

PRODUCTION AND PARTIAL CHARACTERIZATION OF ALKALINE PROTEASE FROM *BACILLUS TEQUILENSIS* STRAINS CSGAB0139 ISOLATED FROM SPOILT COTTAGE CHEESE

Aruna K*.Jill Shah and Radhika Birmole

Department of Microbiology, Wilson College, Mumbai-7, India

*Corresponding author email: arunasam2000@yahoo.co.in Telephone - +919867233673

ABSTRACT: An alkaline protease producing strain was isolated from spoilt cottage cheese sample which was identified as *Bacillus tequilensis* strain SCSGAB0139 on the basis of morphological, cultural, biochemical characteristics and 16S rRNA sequence analysis. Primary screening for protease production was carried out by observing for zone of hydrolysis on skim milk agar, GYEA milk agar and gelatin agar plates. Physicochemical parameters like pH of the medium, incubation time and temperature, aeration and composition of the medium were optimized for maximum protease production by this isolate. Maximum yield of protease (85.67U/ml) was obtained in a medium containing peptone (5% w/v), maltose (5% w/v) and KNO₃, 0.5%; K₂HPO₄, 0.4%; trisodium citrate, 0.4; CaCl₂, 0.0002%; MgSO₄·7H₂O, 0.05%; Na₂CO₃, 1%; 1% (v/v) of a trace element solution (NH₄)₆MO₇O₂₄, 0.01%; FeSO₄·7H₂O, 0.2%; CuSO₄·5H₂O, 0.02%; ZnCl₂, 0.02%) having pH 10, inoculated with 1%(v/v) of pre-grown cell mass and incubated at 30°C on a rotary shaker (100rpm) for 48hrs. Absence of any one of the following salts viz. KNO₃, K₂HPO₄, tri-sodium citrate; MgSO₄, CaCl₂ and Na₂CO₃ from optimized medium reduced the protease production by 80% to 40%. The enzyme has an optimum pH of 9 and maintained its stability over a broad pH range between 6 and 10. Its optimum temperature is 30°C, and exhibited a stability of up to 65°C. Among metal ions only Ca²⁺ and Mg²⁺ ions enhanced the enzyme activity up to 105% and 107% respectively while other metal ions reduced the activity by 40% where as EDTA exhibited the least inhibitory effect upon the enzyme. Protease activity was enhanced in the presence of isopropanol and marginally reduced in the presence of other organic solvents studied. The crude enzyme showed stability towards various surfactants such as Tween-20, Tween- 80, SDS and Triton X-100. It also showed excellent stability and compatibility with commonly used laundry detergents (Ariel, Surf excel and Surf Blue). The enzyme retained its activity in 2% H₂O₂ indicating it to be bleach-stable. The present findings show that the protease from *Bacillus tequilensis* strain SCSGAB0139 is alkali- and detergent- stable and hence, when this protease was applied to remove blood stains from cotton fabrics indicated its potential use in detergent formulations.

Key words: *Bacillus tequilensis*; alkaline protease; detergent- stable

INTRODUCTION

Proteases are degradative enzymes which catalyze the cleavage of peptide bonds in other proteins. They represent one of the three largest groups of industrial enzymes and account for about 60% of the total worldwide sale of enzymes which occupy a pivotal position with respect to their applications (Rao et al 1998; Amoozegara et al., 2004). Proteases execute a large variety of functions and have numerous applications in bakery, brewing, detergent, food, diagnostic reagents, pharmaceutical, leather industries, feeds modification, peptide synthesis, silk, silver recovery from X-ray/photographic film, soy processing, and bioremediation processes (Anwar and Saleemuddin, 1998; Gupta et al., 2002; Rao et al., 1998). Proteases are widespread in nature and among the various proteases; bacterial proteases are the most significant, compared with animal and fungal proteases (Fujiwara et al., 1991). Among bacteria, *Bacillus* sp. are specific producers of extra-cellular proteases (Priest, 1977) and the alkaline proteases produced by *Bacillus* species are by far the most important group of enzymes produced commercially (Puri et al., 2002).

Several *Bacillus* species involved in protease production are e.g. *B. cereus* (Ammar et al., 1991; Banik and Prakash, 2004), *B. stercophilus* (Sookkheo et al., 2000), *B. mojavensis* (Beg and Gupta, 2003), *B. megaterium* (Gerze et al., 2005), *B. subtilis* (Soares et al., 2005), *Bacillus licheniformis* (Claim et al., 2002), *Bacillus clausii* (Christiansen and Nielsen, 2002), *Bacillus brevis* (Banerjee et al., 1999), *Bacillus cereus* BG1 (Ghorbel et al., 2005), *Bacillus anthracis* S-44 and *B. cereus* var. mycoides, S-98 (Ammar et al., 1991), *Bacillus thuringiensis* (Foda et al., 2013), *Bacillus circulans* MTCC 7942 (Patil and Chaudhari, 2013), *Bacillus coagulans* (Asokan and Jayanthi, 2010), *Bacillus marmarensis* (Denizci et al., 2010), *Bacillus firmus* (Vadlamani and Parcha, 2012), *Bacillus stratosphericus* (Bindu and Reddy, 2013), *Bacillus polymyxa* (Lawal et al., 2014), *B. Lentus* (Bettel et al., 1992), *Bacillus alcalophilus sub sp. halodurans* KP 1239 (Takii et al., 1990), *B. amyloliquefaciens* (Wells et al., 1983), *B. subtilis* var. *amylosacchariticus* (Yoshimoto et al., 1988), *Bacillus intermedius* (Itskovich et al., 1995), *B. thermoruber* BT2T (Manachini et al., 1988), *Bacillus clausii* GMBE 22 (Kazan et al., 2005), *Bacillus pumilus* (Wan et al., 2009), *Bacillus cohnii* APT5 (Tekin et al., 2012), *Bacillus fastidiosus* (Shumi et al., 2004), *Bacillus pseudofirmus* (Gessesse et al., 2003), *Bacillus pantotheneticus* (Shikha et al., 2007), *Bacillus aquimaris* strain VITP4 (Shivanand and Jayaraman, 2009), *Bacillus proteolyticus* CFR3001 (Bhaskar et al., 2007), *Bacillus laterosporus* (Usharani and Muthuraj, 2010), *Bacillus amovivorus* (Sharmin et al., 2005), *Bacillus flexus* (Verma et al., 2013) and *Bacillus horikoshii* (Joo et al., 2002).

The present paper reports isolation and identification of alkaline protease producing strain from the spoilt cottage cheese sample. We also describe optimization of parameters for the production of protease along with some characterization of the enzyme and its application as an effective additive in laundry detergents.

MATERIALS AND METHODS

Enrichment, Isolation and identification of protease producing organism

One gram of spoilt cottage cheese sample was inoculated in 2 sterile 100 ml Nutrient broth flasks and incubated under shaker (100 rpm) and static conditions at 30°C for 24 hrs. Enriched broth was then appropriately diluted and isolated on skim milk agar plate containing peptone (0.1%), NaCl (0.5%), agar (2%) and skim milk (10%) incubated at 30°C for 24 hrs. Colonies exhibiting a zone of clearance were further screened for maximum protease production and were maintained on nutrient agar slant (containing 1% casein) at 4°C.

Protease assay

Each isolate was inoculated in 100 ml nutrient broth containing 1% casein and incubated for 24 h on a rotary shaker (100rpm) at 30°C. The cells were separated by centrifugation of growth in broth at 5000 rpm for 20 min. The supernatant contained enzyme and was considered as crude enzyme extract. Protease activity was measured using caseinolytic assay (Zambare et al., 2007) with some modifications. The culture supernatant (0.1 ml) was incubated in 9 ml of 1% casein prepared in phosphate buffer (pH-7) at 30°C for 20 min. The reaction was stopped by 1.5 ml of trichloroacetic acid (5% w/v). After 10 min the entire mixture was centrifuged at 5000 g for 15 min. Absorbance of the supernatant was measured by modified Folin Ciocalteu method (Lowry et al., 1951), against inactive enzyme. A standard graph was generated using standard tyrosine of 10-100 µg ml⁻¹. One unit of protease activity was defined as the amount of enzyme, which liberated 1 µg tyrosine per min at 30°C. The isolate exhibiting maximum protease production was identified on the basis of morphological, cultural and biochemical tests reported by Gatson et al. (2006) and Bonala and Mangamoori (2012) and further confirmed by 16S rRNA sequence analysis carried out by SciGenom Labs Pvt. Ltd. Kerala, India.

Optimization of culture conditions for protease production

Eight different media were investigated for maximum protease production by the selected isolate viz. A- (Khosravi-Darani et al., 2008), B- (Pawar et al., 2009), C- (Mehrotra et al., 1999), D- (Das and Prasad, 2010), E- (Nascimento and Martins, 2004), F- (Adinarayana et al., 2003), G- (Oskouie et al., 2008), H- (Ahmed et al., 2008) and I- (Nadeem et al., 2008). One ml of the culture with (0.1 OD_{530nm}) was inoculated in 50 ml of the specific production medium and incubated at 30°C for 24 h on a rotary shaker (100 rpm). Hundred ml of each of the production medium was inoculated with 2% (v/v) of pre-grown cell suspension, incubated at 30°C for 24 h on a rotary shaker (100 rpm) and the cells were centrifuged at 5000 rpm for 20 minutes to obtain supernatant which was used as a crude enzyme for the study of various parameters. One parameter at-a-time approach was used and all the experiments were carried out in triplicates. The culture conditions (incubation period, aeration, temperature, pH, carbon, nitrogen source and deletion of various mineral salt concentrations) were optimized for maximum protease production by the selected isolate. The enzyme activity was determined for each medium by Folin-Lowry assay. For all the further experiments, the medium which gave maximum protease yield was used to optimize the physicochemical parameters for the production of protease enzyme.

The optimized medium was used for further investigation of various parameters for maximum protease yield by the selected isolate. Optimization of culture conditions for the highest protease production was studied in G medium. The alkaline medium G (pH 9) used for protease production contained: sucrose (1%); yeast extract (0.5%); KNO₃ (0.5%); K₂HPO₄, 0.4%; trisodium citrate, 0.4%; CaCl₂, 0.0002%; MgSO₄·7H₂O, 0.05%; Na₂CO₃, 1%. After autoclaving and cooling the medium, 1% (v/v) of a trace element solution (NH₄)₆MO₇O₂₄, 0.01%; FeSO₄·7H₂O, 0.2%; CuSO₄·5H₂O, 0.02%; ZnCl₂, 0.02%) was added. One salt component such as KNO₃, MgSO₄·7H₂O, K₂HPO₄, CaCl₂, Na₂CO₃ and Trisodium citrate from the production medium was excluded from the optimized medium at a time to determine the effect of its deletion on protease enzyme production by the selected isolate (complete medium was considered as control).

Protease production was determined at various time intervals such as 24, 48 and 72 h which were incubated at 30°C on a rotary shaker (100 rpm). The effect of aeration on protease production was studied by incubating one culture flask on shaker (100rpm) and other under static condition at 30°C for 24 hours. Optimum culture temperature for protease production was determined in the range of 30°C, 35°C, 45°C, 55°C and 65°C on shaker condition (100rpm) for 24 h. Optimum pH for protease production was determined in the culture grown in G medium at different pH range from 7 to 12 (1N NaOH and 1N HCl were used for adjusting pH of the medium). Effect of different carbon sources on protease production was checked by adding 1% w/v of sucrose, glucose, maltose, mannitol, starch, glycerol, xylose and galactose. Optimized sugar (1%-2% w/v at increments of 1%) was tested for higher yield of protease. Different organic nitrogen sources (1% w/v) such as meat extract, yeast extract, peptone and tryptone and inorganic nitrogen sources (0.5%) such as ammonium nitrate, sodium nitrate and potassium nitrate were checked for the maximum protease production. Optimized nitrogen source (1%-2% w/v at increments of 1 %) was tested for maximum yield of protease.

Effect of different physicochemical parameters on protease activity

Protease enzyme from the new isolate was assayed to determine the optimum conditions of temperature and pH. The optimal temperature was determined from 25, 35, 45, 55, 65, 75 and 85°C to determine the optimum temperature for enzyme activity. The protease was assayed at various pH ranging from 6 to 10 in the following buffer systems: Phosphate buffer (pH 6), Tris-HCl buffer (pH 7-9) and glycine-NaOH buffer (pH 10). Effect of metals on enzyme activity was measured with different metals namely (0.1% w/v) HgCl₂, MgCl₂, ZnCl₂, CaCl₂, KCl, NaCl and CdCl₂ and metal chelator EDTA. The presence of organic solvents (10% v/v) such as acetone, benzene, ethanol, methanol, isopropanol and butanol on protease activity was also investigated. The surfactants (1% v/v) such as SDS, Tween 80, Triton X-100 and commercial detergents (1% w/v of Surf Excel, Surf Blue and Ariel) were tested on the protease. To check the effect of an oxidizing agent on protease activity, different concentrations (0.5- 2.0%) of Hydrogen Peroxide was used. The crude enzyme was pre-incubated with the above-mentioned respective solvents, metal ions and surfactants for 30mins at 30°C. The residual activity (%) was measured by standard protease assay. The control was kept with enzyme without metals, solvents and oxidizers (100%).

Enzyme purification

The crude enzyme extract was subjected to various saturation concentrations of ammonium sulphate (40% saturation to 90% saturation) to precipitate the enzyme. The concentration which gave precipitate was then used to precipitate the enzyme from 100 ml of production medium. The precipitate was collected by centrifugation at 5000 rpm for 20 minutes at 4°C. The precipitate pellet was dissolved in 5 ml of tris-HCl buffer pH 9 and dialyzed against the same buffer. Its protein content and enzyme activity was determined after each step.

Application as a potential detergent additive

Wash performance of protease was evaluated by subjecting the blood stain removal test from cotton fabrics. Briefly, application of protease as a detergent additive was studied on white cotton cloth which was cut into 3 cm² pieces and stained with 1 ml of fresh human blood. The stained cotton pieces were allowed to dry overnight. Following overnight drying; the stained pieces were taken in separate flasks (Banerjee et al., 1999; Sharma and Aruna, 2012). The following sets were prepared and studied:

- 1) Flask with distilled water (100 ml) + stained cloth.
- 2) Flask with distilled water (100 ml) + stained cloth + 1 ml of aerial detergent (7 mg/ml).
- 3) Flask with distilled water (100 ml) + stained cloth + 1 ml of aerial detergent (7 mg/ml) + 1 ml (1:5 diluted) purified enzyme.

The above flasks were incubated at 55°C for 15 minutes. After incubation, cloth pieces were taken out, rinsed with water and dried. Visual examination of various pieces exhibited the effect of enzyme in stain removal. Untreated cloth piece stained with blood was taken as control.

RESULTS AND DISCUSSION

Enrichment, screening, isolation and identification of protease producing isolate

One gm of spoilt cottage cheese sample was inoculated in 2 sterile 100 ml nutrient broth with 1% casein flasks and incubated at 30°C for 24hrs under shaker (100rpm) and static conditions. A loopful of enriched broth from each flask was streaked on skim milk agar plates and was incubated at 30° C for 24 hours. After 24 hours 8 different colonies were obtained showing zone of clearance indicating production of protease by the isolates. These 8 isolates (C1- C8) were when further screened for protease activity; colony number 2 (C2) which was confirmed to have protease activity on skim milk agar (Figure 1) also exhibited maximum protease activity (65.56U/ml) as shown in figure 2. This isolate (C2) was selected for further optimization studies and depending upon it's morphological, cultural and biochemical test suggested by Gatson et al. (2006) and Bonala and Mangamoori (2012); it was identified as *Bacillus tequilensis* and strain number SCSGAB0139 was further confirmed by 16S rRNA sequence analysis. It is a sporulating, gram positive bacillus, phylogenetically closely related to *Bacillus subtilis* (Gatson et al., 2006). Microbes particularly spore bearers like *Bacillus* sp. are known to cause spoilage of milk products like cottage cheese which are rich in proteins (Tharmaraj and Shah, 2009; Ternström et al., 1993). This might be the one of the reasons we could isolate *Bacillus* species producing protease from spoiled cottage cheese sample. A thermophilic protease producer *Bacillus subtilis* WIFD5 was also reported to be isolated from milk sample (Sharma and Aruna, 2011).

Optimization of culture conditions for protease production

Bacillus tequilensis exhibited maximum yield of protease in G medium (67.03U/ml) [sucrose (1%); yeast extract (0.5%); KNO₃ (0.5%); K₂HPO₄, 0.4%; trisodium citrate, 0.4%; CaCl₂, 0.0002%; MgSO₄·7H₂O, 0.05%; Na₂CO₃, 1%; pH 9 and 1% (v/v) of a trace element solution (trisodium citrate, 1%; (NH₄)₆MO₇O₂₄, 0.01%; FeSO₄·7H₂O, 0.2%; CuSO₄·5H₂O, 0.02%; ZnCl₂, 0.02%)] which was followed by medium E (44.93U/ml) and H(40U/ml) whereas Medium F supported minimum protease yield (8.84U/ml) as shown in figure 3. Medium F lacked the components such as KNO₃, trisodium citrate, CaCl₂, Na₂CO₃, (NH₄)₆MO₇O₂₄, CuSO₄·5H₂O and ZnCl₂ whereas medium G which supported maximum protease yield contained all the above mentioned chemicals. Several different types of defined as well as undefined media have also been used in the past for production of alkaline protease by alkalophiles viz. Horikoshi-I media (Horikoshi,1996), glucose-yeast extract-asparagine medium (GYA) (Sen and Satyanarayana, 1993), MYGP medium (Laxman et al., 2005; Srinivasan et al.,1983), peptone-yeast extract-glucose medium (PYEG) (Gee et al., 1980), alkaline casein agar medium (Durham, 1987), wheat meal medium (Fujiwara and Yamamoto,1987),Yeast extract casein medium (Tambekar and Tambekar,2013), synthetic medium(Johnvesly and Naik, 2001), modified fermentation medium (MFM) (Rathod and Pathak, 2014), Dye's medium with 0.2 per cent Bengal gram powder (Kanchana and Padmavathy, 2010), Zobell marine broth (Jayaraman and Sridharan, 2012)and GYP (Kumar and Bhalla, 2004).



Figure 1: Colonies C1 and C2 showing zone of hydrolysis on Skim milk agar

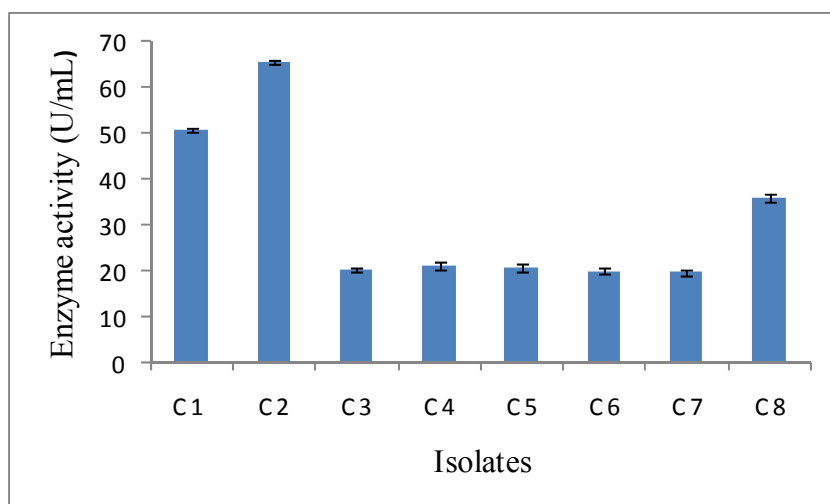


Figure 2: Protease assay of different isolates

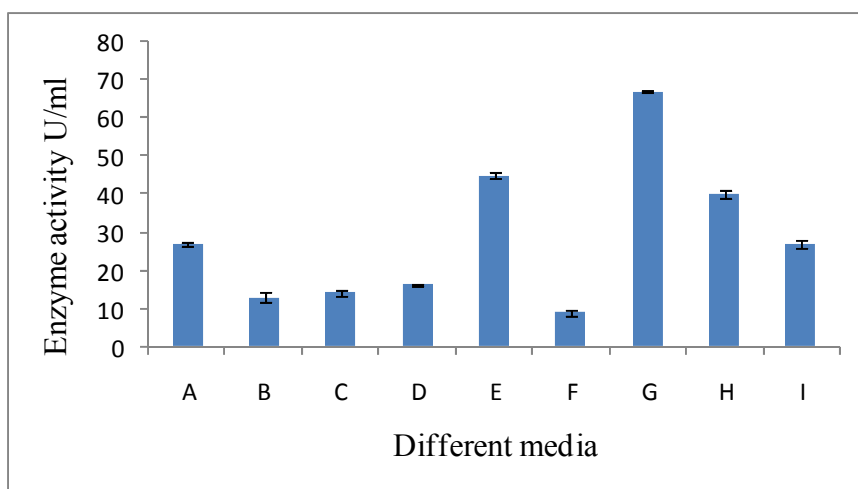


Figure 3: Optimization of media for protease production by *Bacillus tequilensis*

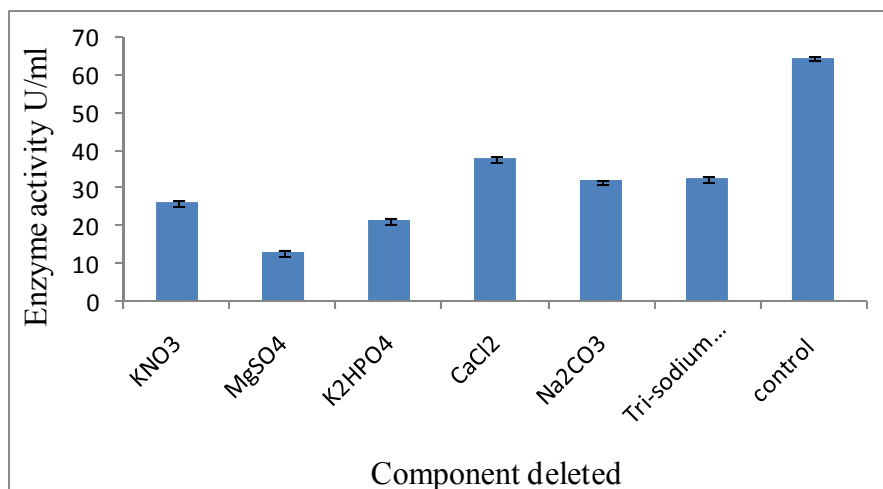


Figure 4: Effect of deleted component on protease production by *Bacillus tequilensis*

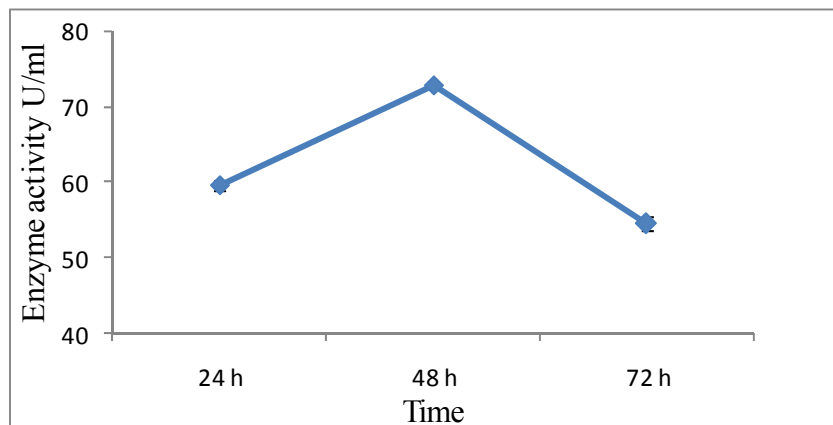


Figure 5: Effect of incubation period on protease production by *Bacillus tequilensis*

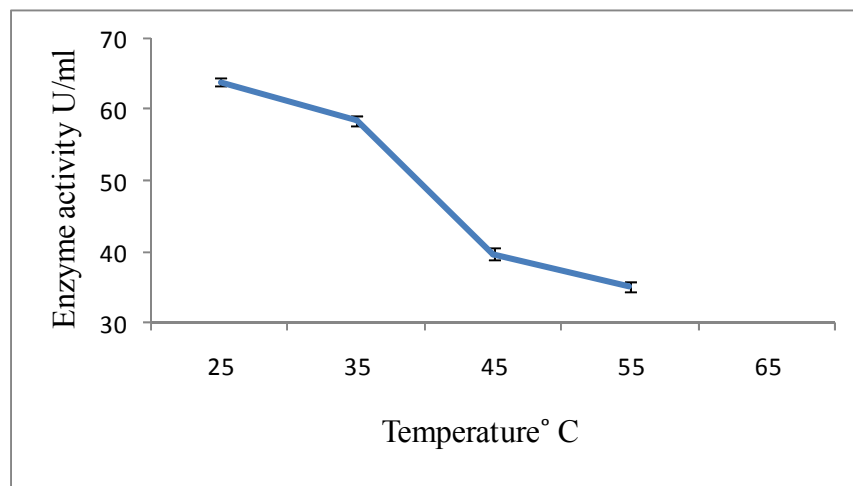


Figure 6: Effect of temperature on protease production by *Bacillus tequilensis*

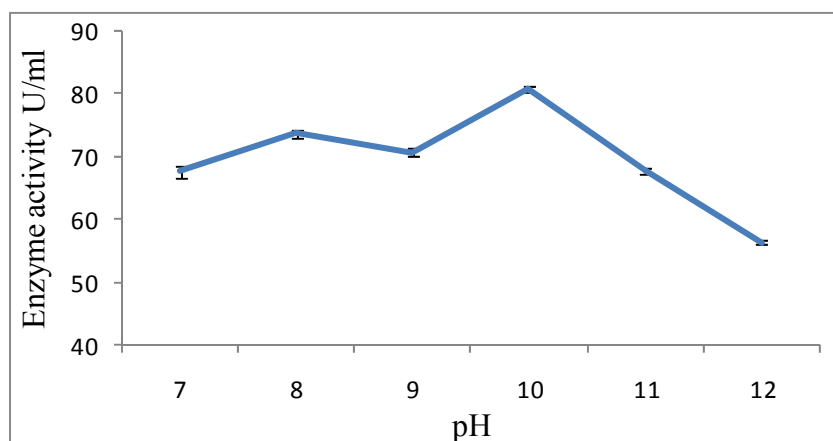


Figure 7: Effect of pH on protease production by *Bacillus tequilensis*

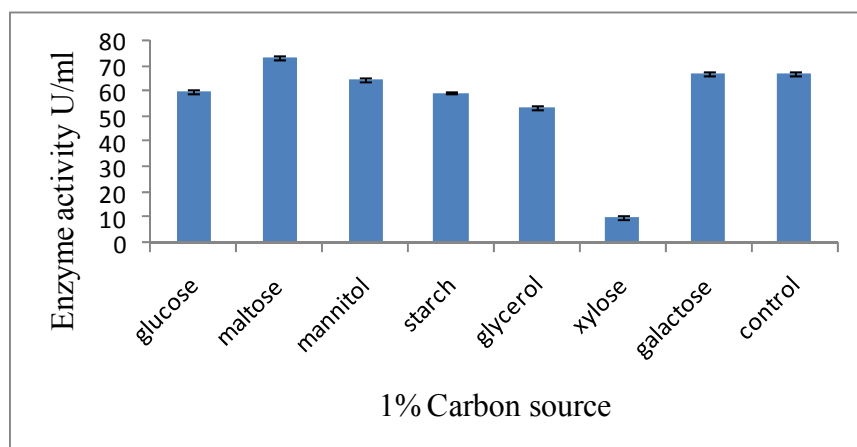


Figure 8: Effect of carbon sources on protease production by *Bacillus tequilensis*

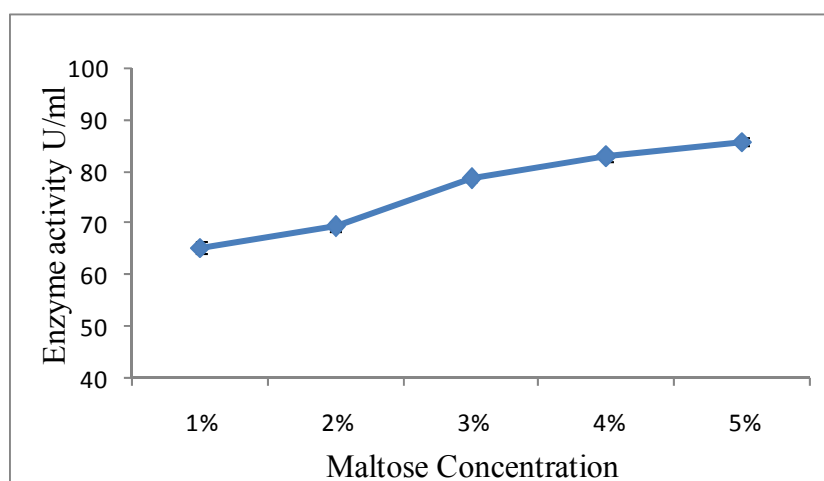


Figure 9: Effect of maltose concentration on protease production by *Bacillus tequilensis*

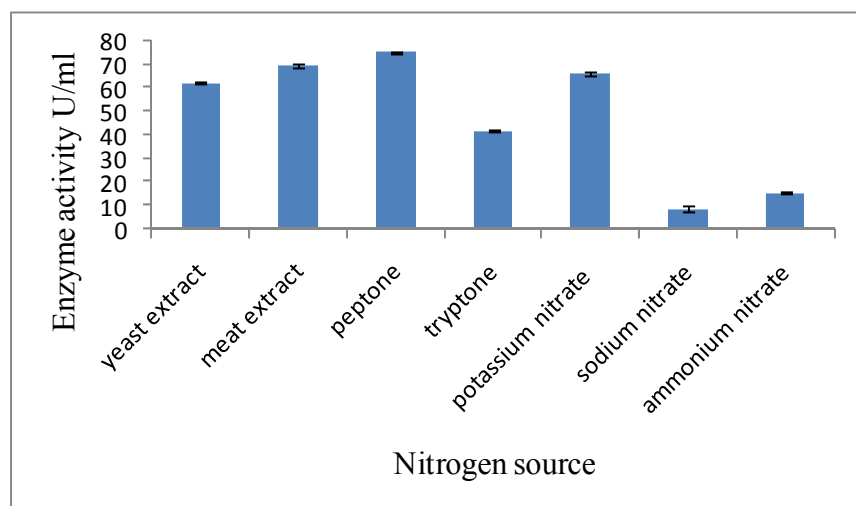


Figure 10: Effect of different nitrogen sources on protease production by *Bacillus tequilensis*

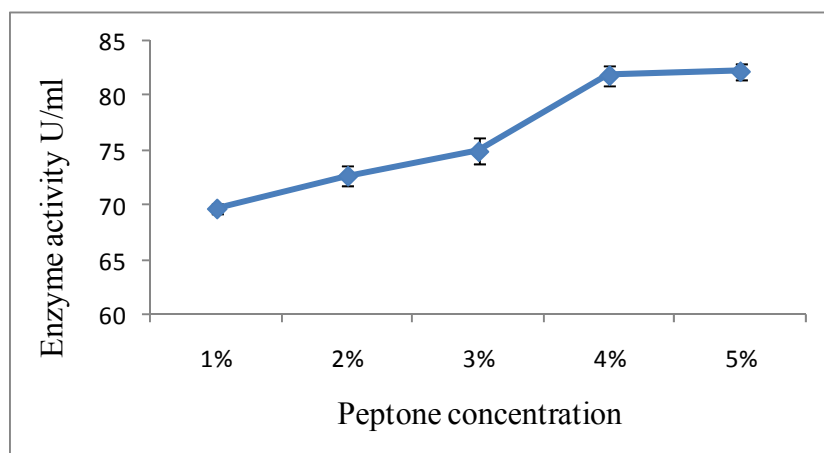


Figure 11: Effect of peptone concentration on protease production by *Bacillus tequilensis*

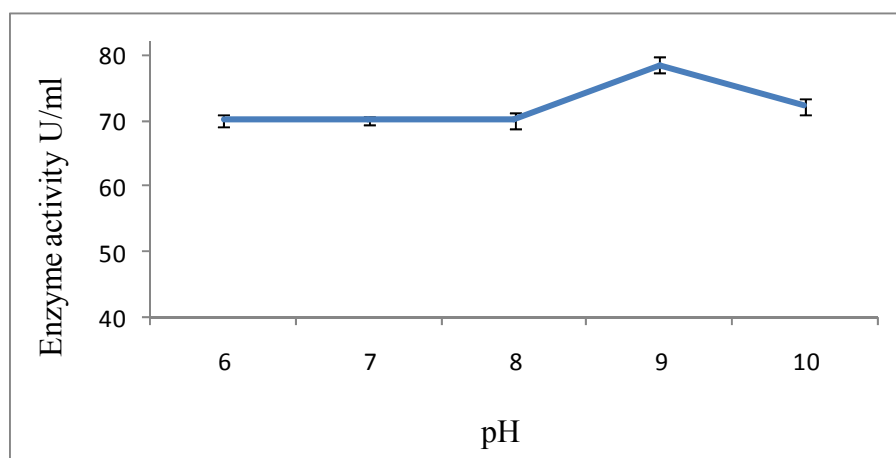


Figure 12: Effect of pH on protease activity of *Bacillus tequilensis*

In the deletion assay studies of medium components, when $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and K_2HPO_4 omitted from the medium G individually the protease yield by *Bacillus tequilensis* was reduced by 80% and 67% respectively as shown in figure 4. However, KNO_3 (0.5%), trisodium citrate (0.4%) CaCl_2 (0.0002%) and Na_2CO_3 (1%) deleted from the medium (one at a time) lowered the protease yield by 40% to 60% (Figure 4). *Bacillus tequilensis* requires metal cations such as Mg^{+2} and Ca^{+2} and also K_2HPO_4 , KNO_3 , trisodium citrate in production medium G for better yield of protease. Divalent metal ions such as calcium, cobalt, copper, iron, magnesium, manganese, and molybdenum are required in the fermentation medium for optimum production of alkaline proteases (Kumar and Takagi, 1999). Our results corroborate the earlier findings of metal ions (Ca^{+2} and Mg^{+2}) along with KH_2PO_4 substantially enhancing the production of protease enzyme while the increase of enzyme production was small when these salts were used individually and it was sufficiently low in the absence of these salts (Janssen et al., 1994; Banerjee et al., 1999; Wang et al., 2005; Haddar et al., 2009; Uyar et al., 2011; Ellaiah et al., 2002; Nadeem et al., 2007; Varela et al., 1996). Magnesium ion was found to be more effective than other metal ions in protease production by *Bacillus licheniformis* NCIM-2042 (Bhunia et al., 2010), *Bacillus cereus* CA15 (Uyar et al., 2011), *B. subtilis* RSKK96 (Akcan and Uyar, 2011), *Bacillus pseudofirmus*, *Cohnella thermotolerans* and *Bacillus odyseyi* (Tambekar and Tambekar, 2013). Hanlon et al. (1982) showed that the depletion in magnesium ion results in decreased rate of enzyme production that ultimately affects the mechanism of protease synthesis. Calcium chloride has also been used by several workers as a source of calcium ions in protease producing media (Qadar et al., 2009). The past studies indicated that incorporation of 0.01% calcium chloride in the fermentation medium produced maximum protease by *Bacillus* sp. PCSIR EA-3 as compared to the medium having no calcium ions (Qadar et al., 2009).

Potassium phosphate has been used as a source of phosphate and buffering agent in the medium for production of alkaline protease in most studies (Mao et al., 1992; Rahman et al., 1994; Moon and Parulekar, 1991; Takii et al., 1990; Rathakrishnan and Nagarajan, 2012; Sarker et al., 2013). Sodium carbonate was found generally to be the major source of alkalinity and its addition to the production media enhanced the growth of alkalophilic microorganisms (Horikoshi and Akiba, 1982; Johnvesly and Naik, 2001; Takagi et al., 1995; Sarker et al., 2013). Many researchers reported that maximum yield of alkaline protease production attained in presence of KNO₃ in the production medium (Lakshmi et al., 2014; Dahot, 1993; Ahmed et al., 2010; Johnvesly and Naik, 2001). Trisodium citrate was shown to have ability to control pH variations in production medium for protease (Kumar and Takagi, 1999; Kumar, 2007). Ferrero et al. (1996) reported the use of trisodium citrate along with MgSO₄, CaCl₂, MnSO₄ and ZnSO₄ for protease production by *Bacillus licheniformis* MIR 29.

Bacillus tequilensis showed protease production starting from 24 h of growth and reached a maximum in 48 h and then it declined with further increase in duration of incubation (Figure 5). Maximum production of protease with 48 to 72 hours of incubation by bacteria was reported by Hoshino et al. (1995) and Shumi et al. (2004). In general, the synthesis of protease in *Bacillus* species is constitutive or partially inducible and is controlled by numerous complex mechanisms operative during the transition state between exponential growth and the stationary phase (Strauch and Hoch, 1993; Priest, 1977). It was suggested that protease production was directly linked to the culture being metabolically active (Kanchana and Padmavathy, 2010). However, Gupta et al. (2002) reported that production of extracellular protease is related to manifestation of nutrient deficiency at the beginning of stationary phase. Ward (1995) reported that *Bacillus* sp. usually produce more protease during the late exponential phase. The function of this enzyme is obscure, but its synthesis is correlated with the onset of a high rate of protein turnover during sporulation in certain bacilli. Similar to our results were reported where alkaline protease production was maximum in 48h for *Bacillus amovivorus* (Sharmin et al., 2005), *Bacillus aquimaris* (Shivanand and Jayaraman, 2009), *Virgibacillus dokdonensis* (Jayaraman and Sridharan, 2012), *Bacillus* sp. I-312 (Joo and Chang, 2005), *Bacillus subtilis* (Ahmed et al., 2010), *B. subtilis* PE-11 (Adinarayana et al., 2003), *B. pumilus* D-6 (Bajaj and Jamwal, 2013), *Bacillus* sp. PCSIR EA-3 (Qadar et al. 2009), *B. subtilis* MTCC 9102 (Kumar et al., 2010), *Bacillus firmus* (Vadlamani and Parcha, 2012), *Bacillus subtilis* PCSIR5 (Nadeem et al., 2006), *Bacillus coagulans* PSB-07 (Olajuyigbe and Ehiosun, 2013), *Bacillus subtilis* SHS-04 (Olajuyigbe, 2013) and *Bacillus licheniformis* P003 (Sarker et al., 2013). However, many researchers recorded maximum protease production time as 18h for *Bacillus horikoshii* (Joo et al., 2002) and 24 h for *Bacillus* sp. strain CR-179 (Sepahy and Jabalameli, 2011), *Bacillus mojavensis* (Beg et al., 2003), *Bacillus fastidiosus* (Shumi et al., 2004), *Bacillus alcalophilus* subsp. halodurans (Takii et al., 1990), *B. pantotheneticus* (Shikha et al., 2007), *B. pumilus* CBS (Jaouadi et al., 2008), *B. pseudofirmus* (Gessesse et al., 2003) and *B. subtilis* P13 (Pillai et al., 2011). Variety of optimum time for maximum yield of protease has been reported as 30h for *B. cereus* (Kanmani et al., 2011), 36h for *Bacillus circulans* MTCC 7942 (Patil and Chaudhari, 2013), 60 h for *Bacillus pumilus* MK6-5 (Kumar, 2002), 72h for *Bacillus flexus* (Verma et al., 2013), *Bacillus licheniformis* BBRC 100053 (Nejad et al., 2009), *Bacillus subtilis* SSD-I (JQ747516) (Pedgeet al., 2013), *Bacillus polymyxa* (Maal et al., 2009), *B. subtilis* 3411 (Pastor, 2001), *Bacillus licheniformis* BI8 (Lakshmi, 2013), *Bacillus clausii* GMBAE 42 (Kazan et al., 2005), 96h for *B. licheniformis* and *B. coagulans* (Asokan and Jayanthi, 2010), *Bacillus proteolyticus* CFR3001 (Bhaskar et al., 2007), *Bacillus* sp. K-30 (Naidu and Devi, 2005) and 120 h for *Bacillus subtilis* RSKK96 (Akcan and Uyar, 2011).

During fermentation, the aeration rate indirectly indicates the dissolved oxygen level in the fermentation broth. Protease production by *Bacillus tequilensis* was found to be the maximum under aeration condition (100rpm) 65U/ml while static condition exhibited 85% lesser yield (10U/ml). This is in agreement with previous report which showed that *B. proteolyticus* CFR3001 (Bhaskar et al., 2007), *Virgibacillus dokdonensis* (Jayaraman and Sridharan, 2012), *Bacillus polymyxa* (Maal et al., 2009) and *Bacillus circulans* MTCC 7942 (Patil and Chaudhari, 2013) needed agitation rate of 100 rpm while *Bacillus firmus* (Vadlamani and Parcha, 2012) required 120rpm agitation speed. Optimum yields of alkaline protease were recorded at 200 rpm for *B. subtilis* ATCC 14416 (Chu et al. 1992), *Bacillus subtilis* SHS-04 (Olajuyigbe, 2013), *Bacillus* sp. I-312 (Joo and Chang, 2005) and *B. licheniformis* (Sen and Satyanarayana 1993). There were reports where lowering the aeration rate had caused a drastic reduction in the protease yields (Moon and Parulekar, 1991). Therefore our results support these findings (Sepahy and Jabalameli, 2011; Nejad et al., 2009; Liu and Tzeng, 1998; Gupta et al., 1999; Banerjee et al., 1999; Joo et al., 2002; Asokraja et al., 2012; Nadeem et al., 2006; Akcan and Uyar, 2011; Kumar, 2002; Beg et al., 2003). This indicates that oxygen supply is an important limiting factor for growth as well as for protease synthesis.

The enzyme was produced at the temperature between 30°C and 55°C but the maximum production of protease by *Bacillus tequilensis* was obtained at 30°C and no growth was observed at 65°C (Figure 6). Similar results were reported for *Bacillus* sp. B21-2 (Fujiwara et al., 1991), *Bacillus pantotheneticus* (Shikha et al., 2007), *Bacillus polymyxa* (Maal et al., 2009), *Bacillus licheniformis* and *Bacillus coagulans* (Asokan and Jayanthi, 2010). Studies by Frankena et al. (1986) showed that a link existed between enzyme synthesis and energy metabolism in bacilli, which was controlled by temperature and it varied from organism to organism. It was reported that the maximum protease production was achieved at 45°C (Manachini et al., 1988; Sen and Satyanarayana, 1993), while 60°C was the best temperature for protease production in case of *B. subtilis* PE-11 and *B. Stearothermophilus* F1 (Adinarayana et al., 2003; Rahman et al., 1994). Subsequently, 37°C was found to be the best temperature for protease production for certain *Bacillus* sp. viz. *B. firmus* (Moon and Parulekar, 1991), *Bacillus circulans* MTCC 7942 (Patil and Chaudhari, 2013), *Bacillus proteolyticus* CFR3001 (Bhaskar et al., 2007), *Bacillus firmus* (Vadlamani and Parcha, 2012), *Bacillus mojavensis* (Beg et al., 2003), *Bacillus aquimaris* (Shivanand and Jayaraman, 2009), *Bacillus subtilis* RSKK96 (Akcan and Uyar 2011), *Bacillus clausii* GMBAE 42 (Kazan et al., 2005), *Bacillus stratosphericus* (Bindu and Reddy et al., 2013), *B. amovivorus* (Sharmin et al., 2005) and *Bacillus coagulans* PSB-07 (Olajuyigbe and Ehiosun, 2013).

The culture pH also strongly affects many enzymatic processes and transport of various components across the cell membrane (Moon and Parulekar, 1980). The enzyme was produced between pH 8 and pH 12 but the maximum production of protease by *Bacillus tequilensis* was obtained at pH 10 (Figure 7). It indicated that that *Bacillus tequilensis* can be classified as alkaliphilic Bacilli, since alkaliphiles are defined as organisms that grow optimally at alkaline pH, with pH optima for growth being in excess of pH 8 and some being capable of growing at pH > 11 (Horikoshi, 1999; Grant and Jones, 2000). For increased protease yields from these alkaliphiles, the pH of the medium must be maintained above 7.5 throughout the fermentation period (Aunstrup, 1980). Past researchers have also reported the pH 10 value similar to our results for maximum protease production by *Bacillus* species viz. *Bacillus circulans* MTCC 7942 (Patil and Chaudhari, 2013), *Bacillus licheniformis* B18 (Lakshmi, 2013), *Bacillus subtilis* strain VV (Vijayaraghavan et al., 2012), *Bacillus licheniformis* BBRC 100053 (Nejad et al., 2009), *B. pantotheneticus* (Shikha et al., 2007), *Bacillus licheniformis* and *Bacillus coagulans* (Asokan and Jayanthi, 2010). However, the maximum yield of protease at pH 9 was reported by some strains of Bacilli such as *Bacillus proteolyticus* CFR3001 (Bhaskar et al., 2007), *Bacillus cereus* (Uyar et al., 2011), *Bacillus flexus* (Verma et al., 2013), *Bacillus subtilis* RSKK96 (Akcan and Uyar, 2011), *Bacillus horikoshii* (Joo et al., 2002), *Bacillus* sp. AGT (Asokraja et al., 2012), while some strains have optimum pH 11 for *Bacillus* sp. I-312 (Joo and Chang, 2005), *B. cohnii* APT (Tekin et al., 2012) and pH 8 and 8.5 for *Bacillus coagulans* PSB-07 (Olajuyigbe and Ehiosun, 2013) and *B. amovivorus* (Sharmin et al., 2005) respectively.

Among the various carbon sources, *B. tequilensis* produced maximum protease (73.60U/ml) in presence of 1% maltose whereas 1% xylose repressed the protease yield (10.30U/ml) while other sugars showed almost the same yield as that of maltose (Figure 8). Parallel results were reported where maltose was optimum sugar for protease yield in case of certain strains of bacteria such as *Bacillus subtilis* strain VV (Vijayaraghavan et al., 2012), *Bacillus licheniformis* NCIM 2044 (Sathyavathan and Kavitha, 2013), *Bacillus* sp. (Prakasham et al., 2006), *Bacillus* sp. strain CR-179 (Sepahy and Jabalameli, 2011), *Bacillus* sp. APP1 (Chu, 2007), *Bacillus licheniformis* BBRC 100053 (Nejad et al., 2009), *B. amyloliquefaciens* PFB-01 (Olajuyigbe and Ogunyewo, 2013), *Bacillus* sp. AGT (Asokraja et al., 2012), *Bacillus licheniformis* B18 (Lakshmi, 2013) and *Thermoactinomyces* sp. HS682 (Tsuchiya et al., 1991). Johnvesly and Nailk (2001) showed that 1% glucose as well as xylose (w/v) repressed completely the synthesis of alkaline protease in *Bacillus* sp. JB-99. However, in the present study glucose was found to be a relatively good carbon source for enzyme production but xylose repressed the protease synthesis. The *Bacillus* sp. SMIA-2 was also reported to be capable of utilizing a wide range of carbon sources but it also produced lower yield of protease in presence xylose (Nascimento and Martins, 2004). On the contrary *Bacillus subtilis* strain AKRS3 was found to produce optimum production of protease in presence xylose (Ravishankar et al., 2012). When different concentrations of maltose used, *B. tequilensis* exhibited maximum yield of protease in presence of 5% maltose (Figure 9).

Results revealed that complex nitrogen sources better supported alkaline protease production over inorganic nitrogen compounds. *B. tequilensis* exhibited maximum yield of protease 75.80U/ml and 66.33U/ml in presence organic nitrogen source 1% peptone and inorganic nitrogen source 0.5% KNO₃ respectively (Figure 10). However, 0.5% of sodium nitrate and ammonium nitrate repressed the yield of protease. To optimize the peptone concentration as nitrogen source for the production of alkaline protease production, experiments were conducted with increase in peptone supplementation in the medium. The results indicated that the increase in protease production was marginal with increase in peptone concentration in the medium (10% increase with peptone concentration from 1 to 5%) (Figure 11). Vijayalakshmi et al. (2013) reported peptone has increased the protease production in *B. subtilis* and *B. licheniformis*.

Similar observations were also noticed in case of protease production by different microbial species (Prakasham et al., 2006; Sathyavathan and Kavitha, 2013; Pandey et al., 2000; Nejad et al., 2009; Lakshmi, 2013; Fujiwara and Yamamoto, 1987; Cheng et al., 1995; Shivanand and Jayaraman, 2009; Chu, 2007). However, peptone was reported to be the poorest organic nitrogen source for alkaline protease production for *Bacillus brevis* (Banerjee et al., 1999), Sinha and Satyanarayana (1991) observed that an increase in protease production by the addition of potassium nitrate in thermophilic *B. licheniformis* which was in accordance with our results. In current studies ammonium nitrate has repressed the protease yield. Readily metabolizable nitrogen ions such as ammonium ion concentrations in medium will repress the enzyme synthesis (Kumar and Takagi, 1999). However, there were reports where replacement of sodium nitrate in the basal medium with ammonium nitrate has increased enzyme production even more (Phadatare et al., 1993). From an industrial prospective, the protease must exhibit considerable activity at high pH(s). The crude protease produced by *B. tequilensis* had maximum enzyme activity (78.40U/ml) at pH 9.0 while it was only marginally low at 6, 7, 8 and 10 pH as compared to pH 9 (Figure 12). These results were in accordance with results from *Bacillus* sp. strain CR-179 (Sepahy and Jabalameli, 2011), *Bacillus licheniformis* RPK (Fakhfakh et al., 2009), *B. subtilis* WIFD5 (Sharma and Aruna, 2012), *G. caldoproteolyticus* (Chen et al., 2004), *Bacillus subtilis* SHS-04 (Olajuyigbe et al., 2013) *Bacillus horikoshii* (Joo et al., 2002), *B. subtilis* 50a and *B. licheniformis* 50b (Azlina and Norazila, 2013), *Bacillus licheniformis* LHSB-05 (Olajuyigbe and Kolawole, 2011) and *B. laterosporus* AK-1 (Arulmani et al., 2007). The optimal pH for other *Bacillus* bacteria reported in literature is pH 10.0 for *Bacillus subtilis* strain VV (Vijayaraghavan et al., 2012), *Bacillus subtilis* (Ahmed et al., 2010) and *Bacillus licheniformis* B18 (Lakshmi, 2013) and pH 7 for *Bacillus mojavensis* (Beg et al., 2003) and *Bacillus laterosporus* (Usharani and Muthuraj, 2010).

Enzyme activity was found to be higher at incubation temperature 30°C and remained almost same at 35°C and 45°C then declined to 50% when temperature was 85°C (Figure 13). In literature, optimum temperature was reported to be 30°C for *B. sphaericus* (Singh et al., 2001a). The optimum temperature of alkaline proteases ranges from 50 to 70°C (Kumar and Takagi, 1999). Past researchers reported optimum temperature for protease activity 45°C for *Bacillus horikoshii* (Joo et al., 2002), 40°C for *Bacillus* sp. SSR1 (Singh et al., 2001b), 55°C for *B. subtilis* WIFD5 (Sharma and Aruna, 2012), 60°C for *Bacillus clausii* I-52 (Joo et al., 2003), 70°C for *Bacillus* sp. SB5 (Gupta et al., 1999) and 85°C for *B. stearothermophilus* F1 (Rahman et al., 1994).

There is a great industrial demand for the organic solvent tolerant proteases for application in the synthesis of useful products in the presence of organic solvents (Gupta and Khare, 2007). Enzymes are usually inactivated by the addition of organic solvents to the reaction solution. Protease activity of *B. tequilensis* was enhanced in the presence of isopropanol (108.54% residual activity). More than 70% activity retained for acetone, Butanol, Methanol and ethanol. The 83% inactivation was observed in case of benzene for protease (figure 14). Parallel results were obtained for a purified protease from *Pseudomonas aeruginosa* PseA strain which was stable in the presence of some organic solvents but unstable in benzene (Gupta and Khare, 2007). However, the protease from *B. amyloliquefaciens* PFB-01 showed good stability in the presence of isopropanol and benzene with residual activity of 63.5 and 62.1% (Olajuyigbe and Ogunyewo, 2013). But the protease from *Bacillus licheniformis* NH1 has earlier been reported as unstable in the presence of isopropanol with 12% residual activity (Hmidet et al., 2009). The protease activity of *B. tequilensis* was enhanced with addition of Ca^{+2} and Mg^{+2} ions resulting in the residual activity of 105 and 107% respectively while other metal ions Zn^{+2} , Cd^{+2} , Na^{+} and K^{+} resulted more than 60% residual activity whereas Hg^{+2} exhibited 12.45% residual activity (Figure 15). Similar results were also reported in case of proteases from *B. cohnii* APT (Tekin et al., 2012), *Bacillus* sp. PCSIR EA-3 (Qadar et al. 2009), *Bacillus licheniformis* RSP-09-37 (Sareen and Mishra, 2008), *B. subtilis* PE-11 (Adinarayana et al., 2003), *B. subtilis* WIFD5 (Sharma and Aruna, 2012) and *Bacillus* sp. strain SMIA-2 (Nascimento and Martins, 2004) where activity of the enzyme was enhanced by Ca^{+2} . However, Bajaj and Jamwal (2013) reported that protease from *Bacillus pumilus* D-6 was not inhibited by Hg^{+2} and Aqel et al. (2012) reported that protease from *Bacillus* strain HUTBS62 was inhibited by Cd^{+2} . Metal chelators like EDTA exhibited 71.5% residual activity suggesting the type of protease produced by *B. tequilensis* was of non metallo protease. The stability and compatibility of novel microbial proteases are important criteria for addition in detergent preparations (Kumar and Takagi, 1999). Studies on the effects of various surfactants on protease activity of *B. tequilensis* revealed that upon incubation with Tween-20, Triton X-100, the enzyme showed enhanced residual activities 102% and 108% respectively while other surfactants including commercially available detergents (Surf excel, Ariel and Surf blue) exhibited more than 90% residual activity except SDS which showed 60% residual activity (Figure 16). There are similar reports, in the case of *Bacillus subtilis*, *Bacillus mojavensis* and *Bacillus* RV.B2.90 giving higher activity with non ionic surfactant and reduction with ionic surfactant (Wang and Yeh, 2006; Beg et al., 2003; Vijayalakshmi et al., 2013). Thus, this protease showed a compatibility with commercial detergents.

For protease based detergent formulations, the enzyme should have the capability to tolerate oxidizing agents. Protease residual activity of *B.tequilensis* was 127% in presence of 2% H_2O_2 (Figure 17). Hence, the enzyme showing extreme stability towards oxidizing agents is of immense commercial significance for detergent industry because peroxides are common ingredients of modern bleach-based detergent. Previous reports on stability of alkaline protease from *Bacillus clausii* I-52 (Joo et al., 2003), *B. licheniformis* N-2 (Nadeem et al., 2008), *Bacillus licheniformis* P003 (Sarker et al., 2013), *Bacillus mojavensis* (Beg et al., 2003), and *Bacillus* sp. I-312 (Joo and Chang, 2005) exhibited enhanced residual activity in presence of H_2O_2 .

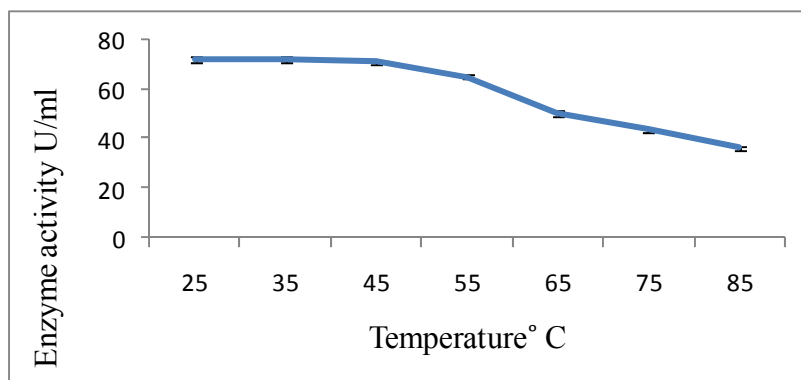


Figure 13: Effect of temperature on protease activity of *Bacillus tequilensis*

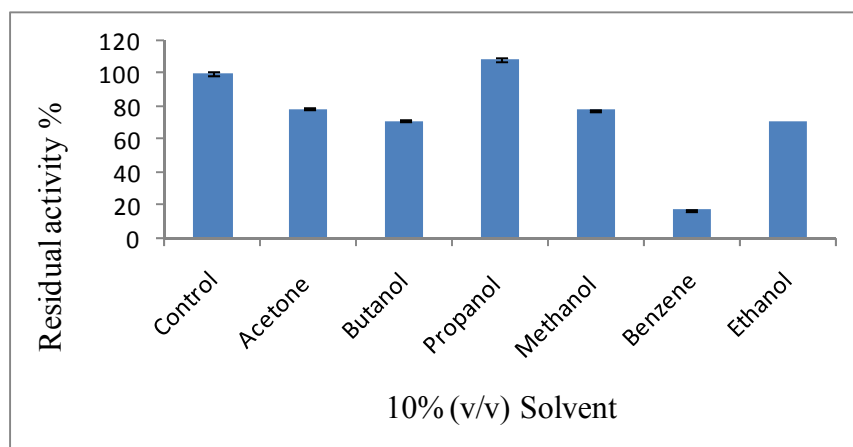


Figure 14: Effect of different solvents on protease activity of *Bacillus tequilensis*

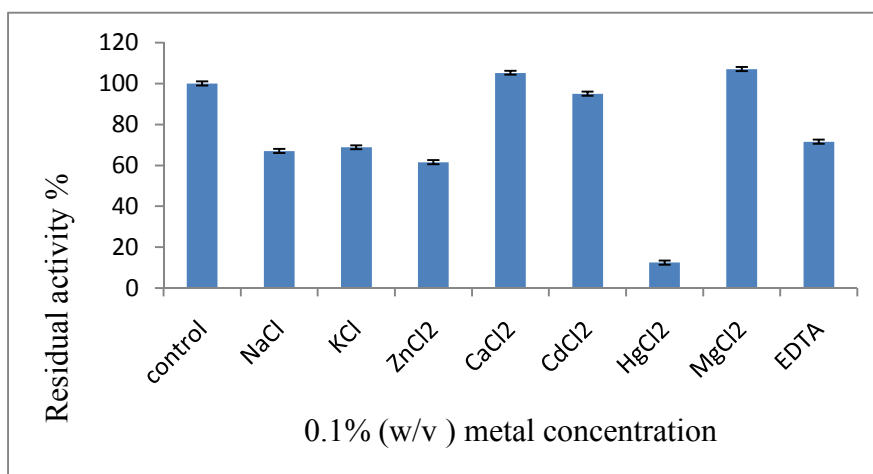


Figure 15: Effect of different metal ions on protease activity of *Bacillus tequilensis*

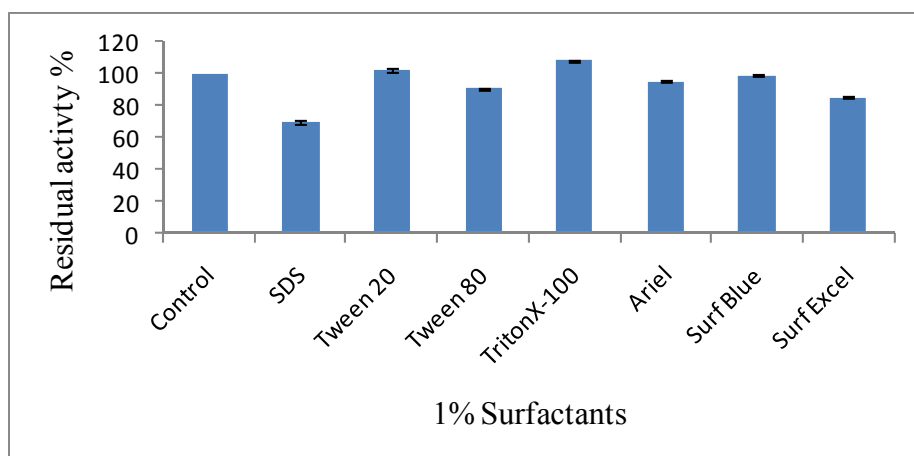


Figure 16: Effect of different surfactants on protease activity of *Bacillus tequilensis*

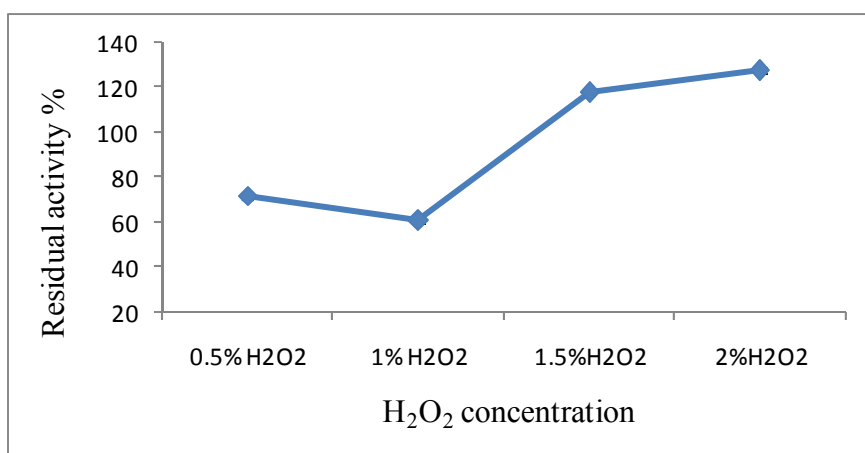


Figure 17: Effect of different concentration of H₂O₂ on protease activity of *Bacillus tequilensis*

Table 1: Purification of Enzyme

Purification step	Protein conc. (mg/ml)	Enzyme activity (U/ml)	Specific activity U/mg	Activity %	Purification fold
Crude enzyme	8.2	80.98	9.86	100	1
Ammonium sulphate precipitate	5.9	69.41	11.76	85.71	1.19

Purification of the enzyme was obtained at 80% saturation using ammonium sulphate after dialysis which was then used to determine its role as a potential detergent additive (Table 1). By applying 80% ammonium sulphate precipitation, over 85.71% of the total protease in the culture filtrate was salted out. Purification achieved with ammonium sulphate was 1.19 fold. This procedure not only facilitated the effective removal of the impurities existing in the culture fluid, it also concentrated the enzyme preparation to a workable volume.

As the protease from *B. tequilensis* showed activity in a broad pH range, broad temperature conditions and compatibility with various surfactants and solvents as well as oxidizing agent; its application as a detergent additive was studied on white cotton pieces stained with human blood (Figure18 A, B, C and D). It was found that there was better stain removal from the stained cotton piece that was supplemented with enzyme plus detergent (brand name Ariel) than the stained cotton piece that was supplemented with the detergent alone after incubation at 55°C for 15 minutes in a water bath (Figure18 C and D).

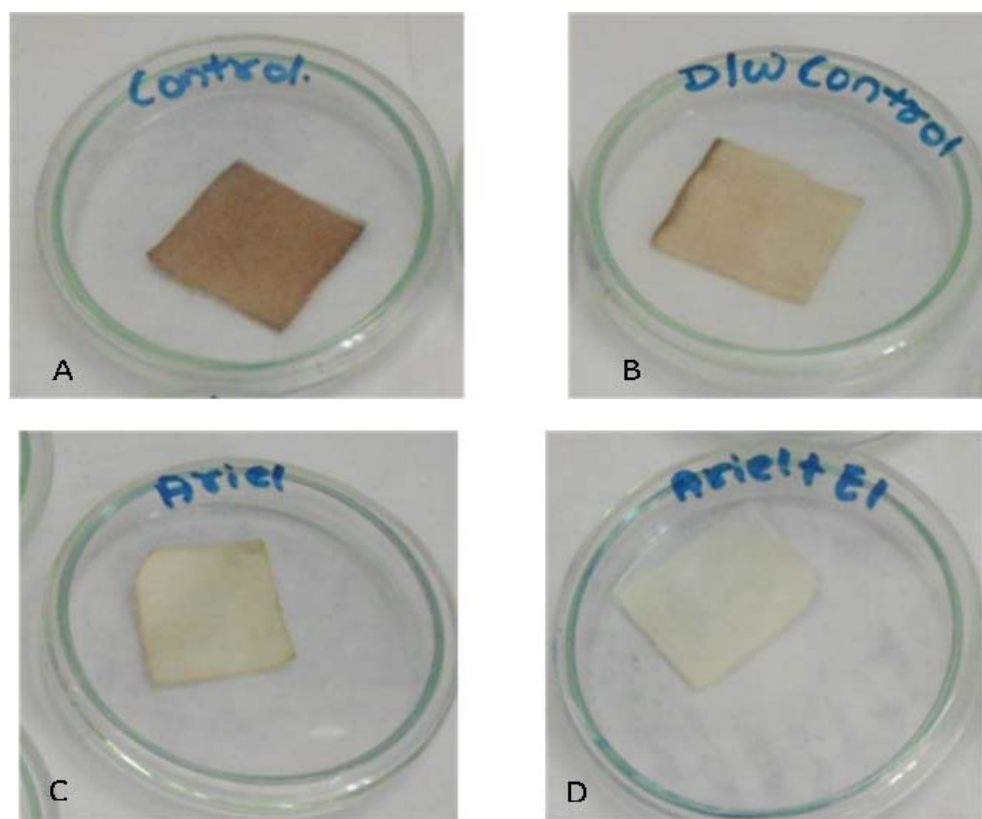


Figure: 18: A- control- Dried blood stained cloth;

B-D/W control- Blood stained cloth rinsed in distilled water; C-Ariel-Blood stained cloth rinsed in Ariel detergent solution; D-Ariel+E1-Blood stained cloth rinsed in Ariel detergent + protease enzyme produced by *B.tequilensi*

This provided with the evidence that the enzyme under consideration has a potential detergent action. The use of proteases in laundry detergents particularly accounts for approximately 25% of the total worldwide sales of enzymes. The detergent proteases work best by hydrolyzing large insoluble proteins. Proteins are initially removed from the fabric surface either by components of detergent matrix or by water alone. Depending upon the size of the resulting fragments, they are either solubilized into the bulk solution, or they deposit themselves back into the fabric. Hence, the best detergent enzyme provides improved substrate hydrolysis, resulting in better stain removal and anti-redeposition benefits (Hmidet et al., 2009). The obtained result demonstrated that crude enzyme from *B.tequilensi* can effectively remove blood stains. Similar observations were done where proteases from *B.Subtilis* WIFD5 (Sharma and Aruna, 2012), *Bacillus cereus* (Kanmani et al., 2011), *Bacillus circulans* (Rao et al., 2009), *Bacillus circulans* BM15 (Venugopal and Saramma, 2007), *Bacillus pumilus* CBS (Jaouadi et al., 2008), *Paenibacillus tezipurensis* sp. (Rai et al., 2010), *Bacillus brevis* (Banerjee et al., 1999), *Bacillus mojavensis* A21 (Haddar et al., 2010), *Bacillus subtilis* DM-04 (Rai and Mukherjee, 2010), *Bacillus licheniformis* RSP-09-37 (Sareen and Mishra, 2008) and *Bacillus subtilis* (Kumar et al., 2012) were used along with detergents to remove the blood stains from fabric. Therefore, *B.tequilensi* enzyme preparation containing protease activity could be considered as a potential candidate for use as a cleaning additive in detergents to facilitate the release of proteinaceous stains. However, caution must also be exercised in the source of enzymes to be used as detergent additives as some of them might cause allergic reactions in humans (Anwar and Salemudin, 1998).

CONCLUSION

From this study, it can be concluded that, the isolate *Bacillus tequilensi* strain SCSGAB0139 obtained from spoilt cottage cheese sample can be a potential source of alkaline protease for use in various industrial applications. The enzyme had its optimum activity at high pH and is stable upto 45°C. In addition, it also exhibited compatibility with different detergents and proved to be excellent in removing stains from fabric. Thus, it can be a potential candidate for detergent formulations.

REFERENCES

- Adinarayana K., Ellaiah Poluri., and Davuluri, Siva Prasad (2003). Purification and partial characterization of thermostable serine alkaline protease from a newly isolated *Bacillus subtilis* PE-11.AAPS. Pharm. Sci. Tech., 4(4): 440-448
- Ahmed I, Irfan M, Nadeem M, Zia M. A, Ahmad B. M. (2010). Optimization of Media and Environmental Conditions for Alkaline Protease Production Using *Bacillus Subtilis* in Submerged Fermentation Process. IJAVMS. 4(4):105-113.
- Ahmed S, Ramadan A, Al-domany, El-Shayeb, Hesham H, Shireen A, Saleh M. (2008). Optimization, Immobilization of Extracellular Alkaline Protease and Characterization of its Enzymatic Properties. Research Journal of Agriculture and Biological Sciences, 4:434-446.
- Akcan N., and Uyar, F. (2011). Production of extracellular alkaline protease from *Bacillus subtilis* RSKK96 with solid state fermentation. EurAsian Journal of Biosciences, 5:64–72.
- Ammar M.S. El-Louboudy, S.S. and Abdulraouf U.M. (1991). Protease (s) from *Bacillus anthracis* S-44 and *B. cereus* var. mycoids, S-98 isolated from a temple and slaughter house in Aswan city. Az. J. Microbiol., (13): 12-29.
- Amoozegara M. A, Fatema A. Z, Ksrbaei, Heidari H. R, Razavic M. R. (2004). Production of extracellular alkaline protease from a newly isolated, moderately halophile saline *Vibrio* sp strains. Afr. J. Microbiol. Res., 162:369-377
- Anwar Aand Salemudin M. (1998). Alkaline Proteases: A Review. Bioresource Technology, 64:175-183.
- Aqel H., Al-quadan, F., and Yousef, T. K. J. (2012). A novel neutral protease from thermophilic *Bacillus* strain HUTBS62. BioSci. Biotech., 1(2): 117-123.,
- Arulmani M., Aparanjini, K., Vasanthi, K., Arumugam, P., Arivuchelvi, M., Kalaichelvan, T. (2007). Purification and partial characterization of serine protease from thermostable alkalophilic *Bacillus laterosporus*-AK 1. World J. Microbiol. Biotechnol., 23:475–481.
- Asokan S., Jayanthi C. (2010). Alkaline protease production by *Bacillus licheniformis* and *Bacillus coagulans*. Journal of cell and Tissue Research, 10(1):2119-2123.
- Asokraja C., Prabhakar C., Saleshrani K., Tharmaraj K. and Senthil Murugan. (2012). Optimization of alkaline protease production from *Bacillus* sp. agt under solid state fermentation. International Journal of Recent Scientific Research, 3(3):145 -148,
- Aunstrup K. (1980). Proteinases In: Rose AH, editor. Economic Microbiology: Microbial Enzymes and Bioconversions, Vol. 5. New York: Academic Press, pp. 50–114.
- Azlina Ibrahim Noor and Yusoff Norazila. (2013). Thermostable Alkaline Serine Protease from Thermophilic *Bacillus* Species. International Research Journal of Biological Sciences, 2(2):29-33.
- Bajaj Kumar B, Jamwal G. (2013). Thermostable alkaline protease production from *Bacillus pumilus* D-6 by using agro-residues as substrates. Adv Enzym Res., 1(2):30–36..
- Banerjee, U. C., Sani, R. K., Azmi, W., and Soni, R. (1999). Thermostable alkaline protease from *Bacillus brevis* 6 is and its characterization as a laundry detergent additive. Proc. Biochem., 35:213–219.
- Banik RM, Prakash M. (2004). Laundry detergent compatibility of the alkaline protease from *Bacillus cereus*. Microbiol Res., 159(2):135–40.
- Beg Q. K., V. Sahai, and R. Gupta, (2003). Statistical media optimization and alkaline protease production from *Bacillus mojavensis* in a bioreactor. Process Biochemistry, 39(2): 203–209.
- Beg QK, Gupta R. (2003). Purification and characterization of an oxidation-stable, thiol-dependent serine alkaline protease from *Bacillus mojavensis*. Enzyme Microb Technol., 32(2):294–304.
- Bettel C, Klupsch S, Papendorf G, Hastrup S, Branner S, Wilson K.S. (1992). Crystal structure of the alkaline protease Savinase® from *Bacillus lentus* at 1.4 angstrom resolution. J Mol Biol., 223:427–45.
- Bhaskar N, Sudeepa E.S, Rashmi H.N, Tamil Selvi (2007). Partial purification and characterization of protease of *Bacillus proteolyticus* CFR3001 isolated from fish processing waste and its antibacterial activities. Bioresour Technol., 98(14):2758–64.
- Bhunia Biswanath, Debjani Dutta, Surabhi Chaudhuri (2010). Production of Alkaline Protease by *Bacillus licheniformis* NCIM-2042. Not Sci Biol., 2 (2): 56-59
- Bindu DRA, Reddy IB. (2013). Extracellular Alkaline Protease from a Newly Isolated *Bacillus stratosphericus* DF□: Isolation and Identification. Biochemistry, 2(6):29–31.
- Bonala KC, Mangamoori LN. (2012). Production and optimization of lipase from *Bacillus tequilensis* NRRL B-41771. International Journal of Biotechnology Applications, 4(1):134–136.

- Chen XG, Stabnikova O, Tay JH, Wang JY, Tay ST.(2004).Thermoactive extracellular proteases of *Geobacillus caldoproteolyticus*, sp. nov., from sewage sludge. *Extremophiles*.8(6):489-98.
- Cheng SW, Hu HM, Shen SW. (1995). Production and characterization of keratinase of a feather-degrading *Bacillus licheniformis* PWD-1. *Biosci Biotechnol Biochem.*, 59:2239-2243
- Christiansen, T., J. Nielsen (2002). Production of extracellular pro- tease and glucose uptake in *Bacillus clausii* in steady-state and transient continuous cultures, *J. Biotechnol.*, 97: 265–273
- Chu IM, Lee C, Li TS.(1992). Production and degradation of alkaline protease in batch cultures of *Bacillus subtilis* ATCC 14416. *Enzyme Microb Technol.*, 14:755-761.
- Chu Wei-Hua J (2007).Optimization of extracellular alkaline protease production from species of *Bacillus*.*Ind Microbiol Biotechnol.*, 34:241–245.
- Claim P, Bilir E, Çalik G, Özdamar T.H. (2002). Influence of pH conditions on metabolic regulations in serine alkaline protease production by *Bacillus licheniformis*. *Enzyme Microb. Technol.*, 31: 685–697.
- Dahot, M. U. (1993). Cultivation of *Penicillium expansum* on rice husk powder for protease Production, *Journal of Islamic Academy of Sciences*. 6(3):193-196.
- Das G and Prasad MP. (2010). Isolation, purification & mass production of protease enzyme from *Bacillus subtilis* .*Int Res J Microbiol.*, 1(2): 26-31.
- Denizci AA, Kazan D, Erarslan A. (2010).*Bacillus marmarensis* sp. nov., an alkaliphilic, protease-producing bacterium isolated from mushroom compost. *Int J Syst Evol Microbiol.*, 60(7):1590–4.
- Durham DR. (1987). Utility of GX as a detergent additive. *J. Appl. Bacteriol* 63: 381-386
- Ellaiah P, Srinivasulu B, Adinarayana K. (2002). A review on microbial alkaline proteases. *Journal of Scientific and Industrial Research*, 61: 690-704
- Fakhfakh Nahed, Safia Kanoun, Laila Manni, Moncef Nasri. (2009). Production and biochemical and molecular characterization of a keratinolytic serine protease from chicken feather-degrading *Bacillus licheniformis* RPK Canadian Journal of Microbiology, 55:427-436.
- Ferrero MA, Castro GR, Abate CM, Baigori MD, Sineriz F. (1996). Thermos table alkaline proteases of *Bacillus licheniformis* MIR 29 isolation, production and characterization. *Appl Microbiol Biotechnology*, 45:327–32.
- Foda, M.S., Safaa M. Ali, Youssef, A.M., Kahil, T., Hoda M. Shata and Amira M. Roshdy(2013).Production Physiology of Alkaline Protease by *Bacillus thuringiensis* spp. under Solid-State Fermentation Conditions *Journal of Applied Sciences Research*, 9(3): 1975-1984,
- Frankena J, Koningstein GM, van Verseveld HW, Stouthamer AH. (1986).Effect of different limitations in chemo- stat cultures on growth and production of exocellular protease by *Bacilluslicheniformis*. *Appl Microbiol Biotechnol*, 24:106–12.
- Fujiwara N, Masui A, Imanaka T. (1993). Purification and properties of the highly thermostable alkaline protease from an alkaliphilic and thermophilic *Bacillus* sp. *J Biotechnol.*, 30:245–56.
- Fujiwara N, Yamamoto K, Masui A (1991). Utilization of a thermostable alkaline protease from an alkalophilic thermophile for the recovery of silver from used X-ray film. *J Ferment Bioeng* 72:306-308.
- Fujiwara N, Yamamoto K. (1987). Production of alkaline protease in a low-cost medium by alkalophilic *Bacillus* sp. and properties of the enzyme. *J Ferment Techno*, 65:345–48.
- Fujiwara NK, VamamotoA, Masui (1991). Utilization of a thermostable alkaline protease from an alkaliphilic thermophile for the recovery of silver from used x ray film. *J. Ferment. Bio. Eng.* 72: 306-308.
- Gatson JW, Benz BF, Chandrasekaran C, Satomi M, Venkateswaran K, Hart ME. (2006). *Bacillus tequilensis* sp. nov., isolated from a 2000-year-old Mexican shaft-tomb, is closely related to *Bacillus subtilis*. *Int J Syst Evol Microbiol.*,56(7):1475–84.
- Gee JM, Lund BM, Metcalf G, Peel JL. (1980). Properties of a new group of alkalophilic bacteria. *J Gen Microbiol*,117:9–17.
- Gerze, A.; Omay, D. and Guvenilir, Y. (2005). Partial purification and characterization of protease enzyme from *Bacillus subtilis* and *Bacillus megataerium*. *Appl Biochem Biotechnol.*, 121-124:335-45
- Gessesse A, Hatti-Kaul R, Gashe B , Mattiasson B. (2003).Novel alkaline proteases from alkaliphilic bacteria grown on chicken feather. *Enzyme Microb Technol*, 32 (5):519–524.
- Ghafoor A, Hasnain S.(2009). Production dynamics of *Bacillus subtrillis* strain AG-1 and EAG-2, producing moderately alkaline proteases, *African J Microbiol*, 3 (5):258-263.
- Ghorbel FB, Sellami KA, Fakhfakh N, Haddar A, Manni L, Nasri M. (2005). Production and purification of a calcium-dependent protease from *Bacillus cereus* BG1. *Journal of Industrial Microbiology and Biotechnology*, 32: 186-194.

- Glesecke, U.E., Bierbaum, G., Rudde, H., Spohn, U. and Wandray, C. (19991). Production of alkaline protease with *Bacillus licheniformis* in a controlled fed-batch process. Appl Microbiol. Biotechnol, 35 :720-724.
- Grant W.D. and B. E. Jones, (2000). "Alkaline environments," in Encyclopaedia of Microbiology, J. Lederberg, Ed., vol. 1, pp. 126– 133, Academic Press, New York, NY, USA, 2nd edition,
- Gupta A., Khare S.K.(2007).Enhanced production and characterization of a solvent stable protease from solvent tolerant *Pseudomonas aeruginosa* PseA. Enzyme and Microbial Technology, 42:11-16.
- Gupta R, Beg QK, Lorenz P. (2002). Bacterial alkaline proteases: molecular approaches and industrial applications. Appl Microbiol Biotechnol., 59(1):15–32.
- Gupta R, Gupta K, Saxena R.K, Khan S, (1999). Bleach-stable, alkaline protease from *Bacillus* sp."Biotechnology Letters, 21:135-138.
- Haddar A, Bougatef A, Agrebi R, Sellami-Kamoun A, Nasri M. (2009). A novel surfactant-stable alkaline serine-protease from a newly isolated *Bacillus mojavensis* A21.Purification and characterization. Process Biochemistry 44: 29-35.
- Hameed A, Keshavarz T, Evans CS. (1999). Effect of dissolved oxygen tension and pH on the production of extracellular protease from a new isolate of *Bacillus subtilis* K2, for use in leather processing. J Chem Technol Biotechnol., 74:5–8
- Hameed A, Natt MA, Evans C.S. (1996). Production of alkaline protease by a new *Bacillus subtilis* isolate for use as a bating enzyme in leather treatment. World J Microbiol Biotechnol.,12:289–291.
- Hanlon, G. W., N. A. Hodges and A. D. Russell. (1982). The influence of glucose, ammonium and magnesium availability on the production of protease and bacitracin by *Bacillus licheniformis*. J. Gen. Microbiol., 128:845-851.
- Hmidet N, Ali N, Haddar A, Kanoun S, Alya S-K, Nasri M. (2009). Alkaline proteases and thermosable α -amylase co-produced by *Bacillus licheniformis* NH1: Characterization and potential application as a detergent additive. Biochemical Engineering Journal, 47:71-79.
- Horikoshi K. (1996). Alkaliphiles – from an industrial point of view. FEMS Microbiol Rev., 18:259–270
- Horikoshi K, Akiba T. (1982). Alkalophilic Microorganisms: A New Microbial World. Tokyo, Japan: Japan Scientific Societies Press and Berlin, Germany: Springer-Verlag,
- Hoshino, E., Maruta, K., Wada, Y., Mori, K.(1995). Hydrolysis of human horny cells by alkaline protease: morphological observation of the process. Journal of American Oil Chemists' Society, 72:785–791.
- Hotha S, Banik RM. (1997). Production of alkaline protease by *Bacillus thuringiensis* H14 in aqueous two-phase systems. J Chem Technol Biotechnol., 69:5–10
- Hübner U, Bock U, Schügerl K. (1993). Production of alkaline serine protease subtilisin Carlsberg by *Bacillus licheniformis* on complex medium in a stirred tank reactor. Appl Microbiol Biotechnol., 40:182–88
- Itskovich EL, Znamenskaya LV, Balaban NP, Ershova TA. (1995). Leshchinskaya IB. Biosynthesis of alkaline proteinase by *Bacillus intermedius*. Microbiol, 64:530–36.
- Janssen PH, Peek K, Morgan HW. (1994). Effect of culture conditions on the production of a extracellular proteinase by *Thermus* sp. Rt41A. Appl Microbiol Biotechnol., 41:400–6
- Jaouadi B., S. Ellouz-Chaabouni, M. Ben Ali, E. Ben Messaoud, B. Naili, A. Dhouib, S. Bejar.(2008). Biochemical and molecular characterization of a detergent-stable serine alkaline protease from *Bacillus pumilus* CBS with high catalytic efficiency. Biochimie., 90(9):1291-1305
- Jayaraman V, D. R., G., & Sridharan, T. B. (2012). Application studies of the halotolerant protease from a newly isolated *Virgibacillus dokdonensis* VIT P14, Research in Biotechnology, 3(6): 59-66.
- Johnvesly, B., & Naik, G. (2001). Studies on production of thermostable alkaline protease from thermophilic and alkaliphilic *Bacillus* sp. JB-99 in a chemically defined medium. Process Biochemistry, 37(2), 139–144.
- Joo H.S, and C.S. Chang (2005). Production of protease from a new alkaliphilic *Bacillus* sp. I-312 grown on soybean meal: optimization and some properties. Proc. Biochem., 40:1263- 1270.
- Joo H-S, Kumar CG, Park G-C, Kim KT, Paik SR, Chang C-S. (2002). Optimization of the production of an extracellular alkaline protease from *Bacillus horikoshii*. Process Biochem., 38(2):155–159.
- Joo, H.S. C.G. Kumar, G.C. Park, S.R. Paik, C.S. Chang (2003). Oxidant and SDS-stable alkaline protease from *Bacillus clausii* I-52: Production and some properties, J. Appl. Microbiol. 95: 267–272.
- Kanchana M. And S. Padmavathy (2010). Optimization of extracellular alkaline protease enzyme from *Bacillus* sp. The Bioscan, 5(1): 85 – 87.
- Kanmani, R. Dhivya S, Jayalakshmi S, Vijayabaskar P. (2011). Studies on detergent additives of protease enzyme from an estuarine bacterium *Bacillus cereus*. International Research Journal of Biotechnology, 2(7):157–163.

- Kazan D, Denizci AA, Oner MNK, Erarslan A. (2005). Purification and characterization of a serine alkaline protease from *Bacillus clausii* GMBAE 42. *J Ind Microbiol Biotechnol.*, 32(8):335–44.
- Khosravi-Darani, K., Falahatpishe, H.R. and Jalai, M.(2008). Alkaline protease production on date waste by an alkalophilic *Bacillus* sp. 2-5 isolated from soil. *African Journal of Biotechnology*. 7(10): 1536-1542.
- Kumar C. G. and H. Takagi (1999). "Microbial alkaline proteases: from a bioindustrial viewpoint," *Biotechnology Advances*, 17(7):561–594.
- Kumar D and Bhalla T C.(2004). Purification and characterization of a small size protease from *Bacillus* sp. APR-4 *Indian Journal of Experimental Biology*,42:515-521
- Kumar R. (2007). Enhanced productivity of serine alkaline protease by *Bacillus* sp. using soybean as substrate.; *Mal J Microbiol.*, 3(1):1–6.
- Kumar CG. (2002). Purification and characterization of a thermostable alkaline protease from alkalophilic *Bacillus pumilus*. *Letters in Applied Microbiology*, 34:13–17.
- Kumar, R., Balaji, S., Uma, T.S., Mandal, A.B. and Shegal, P.K. (2010). Optimization of influential parameters for extracellular keratinase production by *Bacillus subtilis* (MTCC9102) in solid state fermentation using horn Meal—A biowaste management. *Applied Biochemistry Biotechnology*, 160:30-39.
- Lakshmi .G (2013). Optimization of cultural conditions for the over production of alkaline protease from a mutant *Bacillus licheniformis* B18. *The Experiment*, 14(1):864-878.
- Lakshmi B.K.M. , P.V. Ratna Sri, K. Ambika Devi, KP.J.Hemalatha (2014). Media optimization of protease production by *Bacillus licheniformis* and partial characterization of Alkaline protease. *Int.J.Curr.Microbiol. App.Sci.*, 3(5): 650-659
- Lawal, A. K., Itoandon, E. E., Fashola, F., Ajayi, O., Ojo, E. E., Adepoju, F. A., Ugbana, A.I, Elemo, G. N. and Dike, E. N (2014). Production of Alkaline Protease by *Bacillus polymyxa* using Agro-Industrial Waste by Solid State Fermentation. *Global Advanced Research Journal of Microbiology.*,3(3):41–48.
- Laxman, R. S., Sonawane, A. P., More, S. V., Rao, B. S., Rele, M. V., Jogdand, V. V., Rao, M. B. (2005). Optimization and scale up of production of alkaline protease from *Conidiobolus coronatus*. *Process Biochemistry*, 40(9):3152–3158.
- Lowry OH, Rosebrough NJ, Farr AL, Randall RL. (1951). Protein measurement with the folin phenol reagent. *J.Bio. Chem.*, 193: 265-273.
- Lui B-L and Tzeng Y-M, (1998). Optimization of growth medium for the production of spores from *Bacillus thuringiensis* using response surface methodology. *Bioprocess Eng*, 18:413
- Maal, K. B., Emtiazi, G., & Nahvi, I. (2009). Production of alkaline protease by *Bacillus cereus* and *Bacillus polymyxa* in new industrial culture mediums and its immobilization, 3(9), 491–497. *African Journal of Microbiology Research Vol. 3*(9): 491-497,
- Manachini PL, Fortina MG, Parini C. (1988). Thermostable alkaline protease produced by *Bacillus thermoruber*— a new species of *Bacillus*. *Appl Microbiol Biotechnol*, 28:409–13
- Mao W, Pan R, Freedman D. (1992). High production of alkaline protease by *Bacillus licheniformis* in a fed-batch fermentation using a synthetic medium. *J Ind Microbiol*, 11:1–6.
- Mehrotra S, Pandey PK, Gaur R, Darmwal NS. (1999). The production of alkaline protease by a *Bacillus* species isolate. *Bioresour Technol.*, 67(2):201–203.
- Mehta V.J., Thumar J.T., Singh S.P. (2006). Production of alkaline protease from alkaliphilic actinomycete, *Bioresour Technol*, 97:1650-4.
- Moon SH, Parulekar SJ. (1991). A parametric study of protease production in batch and fed-batch cultures of *Bacillus firmus*. *Biotechnol Bioeng.*, 37:467–83
- Rathod Mukundraj G & Anupama P Pathak (2014). Wealth from Waste: Optimized Alkaline Protease Production Using Agro-Industrial Residues by *Bacillus alcalophilus* LW8 and its Biotechnological Applications. *Journal of Taibah University for Science* <http://dx.doi.org/doi:10.1016/j.jtusc.2014.04.002>.
- Nadeem M, Qazi JI, Baig S, Syed Q. (2007). Studies On Commercially Important Alkaline Protease From *Bacillus licheniformis* N-2 Isolated from Decaying Organic Soil. *Turkish Journal of Biochemistry*, 32: 171-177.
- Nadeem M, Qazi JI, Baig S, Syed Q. (2008). Effect of Medium Composition on Commercially Important Alkaline Protease Production by *Bacillus licheniformis* N-2. *Food Technol. Biotechnol.*, 46 (4): 388–394
- Nadeem, M., Baig, S., Syed, Q., & Qazi, J. I. (2006). Microbial Production of Alkaline Proteases by Locally Isolated *Bacillus subtilis* PCSIR-5. *Pakistan J. Zool*, 38(1):109-114.
- Naidu, K.S.B. and K.L. Devi(2005). Optimization of thermostable alkaline protease production from species of *Bacillus* using rice bran. *Afr. J. Biotechnol.*, 4:724-726.

- Nascimento W. C. A. and Martins M.L.L. (2004). Production and properties of an extracellular protease from thermophilic *Bacillus* sp., Brazilian Journal of Microbiology, 35(1-2): 91–96.
- Nejad ZG, Yaghmaei S, Hosseini RH. (2009). Production of extracellular protease and determination of optimal condition by *Bacillus* Archive of SID., 22(3):221–228.
- Olajuyigbe FM, Ehiosun KI. (2013). Production of thermostable and organic solvent- tolerant alkaline protease from *Bacillus coagulans* PSB- 07 under different submerged fermentation conditions, African Journal Of Biotechnology, 12(21):3341–3350.
- Olajuyigbe FM, Kolawole AO. (2011). Purification and partial characterization of a thermostable alkaline protease from *Bacillus licheniformis* LHSB-05 isolated from hot spring. Afr J Biotechnol., 10:11703-10.
- Olajuyigbe FM, Ogunyewo OA. (2013). Enhanced Production and Physicochemical Properties of a Commercially Viable Alkaline Protease from *Bacillus amyloliquefaciens* PFB-01. Current Biotechnology, 2:73-80.
- Olajuyigbe, F. M. (2013). Optimized production and properties of thermostable alkaline protease from *Bacillus subtilis* SHS-04 grown on groundnut (*Arachis hypogaea*) meal. Advances in Enzyme Research, 01(04):112–120.
- Oskouie SFG, Tabandeh F, Yakhchali B, Eftekhari F. (2008). Response surface optimization of medium composition for alkaline protease production by *Bacillus clausii*. Biochem Eng J., 39(1):37–42.
- Pandey A., P. Nigam, C. R. Soccol, V. T. Soccol, D. Singh, and Mohan R. (2000). Advances in microbial amylases. Biotechnol. Appl. Biochem., 31(2):135-152.
- Pastor M D, Lorda G S & Balatti A. (2001). Protease obtention using *Bacillus subtilis* 3411 and Amaranth seed meal medium of different aeration rate. Braz J Microbiol, 32: 6-9.
- Patil U, Chaudhari A. (2013). Production of Alkaline Protease by Solvent-Tolerant Alkaliphilic *Bacillus circulans* MTCC 7942 Isolated from Hydrocarbon Contaminated Habitat: Process Parameters Optimization. ISRN Biochemistry, 3:1-10.
- Pawar R., Zambare V., Barve S., Paratkar G. (2009). Application of protease isolated from *Bacillus* sp.-158 in enzymatic cleaning of contact lenses. Biotechnol, 8 (2): 276-280.
- Pedga Sudarshan S., Sunil D. Ahirrao, Vasant B. Garad (2013). Optimization on protease enzyme production using *Bacillus subtilis* SSD-I (JQ747516) isolated from Ionar meteorite crater in India International Journal of Biological & Pharmaceutical Research., 4(12):885–890.
- Phadatare SU, Deshpande VV, Srinivasan MC. (1993). High activity alkaline protease from *Conidiobolus coronatus* (NCL 86.8.20): Enzyme production and compatibility with commercial detergents. Enzyme Microb Technol, 15:72–76.
- Pillai P., Mandge, S. and Archana, G. (2011). Statistical optimization of production and tannery applications of a keratinolytic serine protease from *Bacillus subtilis* P13. Process Biochemistry, 46:1110-1117.
- Prakasham, R. S., Rao, C. S., & Sarma, P. N. (2006). Green gram husk--an inexpensive substrate for alkaline protease production by *Bacillus* sp. in solid-state fermentation. Bioresource Technology, 97(13):1449–54.
- Priest FG. (1977). Extracellular enzyme synthesis in the genus *Bacillus*. Bacteriol Rev., 41:711-753.
- Puri S, Beg QK, Gupta R. (2002). Optimization of alkaline protease production from *Bacillus* sp. using response surface methodology. Curr Microbiol., 44:286–290.
- Qadar Shah Ali Ul, Erum Shireen, Samina Iqbal and Abida Anwar (2009). Optimization of protease production from newly isolated strain of *Bacillus* sp. PCSIR EA-3. Indian Journal Biotechnology, 8: 286-290.
- Rahman R N Z A, Razak C N, Ampon K, Basri M, Yunus W M Z W et al. (1994). Purification and characterization of heat stable alkaline protease from *Bacillus stearothermophilus* F1. Appl Microbiol Biotechnol, 40: 822-827.
- Rai SK, Roy JK, Mukherjee AK. (2010). Characterisation of a detergent-stable alkaline protease from a novel thermophilic strain *Paenibacillus tezipurensis* sp. nov. AS-S24-II. Appl Microbiol Biotechnol., 85(5):1437–50.
- Rai SK, Mukherjee AK. (2010). Statistical optimization of production, purification and industrial application of a laundry detergent and organic solvent-stable subtilisin-like serine protease (Alzwiiprase) from *Bacillus subtilis* DM-04. Biochem Eng J, 48 (2):173–180.
- Rao MB, Tanksale a M, Ghatge MS, Deshpande V V. (1998). Molecular and biotechnological aspects of microbial proteases. Microbiol Mol Biol Rev., 62(3):597–635.
- Rathakrishnan, P., and Nagarajan, P. (2012). Optimizing factors affecting protease production by a *Bacillus cereus* using groundnut shell under solid substrate fermentation, IJSIT, 1(2):114–129.
- Ravishankar K, Kumar MA, Saravanan K. (2012). Isolation of alkaline protease from *Bacillus subtilis* AKRS3. African J Biotechnol., 11(69): 13415-13427.
- Tambekar Sandhya D and D H Tambekar (2013). Optimization of the production and partial characterization of an extracellular alkaline protease from thermo-halo-alkalophilic Ionar lake bacteria. Bioscience Discovery, 4(1): 30-38.

- Sareen R, Mishra P. (2008). Purification and characterization of organic solvent stable protease from *Bacillus licheniformis* RSP-09-37. *Appl Microbiol Biotechnol.*, 79(3):399–405.
- Sarker, P. K., Talukdar, S. A., Deb, P., Sayem, S. M. A., and Mohsina, K. (2013). Optimization and partial characterization of culture conditions for the production of alkaline protease from *Bacillus licheniformis* P003. *Springer Plus*, 2:506.
- Sathyavathan P, Kavitha M. (2013). Production of Alkaline Protease from *Bacillus licheniformis* (NCIM 2044) and Media Optimisation for Enhanced Enzyme Production. *Int J Chem Tech Res.*, 5(1):550–553.
- Sen S, Satyanarayana T. (1993). Optimization of alkaline protease production by thermophilic *Bacillus licheniformis* S-40. *Ind J Microbio.*, 33:43–47.
- Sepahy AA and Jabalameli L. (2011). Effect of Culture Conditions on the Production of an Extracellular Protease by *Bacillus* sp. Isolated from Soil Sample of Lavizan Jungle Park. *Enz Res*, Article ID 219628, 2: 1-7.
- Sharma Sakshi, Aruna K. (2011). Optimization of Protease Production by Thermophilic *Bacillus subtilis* WIFD5 Isolated from a Milk Powder Sample. *Journal of Microbial World*; 13(2): 241-247.
- Sharma S, Aruna K. (2012). Optimization of protease activity of thermophilic *Bacillus subtilis* WIFD5 isolated from a milk powder sample. *Biomedical and pharmacology Journal*, 5(1): 57-64.
- Sharmin S, Towhid Hossain MD, Anwar MN. (2005). Isolation and characterization of a protease producing bacteria *Bacillus amovivorus* and optimization of some factors of culture condition for protease production. *J Biol Sci.*, 5:358-62.
- Shikha, Sharan, A., & Darmwal, N. S. (2007). Improved production of alkaline protease from a mutant of alkalophilic *Bacillus pantotheneticus* using molasses as a substrate. *Bioresource Technology*, 98(4), 881–5.
- Shimogaki H, Takeuchi K, Nishino T, Ohdera M, Kudo T, Ohba K, Iwama M, Irie M. (1991). Purification and properties of a novel surface-active agent and alkaline-resistant protease from *Bacillus* sp. *Y. Agric Biol Chem*, 55:2251–58.
- Shivanand, P and Jayaraman, G. (2009). Production of extracellular protease from halotolerant bacterium, *Bacillus aquimaris* strain VITP4 isolated from Kumta coast. *Process Biochemistry*, 44(10), 1088–1094.
- Shumi W, Hossain MT, Anwar MN (2004). Proteolytic activity of a bacterial isolate *Bacillus fastidious*. *Journal of Biological Science*. 4: 370-374.
- Singh J, Batra N, Sobti R.C. (2001a). “Serine alkaline protease from a newly isolated *Bacillus* sp.SSR1” *Process Biochemistry*, 36:781-785.
- Singh J., N.Batra, Sobti C.R. (2001b). Serine alkaline protease from a newly isolated *Bacillus* sp. SSR1. *Proc.Biochem*, 36:781-785.
- Sinha, N.; Satyanarayana, T. (1991). Alkaline protease by thermophilic *Bacillus licheniformis*. *Indian J. Microbiol.*, 31: 425-430.
- Soares VF, Castilho LR, Bon EP, Freire DM. (2005). High-yield *Bacillus subtilis* protease production by solid-state fermentation. *Appl. Biochem. Biotechnol*, 121-124: 311-319
- Sookkheo B, Sinchaikul S, Phutrakul S, Chen ST. (2000). Purification and characterization of the highly thermostable proteases from *Bacillus stearothermophilus* TLS33. *Protein Expr Purif.*, 20(2):142–51.
- Srinivasan MC, Vartak HG, Powar VK, Sutar. (1983). High activity alkaline protease production by a *Conidiobolus* sp. *Biotechnol Lett.*, 5:285–88.
- Strauch MA, Hoch JA. (1993). Transition-state regulators: sentinels of *Bacillus subtilis* post-exponential phase gene expression. *Molecular Microbiol*, 77:337-342
- Takagi H, Tsai YC, Nakamori S, Yamasaki M. (1995). Improved production and recovery of alkaline elastase from alkalophilic *Bacillus* strain by a change of medium composition. *Biosci Biotechnol Biochem.*, 59:1591–92.
- Takami H, Akiba T, Horikoshi K. (1989). Production of extremely thermostable alkaline protease from *Bacillus* sp. no.AH-101. *Appl Microbiol Biotechnol*, 30:120–24.
- Takii Y, Kuriyama N, Suzuki Y. (1990). Alkaline serine protease produced from citric acid by *Bacillus alcalophilus* subsp. halodurans KP1239. *Appl Microbiol Biotechnol*, 34:57–62.
- Tambekar Sandhya D. and Tambekar D. H. (2013). Optimization of the production and partial characterization of an extracellular alkaline protease from thermo-halo-alkalophilic *Ionar*. *Bioscience Discovery*, 4(1): 30-38.
- Tekin, Nilgün Arzu Çöleri Cihan, Zekiye Serpil Takaç, Canan Yağci Tüzün, Kenan Tunç, Cumhuri Çökmüş (2012). Alkaline protease production of *Bacillus cohnii* APT5 *Turk J Biol.*, 36: 430-440.
- Ternström A, Lindberg AM, Molin G. (1993). Classification of the spoilage flora of raw and pasteurized bovine milk, with special reference to *Pseudomonas* and *Bacillus*. *J Appl Bacteriol.*, 75(1):25-34.

- Tharmaraj, N. and Shah, N. P. (2009). Antimicrobial effects of probiotics against selected pathogenic and spoilage bacteria in cheese-based dips International Food Research Journal, 16: 261-276.
- Tsuchiya K, Sakashita H, Nakamura Y, Kimura T. (1991). Production of thermostable alkaline protease by alkalophilic *Thermoactinomyces* sp. HS682. Agric Biol Chem., 55:3125–27.
- Usharani B., Muthuraj M. (2010). Production and characterization of protease enzyme from *Bacillus laterosporus*, African Journal of Microbiology Research., 4(11):1057-1063.
- Uyar F, Porsuk I, Kizil G, Yilmaz EI. (2011). Optimal conditions for production of extracellular protease from newly isolated *Bacillus cereus* strain CA15. EurAsian Journal of BioSciences, 5: 1-9
- Uyar, F., & Baysal, Z. (2004). Production and optimization of process parameters for alkaline protease production by a newly isolated *Bacillus* sp. under solid state fermentation. Process Biochemistry, 39(12), 1893–1898.
- Vadlamani S, Parcha S.R. (2012). Optimization Of Alkaline Protease Production From Locally Isolated *Bacillus* Sp. *Bacillus Firmus* From Soil Microorganisms in Batch Culture Using Statistical Design. International Journal of Engineering Research and Applications, 2(2):917–924.
- Varela H, Ferrari MD, Belobradjic L, Weyrauch R, Loperena ML. (1996). Effect of medium composition on the production by a new *Bacillus subtilis* isolate of protease with promising unhairing activity. World J Microbiol Biotechnol., 12:643–645.
- Venugopal M, Saramma a V. (2007). An alkaline protease from *Bacillus circulans* BM15, newly isolated from a mangrove station: characterization and application in laundry detergent formulations. Indian J Microbiol, 47(4):298–303.
- Verma Jyoti, Sangeeta Saxena, and Shikha (2013). 16S rDNA Based Identification of Alkaline Protease Producing Alkaliphilic *Bacillus* Sp Isolated from Dairy Industry Soil and Evaluation of the Enzyme Potential In Detergent Formulation. Research Journal of Pharmaceutical, Biological and Chemical Sciences, 4(4): 1339-1349.
- Vijayalakshmi S, J Ranjitha, V Devi Rajeswari, (2013). Enzyme production ability by *Bacillus subtilis* and *Bacillus licheniformis* - a comparative study Asian J Pharm Clin Res., 6 (4): 29-32.
- Vijayaraghavan, P., Vijayan, A., Arun, A., Jenisha, J. K., & Vincent, S. G. P. (2012). Cow dung: a potential biomass substrate for the production of detergent-stable dehairing protease by alkaliphilic *Bacillus subtilis* strain VV. Springer Plus, 1(1): 76.
- Wan, M.Y., Wang, H.Y., Zhang, Y.Z. and Feng, H. (2009). Substrate specificity and thermostability of the dehairing alkaline protease from *Bacillus pumilus*. Applied Biochemistry Biotechnology, 159:394-403.
- Wang S, Chen Y, Wang C, Yen Y, Chern M (2005). Purification and characterization of a serine protease extracellularly produced by *Aspergillus fumigatus* in a shrimp and crab shell powder medium. Enzyme Microbiology and Technology, 36: 660-665.
- Ward O.P. (1995). Proteolytic enzymes In: M.Moo-Young Editor, Comprehensive Biotechnology., 3: 789-818.
- Wells J.A., Ferrari, E., Henner, D.J., Estell, D.A. and Chen, E.Y. (1983). Cloning, sequencing and secretion of *Bacillus amyloliquefaciens* subtilisin in *Bacillus subtilis*. Nucleic Acid Research, 11(22):7911-7925.
- Yoshimoto T, Oyama H, Honda T, Tone H, Takeshita T, Kamiyama T, Tsuru D. (1988). Cloning and expression of subtilisin amylosacchariticus gene. J Biochem, 103:1060–1065.
- Zambare V.P., S.S. Nilegaonkar, P.P. Kanekar, (2007). Production of an alkaline protease by *Bacillus cereus* MCM B-326 and its application as a dehairing agents. World J. Microbiol. Biotechnol, 23: 1569–1574.