{2+}. In addition, the alkaline protease exhibited higher tolerance to wide ranges of physical conditions (pH and temperatures), surfactants (SDS, Triton X-100 and Tween 80), multisolvents (ethanol, methanol, chloroform, acetone and hexane) and inhibitors (EDTA and β- mercaptoetahnol). These potential features make this enzyme as an efficient candidate for commercial production of alkaline protease at cost effective manner and their application in various industrial sectors like detergents, leather, food, etc.

Conflict of interest: All the authors declare that they don't have conflict of interest.

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