CHAPTER 4

DISCUSSION

4.1 Isolation, identification and characterization of amylase producing bacteria from the waters of Bay of Bengal, Coastal area of Visakhapatnam.

In the present study of isolation of amylase producing bacteria, 1618 isolates were identified depending on the size of the zone of starch hydrolysis in four areas (Rushikonda, Appughur, Fishing harbor and Gangavaram) during 2 years. There was negligible effect of pollutants and seasonal fluctuations in the availability of amylase producing bacteria. Out of 1618 isolates, 143 isolates were exhibiting 3-11 mm, 17 isolates exhibited above 11 mm and the rest of them yielded less than 3 mm clear zone of starch hydrolysis. Seventeen isolates of high potent amylase producing bacteria were identified based on the enzyme production potentials ranging from 400 to 1800 U/ml.

Nizioldek (1998) had studied 41 strains of the genus Bacillus representing 13 species using different liquid media. It was reported that 19 strains were low productive and 12 were medium productive strains (10-25 U/ml). Bacillus subtilis AS-1-108, Bacillus subtilis NCIB 8159 and Bacillus licheniformis NCIB 7198 strains were included among the higher productive as they produced about 370, 170 and 40 U/ml of α-amylase activity respectively. The enzymes from Bacillus subtilis AS-1-108 and NCIB 8159 strains were more thermo sensitive than those of the medium productive strains of Bacillus subtilis. Similar work with fungi was done by Tokhadze et al. (1975) who isolated 86 strains of the Aspergillus, producing maximum acid stable α-amylase. Bacilli species like B. amyoliquefaciens, B. licheniformis, B. stearothermophilus and B. subtilis were the major producers of α-amylases (Tomazie & Klibanov 2003). Identification of all isolates reported that they belong to the genus Bacillus. Out of all,
three strains of *Bacillus* designated as B1, B2, and B11 were chosen for amylase production (Salwa Elamin Ibrahim *et al.*, 2012).

Spread plate technique was adopted to isolate discrete colonies (Kriss, 1963). Bacterial strains were screened for their ability to produce α-amylase by streaking them on starch agar plates. Screening and isolation of alpha-amylase producing fungi and bacteria from marine water and soil by using enrichment techniques was reported by Medda & Chandra (1980) and Pretorius *et al.* (1986). Different sources of fungi (seventy-eight strains of *Aspergillus oryzae*) were reported to be isolated from soils of different habitats by serial dilution method (Clark *et al.*, 1958).

The high yielding 17 isolates were partially identified using protocols given in Bergy’s manual of determinative bacteriology. Similarly by using protocols given in Bergy’s manual of determinative bacteriology, the partial identification of bacteria was performed and identified as *Bacillus* *spp.* (Sneath *et al.*, 1986). Ajayi & Fagade (2003) have reported several bacterial isolates, based on the zone of starch hydrolysis. They were reported as *Bacillus* sp.1 and *Bacillus* sp.2, which produced more α-amylase enzyme. Bacterial identification was performed on randomly selected colonies using standard bacterial taxonomy procedures according to the 8th edition of Bergey’s Manual of Determinative Bacteriology (Buchanan & Gibbons, 1974).

The identification of bacteria was carried out by observing colony, morphological and biochemical characterization. The isolates were classified in to 5 groups: *Brevibacillus borstelensis*, *Bacillus cereus*, *Bacillus mycoides*, *Bacillus licheniformis* and *Bacillus cogulans*. Pretorius *et al.* (1986) have isolated 134 α-amylase producing strains of *Bacillus* *spp*. The strains were divided into 12 groups and their biochemical and morphological characterizations were carried out. It was reported that the isolates were related to *Bacillus subtilis*, *Bacillus licheniformis* and *Bacillus amyloliquefaciens*. 

201
Highly active α-amylase is preferred for the conversion of starch into oligosaccharides. So, it is worthwhile to select a potent microbial isolate for α-amylase production.

All the 17 isolates were Gram positive, showed amylase activity, blood agar hydrolysis, and exhibited sugar fermentation. Many of them were sporulated, motile and capsulated. Exhibited activities of protease, lipase, catalase, oxidase and urease. Morphological and biochemical characterization was reported by many earlier workers, such as Gram staining (Salle, 1948), coagulation and peptonization of milk (Salle, 1948), starch hydrolysis (Lyons & Pridham, 1962; Hugo et al., 2003), H₂S production (Shirling & Gottlieb, 1966), lipolytic assay (Mielmann, 2006), nitrate reduction test (Salle, 1948) and gelatin hydrolysis (Gordon & Mihm, 1957).

4. II Optimization of α-amylase production of Brevibacillus borstelensis R1

The present work was carried out in one medium selected from ten media to optimize the physical and chemical parameters. The highest α-amylase production was found in Pikovskaya’s Medium (PK) and the lowest in Nutrient broth and Luria bertain broth. The highest α-amylase production (1,800U/ml) was found during the stationary phase (18-24hrs). The production of amylase was increased with the increase in the level of inoculum at 2% (1813±23U/ml). The highest amylase activity was recorded at 37°C (2086±71U/ml), pH 7.0 (2083±56U/ml) and at salinity 1.0% (2082±58U/ml) and the lowest at 4°C, pH 11.0 and at salinity 2.0%.

The amylase production after 72 hours of growth (Bacillus subtilis) was maximum (370U/mg). The effects of incubation period, pH of the medium and incubation temperature were optimized. The maximum production of enzyme was obtained at 30°C and pH 7.0 (Harikrishna yadav et al., 2012). The production of the enzyme by Bacillus sp. was maximum at 10hrs after inoculation (Pushpendra Singh et al., 2012). The enzyme production largely dependent on the type of strain, composition
of medium, cell growth, initial pH and thermo stability (Haq et al., 2002 & 2005). Increased incubation period decreased the α-amylase production. It might be due the depletion of nutrients, accumulation of toxic byproducts in the medium, proteolysis of α-amylase and microorganisms (Chamber et al., 1999; Yang & Liu, 2004). To obtain maximum enzyme yield, development of a suitable medium and culture conditions were obligatory (Narang & Satyanarayana, 2001; Ray et al., 2008).

Abate et al., (1999) and Raj Devi & Yogeesvaran (1999) reported that the maximum enzyme production obtained after 24hrs of incubation in case of Bacillus amyloliquefaciens and 48hrs of incubation with Micrococcus halobius. The exponential growth phase was 14-20hours for Pyrococcus furiosus (Schicho et al., 1993). The production of the enzyme was maximum at 10hrs after inoculation with Bacillus sp. (Pushpendra Singh et al., 2012). Maximum growth was shown at 48hrs and amylase activity at 24hrs (Srividya Shivakumar, 2012). Bradyrhizobium and Bacillus stearothermophilus exhibited positive correlation between amylase production and bacterial biomass in the stationary phase (Davies et al., 1980). Lee et al. (2006) reported more amylase production in exponential growth phase of Bacillus licheniformis. Sunna & Hashwa (1990) reported amylase production in decline phase of growth in Bacillus species. Herbert (1961) correlated the amylase production and dry weight.

In the literature survey the optimum amylase production was delineated by several authors in different media. In acidic conditions (pH 4.0 - 6.5) the amylase production was reported in Bacillus spp. (Anyangwa et al., 1993; Wind et al., 1994; Mck-Tigue et al., 1994; Takasaki et al., 1994; Zhang et al., 1994; Kim et al., 1995; Mamo & Gessesse, 1999b; Ohdan et al., 1999; Malhotra et al., 2000; Deutch, 2002; Gupta et al., 2003; Soni et al., 2003; Konsula & Liakopoulou-kyriakides, 2004; Sodhi et al., 2005;
Sajedi et al., 2005; Zlem Kirani et al., 2005; Sivarama Krishnan et al., 2006; Rajagopalan & Krishnan, 2008; Schwab et al., 2009; Asoodeh et al., 2010).

In neutral conditions (pH 6.5 - 7.5) the amylase production was reported in Bacillus spp. (Ramesh & Lonsane, 1991; Castro et al., 1992; Lealem & Gashe, 1994; Syu & Chen, 1997; Castro et al., 1999; Malhotra et al., 2000; Cordeiro et al., 2002; Haq et al., 2002; Burhan et al., 2003; Baysal et al., 2003; Tanyildizi et al., 2005; Haq et al., 2005; Tanyildizi et al., 2005b; Nagarajan et al., 2010; Goyal et al., 2005; Agrawal et al., 2005; Asgher et al., 2007; Tanyildizi et al., 2007; Konsoula & Liakopoulou-Kyriakides, 2007). In alkaline conditions (pH 7.5 - 11) the α-amylase production was reported in Bacillus spp. (Mc-Tigue et al., 1994; Horikoshi, 1996; Igarashi et al., 1998; Hagihara et al., 2001; Burhan et al., 2003; Saxena et al., 2007).

The production was highest in Pikovskaya’s Medium at 37°C. The amylase production in different media was reported in psychrophilic conditions (Temp. 4°C) in Alteromonas haloplanktis (Feller et al., 1998), Nocardiopsis sp. 7326 (Feller et al., 1999, Groudieva et al., 2004, D'Amico et al., 2006; Siddiqui et al., 2006) and Arthrobacter psychrolactophilus (Michael et al., 2005). The zenith amylase production of mesophilic Bacillus spp. (Temperature 30°-45°C) was communicated by Saito & Yamamoto (1975), Fogarty (1983), Philip et al., (1988), Krishna & Chandrasekaran (1990), Salva & Moraes (1994), Lealem & Gashe (1994), Castro et al. (1999), Haki & Rakshit (2003), Declerck et al. (2003), Baysal et al. (2003), Soni et al. (2003), Konsoula & Liakopoulou-Kyriakides (2004), Zlem kirani et al. (2005), Tanyildizi et al. (2007), Rajagopalan & Krishnan (2008) and Nagarajan et al. (2010).

Peak production of amylase in Bacillus spp. in thermophilic condition (Temp. 50°-100°C) was documented by Malhotra et al. (2000), Narang & Satyanarayana (2001), Fitter et al. (2001), Hagihara et al. (2001), Deutch (2002), Burhan et al. (2003), Gomes
et al. (2003), Aditi et al. (2004), Konsula & Liakopoulou-Kyriakides (2004), Goyal et al. (2005), Mishra et al. (2005), Mendu et al. (2005), Agrawal et al. (2005), Sodhi et al. (2005), Sajedi et al. (2005), Saxena et al. (2007), Konsoula & Liakopoulou-Kyriakides (2007), Mukherjee et al. (2009), Schwab et al. (2009) and Asoodeh et al. (2010).

The amylase production of *Brevibacillus borostelensis* R1 was most favorable in Pikovskaya’s medium with augment of 1% NaCl. The NaCl % source of metal ion was reported to have a stirring effect on the production of amylase was disclosed in *Bacillus* spp. by Fukumoto (1944), Proom & Knight (1955), Jana & Pati (1997), Shinke et al. (1977), Chandra et al. (1980), Wu et al. (1999), Haq et al. (2002b), Bernharsdotter (2005) and Ashabil Aygan (2008). Parallel work with different NaCl concentrations (0.5-1.5%) was carried out in *Bacillus* spp. (Ventosa et al., 1989; Ikram-ul-haq, 2009; Bal et al., 2009). But subdued effect was reported in *Bacillus* sp. (Muhammad Hamad Ashraf, 2004).

The production of α-amylase by *B. Borostelensis* R1 was found to be highest in natural carbon supplements in PK medium than synthetic carbon sources. The optimum production was found by natural additive of *Saccharum officinarum* (5%) when compared with other sources. In mannitol (3%) the production was least. The addition of 2% *Metroxylon sago* yielded maximum amylase in *Bacillus* sp. (Yang & Liu, 2004). *Triticum vulgare* as supplement in optimized enzyme production with *Pencillium citrinum* HBF62 was reported by Fogarty & Kelly (1990), Chung et al. (1995), Lefuji et al. (1996), Swamy & Seenayya (1996) and Djekri-Dakhmouche et al. (2005). Corn starch was used as carbon source in *Bacillus circulans* (Tsurikova et al., 2002), *Bacillus licheniformis*, *Bacillus megaterium* and in *Bacillus subtilis* (Ajayi & Fagade, 2006). Potato starch as carbon source was used in *Bacillus subtilis* JS (72) (Asgher et al., 2007).
Dettori et al. (1992), Tanyildizi et al. (2005), Arunava et al. (1993) and Narang & Satyanarayana (2001) reported maltose (0.5-1.0%) as a provoking additive in some Bacillus spp.. Different concentrations of maltose were reported by Qixian Zhang et al. (1983), Dettori et al. (1992), Verhagen et al. (2001), Adams et al. (2001), Schut et al. (2003) and Tanyildizi et al. (2005). In contrast it was reported that maltose suppressed the production of α-amylase in Bacillus coagulans (Deutch, 2002; Babu & Satyanarayana, 1995). Sucrose as stimulant was described by Babu and Satyanarayana (1993). In contrast Deutch (2002) reported the inhibitory action of sucrose in Bacillus spp.

Starch in Bacillus spp. was found to be better augment to enhance amylase production as inquired into by Velcheva & Galabova (1984), Srivastava & Baruah (1986), Arunava et al. (1993), Mc-Tigue et al. (1994), Wind et al. (1994), Salva & Moraes (1995), Hamilton et al. (1999), Narang & Satyanarayana (2001), Goyal et al. (2005), Tanyildizi et al. (2005) and Sodhi et al. (2005). Starch was reported to be an ideal carbon source for the production of amylase from Bacillus thermooleovorans (Sailas Benjamin et al., 2013). Starch supplement (2%) in PK medium gave maximum production in our studies. Similar findings were observed by Malhotra et al. (2000) and Aditi et al. (2004).

Dextrose enhanced amylase production by Bacillus sp. in the circulate of Babu & Satyanarayana (1993), Arunava et al. (1993) and Narang & Satyanarayana (2001). Optimal production of amylase in different concentrations of dextrose (0.075% and 1.0%) was examined by Salva & Moraes (1995) and Lin et al. (1998) respectively. In contrast to these observations Babu & Satyanarayana (1995) and Deutch (2002) reported the inhibitory effect of dextrose on Bacillus sp.. Chandra et al. (1980) and
Babu & Satyanarayana (1993) showed galactose as stimulator in the amylase production in *Bacillus* species.

Lactose in *Bacillus* sp. was reported to enhance the production as investigated by Arunava *et al.* (1993), Narang & satyanarayana (2001), Salva & Moraes (1995) and Hillier *et al.* (1996) but it was also reported that lactose inhibited the amylase production in *Bacillus* *dipsosauri* strain DD1 (Deutsch, 2002). Arunava *et al.* (1993), Hamilton *et al.* (1999) and Carlsen & Nielsen (2001) had shown accelerating effect of mannitol in the amylase production by *Aspergillus oryzae*.

The production of α-amylase (2826±37) was found to be highest in 1% beef extract. The enhancing effect of beef extract on amylase production by bacterial strains was circulated by Rukhaiyar & Srivastava (1995), Suk *et al.* (1995b) and Okolo *et al.* (1996). Lachmund *et al.* (1993), Pederson & Nielsion (2000) and Kammoun *et al.* (2008) have shown the positive effect of casein on amylase production by *Aspergillus oryzae*. The summit production was found in natural addition of soybean meal (1%). Corresponding studies in enhancing amylase production in *Bacillus* sp. were reported by Francis *et al.* (2003), Sodhi *et al.* (2005) and Goyal *et al.* (2005).

Babu & Satyanarayana (1993), Haq *et al.* (2002), Aiyer (2004), Swain *et al.* (2006) and Elif Demirkan & Demirkan (2011) reported the stimulating effect of tryptone in *Bacillus* sp. Malhotra *et al.* (2000) observed that tryptone (0.3%) showed optimum amylase production. Tryptone was reported to be as nitrogen source for the production of amylase in *B. thermooleovorans* (Saias Benjamin *et al.*, 2013).

Calcium chloride (0.8%) was found to produce maximum amylase activity (2962 ± 2U/ml) among 10 natural and 10 synthetic mineral sources used. Burhan *et al.* (2003) have reported that *Bacillus* sp. in the presence of calcium increased enzyme production. Effect of different concentrations of calcium was reported by Proom &

Agar-agar is found effective in increasing thermal stability of immobilized Brevibacillus borostelensis R1. Cell immobilization in alginate was reported in Bacillus licheniformis (Dobreva et al., 1996) and in Bacillus circulans GRS 313 (Gargi et al., 2003). Jamuna & Ramakrishna (1992) reported maximal enzyme yield (90.0 U/ml) at 4% (w/v) alginate concentration. This concentration was recommended for the production of amylase by encapsulated Bifidobacterium Bifidum No.1, 791 cells. Gomboz & Wee (1998) and Goksungur & Zorlu (2001) studied that Bacillus subtilis had produced thermostable α-amylase by entrapping in calcium alginate gel capsules. Lamas et al. (2001) reported that Bacillus sp. when immobilized in 1% sodium alginate gave highest enzyme yield. Audet et al. (1990) and Konsoula & Liakopoulou-Kyriakides (2006) reported the increase of thermal stability of cells immobilized in carrageenan by using entrapment technique. Chevalier & Nouee (1987) have obtained optimum production of amylase with increased optimum temperature to 50°C by using carrageenan as immobilized media.

Alpha-amylase immobilized at 3% concentration of agarose, sodium alginate and acrylamide (10%) media have produced increased production of amylase with increased optimum temperature to 60°C. Mahajan et al. (2010) checked the suitability of amylase entrapped in agarose beads for use in pharmaceutical industry. Raviyan et
al. (2003) reported the thermal stability of *Bacillus licheniformis* in agar medium. Dobreva *et al.* (1996) and Abou-Elela *et al.* (2009) studied thermal stability of α-amylase from *Aspergillus oryzae* entrapped in polyacrylamide gel (50-70 °C). The thermal stability of amylase was increased when immobilized in sodium alginate to 50°C (Krajewska *et al.* 1990) and 60°C (Dey *et al.* 2003) in *Bacillus* species.

4. III Isolation of mutants by using physical and chemical mutagens

The percent of survival of *Brevibacillus borstelensis* R1 in Pikovskaya’s medium was 25.75% after 120 minutes of exposure to UV radiation. The potent UV mutants which showed more than 20mm zone of starch hydrolysis were screened and selected at 42% survival time after 80 minutes exposure. The potent UV mutants and chemical mutants which showed more than 20mm zone of starch hydrolysis were screened and selected. The wild strain with fixed physical parameters and chemical parameters yielded 3000U/ml enzyme activity. Out of ten UV mutants, two (UV-3 and UV-10) showed amylase activity more than the wild strain. Out of fifty chemical mutants, ten mutants (HNO₂-10, HNO₂-30, EMS-4, EtBr-40, EtBr-50, Acr-1, Acr-20, Acr-30, Acr-4 and 5-FU-50) showed amylase activity more than the wild strain.

Baily & Markkanen (1979) reported the mutant of *Bacillus subtilis* yielded amylase, double that of parent strain. In extraordinary way Jin *et al.* (1998) have developed a hyper producing α-amylase mutant of *Bacillus licheniformis* which yielded 50 times higher enzyme activity than the parental strain. The α-amylase production was decreased in 48 mutant strains out of 60mutant strains isolated when compared to parent strain/wild strain. Similarly decrease in production of α-amylase was reported by Haq *et al.* (2010) in mutant strain of *Bacillus amyloliquefaciens* UNG-16 when compared to the wild parental strain.
The effect of UV irradiation on *Bacillus* spp. was most effective in the production of α-amylase (Demain & Hendlin, 1957; Buchanan & Gibbons, 1974; Markkanen & Suihko, 1974; Markkanen & Suihko, 1974b; Yoneda & Maruo, 1975; Baily & Markkanen, 1979; Qirang & Zhao 1994; Sarikaya & Gurgun, 2000; Haq et al., 2002b; Pandey et al., 2000b; Varalakshmi, 2008; Haq et al., 2010).

The studies of Adelberg *et al.* (1965), Konishi *et al.* (1990), Sidhu *et al.* (1997), Bin *et al.* (1999) and Haq (2002) reported that nitrous acid was found to be the suitable mutagen for improvement of α-amylase production in *Bacillus* spp. Similarly, maximum production of α-amylase (285 U/ml) was reported in *Aspergillus oryzae* NA17 and also in *Aspergillus oryzae* by Szafraniec *et al.* (2003) and Roheena Abdullah (2005).

Haq *et al.* (2010) screened mutant strains of *Bacillus amyloliquificans* EMS-6 which gave 102.78±2.22 U/ml/min α-amylase activity which was around 1.4 fold higher than the parental strain *Bacillus amyloliquificans* UNG-16. Similarly, Szafraniec *et al.* (2003) and Roheena abdullah (2005) worked with *Aspergillus oryzae* (EMS-18) isolate which gave maximum production of α-amylase 347 U/ml. Sobiya Shafique *et al.* (2009) described the *Aspergillus tenuissima* FCBP-252 for hyperactivity of α-amylase enzyme through EMS mutagenesis. They reported that EMS (250μg/mL⁻¹) treatment gave maximum α-amylase activity (76.75 Units mL⁻¹). It was a superior mutant strain and was assigned a code At-Ch-5.6. Qirang & Zhao (1994), Spohr *et al.* (1998), Azin & Noroozi (2001) worked on *Aspergillus oryzae* and reported it to be the best mutant for α-amylase production by successive treatments with mutagenic EMS.

mutagenesis for improvement of α-amylase production of bacterial strains with EMS. Similarly Sarikaya & Gurgun (2000) worked on mutants of *Bacillus subtilis* and Ikram-ul-haq *et al.* (2009) reported on mutants of *Bacillus licheniformis* GCB-30UCM strain. Sarikaya & Gurgun (2000) reported about the mutants of *Bacillus subtilis* obtained by ethidium bromide which increased amylase production.

4. IV Partial purification of α-amylase produced by *Brevibacillus borstelensis* R1

The 70% (NH₄)₂SO₄ precipitation fraction with 1.94fold purification was selected for further purification. Haider (2004) reported the precipitation of the amylase by ammonium sulfate (40-60%) saturation in *Bacillus subtilis*. Ventosa *et al.* (1998) precipitated amylase (halophilic *Bacillus spp.*) using 40 to 90% ammonium sulfate and the active enzyme fraction was recovered at 70-80%. Ikram-ul-haq *et al.* (2010) recovered precipitated amylase with purification fold (1.815) in *Bacillus licheniformis* EMS-6. Varalakshmi *et al.* (2008) reported 1.06fold purification fold with ammonium sulphate fractionation.

The 38th fraction of Sephadex G-100 with 3.9 fold purification, yielded (30.4%) with specific activity (790.21U/mg protein). Chung *et al.* (1995) purified α-amylase (*Thermococcus profundus*) by gel filtration of sephadex 200. Haider (2004) purified *Bacillus subtilis* α-amylase by gel filtration chromatography on Sephadex column (G-100) and obtained purification fold and recovery 67.92 and 43.9% respectively. The specific activity of purified enzyme was 49.3 fold of the crude enzyme in *Bacillus amyloliquefaciens* strain (Zeily Nurachman *et al.* 2010).

In the present study, the reducing sugar released by the α-amylase activity was maltose under assay conditions. Similar studies were conducted on *Bacillus spp.* by Ashabil Aygan *et al.* (2008) and Gashaw Mamo & Amare Gessesse (1999a). Hansen
(1975) employed thin layer chromatography (TLC) for identification of maltose and oligosaccharides of starch hydrolysates. Jin & Run (2007) reported that the concentration of the resulting sugars increased with increase of the incubation time. These hydrolysis patterns revealed that amylase functioned as a typical amylase to hydrolyze the α-(1, 4)-glycosidic linkage in *Bacillus* strain 7326. Vaseekaran *et al.* (2010) reported that the α-amylase activity resulted in releasing glucose, under assay conditions.

Native PAGE (polyacrylamide gel electrophoresis) conducted to determine the homogeneity of α-amylase at pH 8.3 in 12% slab gel. The zymogram of native-PAGE result of partially purified sample was identified as single protein.

The protein band obtained by partial purified fraction was found to be α-amylase by conducting specific staining methods in polyacrylamide gel electrophoretic pattern at pH 8.3 in 12% slab gel: iodine staining. Silver staining was done to separate less amount of protein (Sammons *et al*., 1981). The α-amylolytic activity was confirmed by halo zone of starch hydrolysis (Hashim *et al*., 2004; Ashabil Aygan *et al*., 2008). Hmidet *et al.* (2008) purified amylase with Sephadex G-100, with activity 178.5(U/mg protein), Yield 15.9% and purification (fold) 3.08 in *Bacillus licheniformis*.

The optimum temperature of partially purified α-amylase of *B. borostelensis* R1 was studied. The highest amylase activity was observed at 37°C (6133±58U/ml). Only 38.47% activity remained at 70°C. The optimum temperature of partially purified enzyme was similar to the crude enzyme. Optimum temperature range (30-50°C) was reported in *Halomonas meridian* (Coronado *et al*., 2000), *Clostridium perfringenes* (Shih *et al*., 1995), *Micrococcus luteus* (Fergus, 1969), *Halomonas meridian* (Coronado *et al*., 2000), *Mycococcus varians* (Stevens *et al*., 1994), *Bifidobacterium adolescentis* (Lee *et al*., 1997) and *Streptococcus bovis* (Freer *et al*., 1993).
The partially purified enzyme was tested for its stability for 1-10 hours at temperatures ranging from 4°C-70°C. The α-amylase retained its activity after 10 hrs exposure to 4°C with negligible variations in activity, at 20°C it retained 34.2% activity, at 25°C it retained 44.1% activity, at 37°C it retained 34.7% activity, at 50°C it retained 56.2% activity, at 60°C it retained 38.2% activity and at 70°C it retained 30.8% activity upon exposure to about 10 hrs. Similar reports on thermal stability were reported (Kong et al., 1993; Xianliang et al., 1993; Salva & Moraes, 1994; Goyal et al., 1995; Chung, Sang-Jin & Baik Hwang, 1996; Schokker & Van Boekel, 1999; Malhotra et al., 2000; Huei-Gen et al., 2001; El-Safey & Ammar, 2002; Pimpa, 2004; Alva et al., 2007). The enzyme was optimally active with a half-life of 3 hrs at 100°C in Bacillus thermooleovorans NP5 (Malhotra et al., 2000). Enzyme activity increased as the temperature rose gradually from 75°C to 100°C. The enzyme was reported to be fairly stable retaining more than 90% of its original activity after 60 min exposure at 90°C and 20 min exposure at 95°C of Bacillus licheniformis mutant 7902 (Kong et al., 1993; Xianliang et al., 1993). In Bacillus spp. the enzyme was reported to be not very stable at 60°C and was completely inactivated after 10 minutes at 80°C (Salva & Moraes, 1994). The enzyme was stable at 90°C for 10 min and at 100°C it still retained 26% of its initial activity (Goyal et al., 1995). Amylase from Bacillus licheniformis CUMC305 showed many interesting features. The purified enzyme showed maximal activity at 90°C, pH 9.0 and 91% of it’s activity remained at 100°C (Krishnan & Chandra, 1983). The stability of the amylase from Bacillus stearothermophilus could be enhanced if liquefied thick starch slurries (at 80°C and pH 6.9) were provided as feed/substrate in the presence of Ca²⁺ (Srivastava & Baruah, 1986).
The optimum pH of partially purified α-amylase was 7.0. Similar reports were given by Loginova et al. (1970), Ladermann et al. (1993), Syu & Chen (1997), Malhotra et al. (2000), Haq et al. (2002), Tanyildizi et al. (2005) and Haq et al. (2005).

The effect substrate on the rate of catalytic activity of partially purified α-amylase was studied by Michaelis-Menten equation. The Vmax (17.73±0.5612 U/ml) was observed at starch concentration of 3.5mg/ml. The Km value of the enzyme was (1.368±0.1292mg/ml). The Km values of partially purified amylases were reported to be within the range of 0.35 to 4.7mg/ml. Several authors reported Km value of α-amylase by using starch as substrate: 2.38 g/l in Lactobacillus plantarum (Giraud et al., 1993), 3.85 mg/ml in Bacillus subtilis (Macro et al., 1996), 2.2 mg/ml in Bacillus flavothermus (Boltan et al., 1997), 2.4g/l in Bifidobacterium adolescentis (Lee et al., 1997), 5.0mg/ml in Thermococcus filiformis (Egas et al., 1998), 1.4 mg/ml in Bacillus cereus GUF8 (Aguilar et al., 2000), 2.6mg/ml in Bacillus subtilis (Nagarajan et al., 2010), 3.05mg/ml in Geobacillus thermodenitrificans (Ezeji & Bahl, 2006), 11.7 mg/ml for Bacillus acidocaldarius strain RP1 (Natalia et al., 2006), 1.08mg/ml/minute in Bacillus licheniformis (Krishnan & Chandra, 1983), 909U/mg in Bacillus subtilis (Nagarajan et al., 2010), 0.051µmol/min for a species of an alkalophilic Bacillus (Bernhardsdotter et al., 2005), 11.176 mg/ml/h in thermophilic Bacillus subtilis strain (Konsula & Liakopoulou-Kyriakides, 2004), 600milliunits/mg in Bacillus acidocaldarius strain RP1 (Natalia et al., 2006), 7.35U/mL Geobacillus thermodenitrificans (Ezeji & Bahl, 2006) and 11.7mg/mL in Bacillus acidocaldarius strain RP1 (Natalia et al., 2006). Hanumanthu Prasanna Lakshmi (2013) reported Km (11.57±0.409mg/ml), Vmax (0.032±0.0036 (mg/ml)/mg/min) and Kcat (1.6517±0.055mg/ml) in Staphylococcus aureus. Amico et al. (2002) reported Km (234 µM) of amylase in
*Pseudoalteromonas haloplanktis* using 3.5 mM 4-nitrophenyl-α-D-maltoheptaoside-4, 6-O-ethylidene as substrate.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) in 12% slab gel was run at pH 8.3 with molecular weight markers. Our findings suggested that the quaternary structure of the α-amylase of the *Brevibacillus borstelensis* R1 was monomeric with 43kDa molecular weight. Approximate molecular mass (42kDa) was reported by Koch *et al.*, 1991 & Chung *et al.*, 1995 (*Thermococcus profundus*), Elaassar *et al.*, 1992 (*Bacillus lentus*), Salva *et al.*, 1995 (*Bacillus subtilis*), Schokker & van Boekel (1999) and Alva *et al.*, 2007. In *Bacillus cereus* GUF8 (Aguilar *et al.*, 2000) purified α-amylase appeared as a single polypeptide with a molecular weight of about 56kDa similar to *Bacillus licheniformis* and *Bacillus amyloliquefaciens* α-amylases.

The percentage of increase in α-amylase activity when treated with (0.5M CaSO₄), (0.5M CaCO₃), (0.5M NaCl), (0.5M CaCl₂), (0.2M MgSO₄) and (0.1M MgCl₂) was 23.72%, 22.03%, 21.08%, 20.53%, 18.8% and 5.93% respectively.

Stimulatory effect of MgCl₂ on α-amylase was studied in *Bacillus spp.* (Kong *et al.*, 1993; Zhang *et al.*, 1994; Yuguo *et al.*, 1994; Hori *et al.*, 1997; Kelly *et al.*, 1997; Pandey *et al.*, 2000; Gupta *et al.*, 2003; Sindhu, 2005; Polaina MacCabe, 2007; Sun *et al.*, 2010; Elif Demirkan & Demirkan, 2011). Inhibition of amylase activity by MgCl₂ was also reported in *Bacillus spp.* (Babu & Satyanarayana, 1993b; Chung *et al.*, 1995; Abou Zeid, 1997). Stimulatory effect of magnesium sulphate on α-amylase was reported in *Bacillus spp.* (Saito & Yamamoto, 1974; Abou Zeid 1997; Roheena abdullah, 2005; Ikram-ul-haq *et al.*, 2010).

The effect of inhibitors (AgNO₃, HgCl₂, EDTA, CuSO₄, L-Glutamic acid and ZnCl₂) on partially purified enzyme activity was studied. All the inhibitors inhibited the activity when compared with the control. The % of amylase activity retained after treating with 0.5M of AgNO₃, HgCl₂, EDTA, CuSO₄, L-Glutamic acid and ZnCl₂ was 24.58%, 27.79%, 19.03%, 20.19%, 31.59% and 23.69% respectively.


It was reported that the α-amylase activity of *Bacillus spp.* was inhibited in the presence of zinc ions (Brown *et al.*, 1990; Hn *et al.*, 1992; El-Aaasr *et al.*, 1992; Fengxie *et al.*, 1992; Chunzhi *et al.*, 1992; Abou Zeid, 1997; Lin *et al.*, 1998; Elif & Velittin 2000; Cordeiro *et al.*, 2002; Burhan *et al.*, 2003; Rohcena abдullah, 2005). *Bacillus dipsosauri* strain DD1 (Deutch, 2002), *Bacillus stearothermophilus* (Chakraborty *et al.*, 2000), Bifidobacterium adolescentis (Lee *et al.*, 1997), *Bacillus pumilus* (Ming *et al.*, 1992), *Bacillus coagulans* (Hawary, 1991), *Bacillus licheniformis* A-4041 (Yungo *et al.*, 1994) and *Bacillus firmus* (Igarashi *et al.*, 1998) were also
reported to be inhibited by zinc ions. Zinc chloride reported to increase the activity of amylase in *Bacillus* spp. (Zhang et al., 1994; Chung et al., 1996; Pandey et al., 2000; Savchenko et al., 2001; Gupta et al., 2003; Sindh, 2005; Sun et al., 2010).

The stimulatory effect of Cu$^{2+}$ was reported in *Bacillus licheniformis* CUMC-305 (Krishnan & Chandra, 1983).

4. V Applications

In the present study, the application of α-amylase produced from *Brevibacillus borostelensis* R1 had improved taste, crust color, softness and toasting qualities of the bread. In bakery industry, the shelf life of bread and buns was increased from 5 days to 11 days. Similar work on α-amylase produced by *Bacillus* spp. was reported in improving the quality and shelf life of the bread in bakery industry by Hebeda et al. (1991), Van Damme et al. (1992), Hamer (1995), Armero & Collar (1996), Qi Si (1996), Sahlstrom & Brathen (1997), Christophersen et al. (1998), Dauter et al. (1999), Martinez et al. (1999), Nielsen & Borchert (2000) and Van der Maarel et al. (2002).

Alpha-amylase along with detergent worked more effectively in automatic dishwashing in removing tough stains. Similar work on the role of amylases in modern detergents was carried out by Olsen & Falholt (1998), Kirk et al. (2002), Maarel et al. (2002), Gupta et al. (2003), Mitidieri et al. (2006), Chi et al. (2009), Hmidet et al. (2009) and Mukherjee et al. (2009).

In dual fermentation by using co-strains, *B. borostelensis* R1 (α-amylase producing) along with *Saccharomyces cerevisiae* gave the best results on 21$^\text{st}$ day in ethyl alcohol production than with *S. cerevisiae* alone. Similarly dual fermentation with *Bacillus* spp. in ethyl alcohol production was reported by Matsumoto et al. (1982), De Moraes et al. (1995), Moraes et al. (1999), Santa Maria et al. (1999), Öner (2006) and Chi et al. (2009).
*B. borostelensis* R1 was used in treatment of sago, rice industries effluent and kitchen sewage water. The test sample treated with 5% *B. borostelensis* R1 was proved to be the best in degrading the starch and also increased the biomass production subsequently. Aiyer (2005) reported about the treatment of food processing starch in waste water and standard sewage treatment with amylases and amylase producing microorganisms which produced valuable microbial biomass protein (used as fodder), solving pollution problem and also used in effluent purification.

In textile industry, α- amylase produced from *B. borostelensis* R1 used as a good desizer when applied on the sample pieces of sari, towel and navaar. Similarly, amylases from *Bacillus* strain employed in textile industry were reported to show promising results (Chengyi *et al.*, 1999; Maarel *et al.*, 2002; Feitkenhauer 2003; Gupta *et al.*, 2003; Thippeswamy *et al.*, 2006; Arikam, 2008; Shaw *et al.*, 2009; Ahlawat *et al.*, 2009; Indira *et al.*, 2012).