Hydrolytic enzymes specifically amylases are among the most important biocatalysts for anaerobic digestion of organic waste. The current research is aimed at isolation, partial characterization and purification of α-Amylase by microorganisms from anaerobic digester carrying anaerobic co-digestion of cow manure (CM) with fruit-vegetable waste (FVW), and agricultural residues (AR) in continuous stirred tank reactor (CSTR). Six bacterial strains were isolated from anaerobic digester operating at mesophilic condition. With large hydrolytic zone producing two strains (RAS-1 and RAS-4) were selected for further study among the six isolates by plate assay. Selected isolates were identified as Bacillus subtilis (RAS-1) and Clostridium perfringens (RAS-4) by 16s rRNA gene sequencing. Temperature, pH, effect of inhibitor and metal ions on the stability of purified α-Amylase were studied. The enzyme was relatively stable between pH 7 to pH 9 and at temperatures ranging from 37°C to 45°C. The α-Amylase enzyme was purified by ammonium sulphate precipitation and column chromatography. The α-Amylase molecular weight estimation was confirmed by SDS-PAGE with a band of 52 kDa for B. subtilis (RAS-1) and 73 kDa of C. perfringens (RAS-4). The results suggested the cost effective production of commercially important enzyme α-Amylases from organic waste during biogas production.

**Keywords:** Amylase, Bacillus subtilis, Clostridium perfringens, agricultural residues, column chromatography.

**INTRODUCTION**

Hydrolytic bacteria like Clostridia and Bacteriocides hydrolyze insoluble complex polymeric compounds to simple monomers such as amino acids, fatty acid and glucose by the action of their hydrolytic enzymes, including; amylase, protease, lipase and cellulase (Bagi et al., 2007). α-Amylases are the group of hydrolytic enzymes which catalyses the breakdown of α-1,4 glycosidic linkage of the starch molecule and release monosaccharaides (glucose, maltose and maltotriose). Bacterial strains are the significant source of amylase production (α, β, γ). The microbes can be isolated from soil, water, plants and animal sources. Microorganisms are the ubiquitous source of α-Amylase production because of accessibility, optimum growth requirements, efficient, ecofriendly and cheap substrate requirements as compared to animal and plant resources. In the starch liquefaction process other than chemical hydrolysis enzymatic hydrolysis through amylases are preferable, and contributing a major share (25%) of total enzymes presently use in the world (Burhan et al., 2003; Rajagopalan and Krishnan, 2008). A number of microorganisms are reported for production of α Amylase like, Bacillus subtilis, B. flavothermus, B. amylobiolutefaciens, Escherichia coliand Clostridium species (Pandey et al., 2000). Recently amylase producing Bacillus subtilis strain was isolated from garden soil responsible to hydrolyze polysaccharides into monosaccharaides (Panneerselvam and Elavarasi, 2015). By solid state fermentation alpha amylase was produced and purified from Bacillus subtilis (MTCC121) providing wheat bran as a substrate (Dibyangana et al., 2014).

Production of enzymes at industrial scale is sold out at market for various applications. In 2012 cost estimated on production was US $2.7 billion, which will increase to 4% annually according to estimation. Major industries which use 75% of industrially produced enzymes are textiles (12%), baking (8%), detergents (37%), animal feed 96%, and starch (11%). Amylase deals with the 25% of industrial total enzyme market requirement. Alpha Amylase has a significant application in bioconversion of starch based substrates, clinical, food, paper, Bakery, anti-salting Ethanol production and biotechnological applications (Panneerselvam and Elavarasi, 2015).

Energy has been considered as mainstay of social, industrial and economic development of the world. Biofuels such as biogas and bioethanol are among the most significant outcome of the global research on alternative fuels. Basically, these fuels are produced using agro-industrial waste, thus considered as sustainable and ecofriendly (Evans, 2007).

The municipal solid waste, including various agricultural leftovers and industrial wastes are being generated approximately two billion tons per year globally with an increase rate of 50% till the end of 2025 (Charles et al., 2009). Being an agricultural country Pakistan produces a
huge amount of cellulosic solid waste materials which could be used for generating biofuels. These waste materials contain high amount of organic compounds, therefore, considered as potential raw material for the production of biogas. On the other hand, such enormous amount of solid waste could be an additional load on the environment (Garcia et al., 2005; Neves et al., 2009). Various techniques have been employed for the management of aforementioned solid wastes such as composting, thermolysis and anaerobic digestion (Adhikari et al., 2009). Among these methods, anaerobic digestion technology is considered as the most promising as it generates biogas from the breakdown of complex organic pollutants. Anaerobic digestion technology is being used to generate the biogas from various organic waste materials. Biogas is considered as a potential substitute of fossil fuels for heat and power generation (Borjesson and Mattiasson., 2008; Tippayawong and Thanompongchart, 2010).

Anaerobic digestion is the sequential and complex series of biochemical processes that capitalize indigenous microorganisms for the conversion of organic matter to biogas under anaerobic conditions. Generally, process of anaerobic digestion can be divided into four phases, i.e., hydrolysis, acidogenesis, acetogenesis and methanogenesis (Salminen and Rintala, 2002).

Currently, the bioconversion of organic waste in Pakistan is impeding owing to less productive microbial strains, process optimization and knowledge regarding the process specific conditions. The current study is therefore, aimed for isolation, characterization and purification of α amylase from indigenously isolated bacterial strains treating organic waste in an anaerobic digester.

MATERIALS AND METHODS

Characterization of organic waste: Physiochemical characterization, including, total solids (TS), volatile solids (VS), moisture content (MC), of cow manure, fruit-vegetable waste and agricultural residues were analyzed in accordance with standard methods for the examination of water and wastewater treatment (APHA, 2005). The reactor treating the organic waste was CSTR operating at 37°C for 35 days of hydraulic retention time (HRT) with an organic loading rate (OLR) 12g-VS/L of substrate. Digestion set up: A 20L stainless steel dome shaped continuous digester was designed. The dome shaped biogas reactor ensures the gas storage to a single dimension. It reduces the surface area for a given volume of the reactor while elevating the biogas production efficiency. For feeding and waste mechanisms, 3/4 inch diameter opening fitted with pressure valves was utilized. For biogas determination, water displacement method was used. Mixing was performed by stirring rod attached in digester with baffles. For maintaining oxygen free conditions inside the reactor nitrogen gas was purged for 30 seconds.

Isolation and screening of α-Amylase producing bacteria: The samples were collected aseptically from the anaerobic CSTR bioreactor co-digesting fruit-vegetable waste, agricultural residues and cow manure at mesophilic temperature. The homogenized collected samples were diluted with 100 ml distilled water for further analysis. The samples were serially diluted and 0.1 ml from each tube was spread over freshly prepared nutrient agar plates. The plates were placed in an incubator at 37°C for 24 hours. For the screening of α-Amylase, Suman and Ramesh (2010), method was followed. The colonies showed clear zone on starch agar plates were maintained on nutrient agar slants and was stored at 4°C for further investigation.

Identification of bacterial isolates: The bacterial species isolated from anaerobic digester were identified by standard bacteriological identification procedure (Holt and Krieg, 1994). Morphological and biochemical characterization was carried out. Biochemical test includes, Catalase, H₂S, Voges Proksaure, Mannitol, Sugar fermentation (sucrose, lactose, and glucose) and starch hydrolysis were performed. For molecular identification, DNA was extracted from selected strains, RAS-1 and RAS-4 by CTAB method (Wilson, 1987). The Polymerase chain reaction was carried out using universal 16s rRNA Primers (518F and 800R) (Sekiguchi et al., 1998). The sequencing data were analyzed by using BLAST toll. Phylogenetic trees were constructed using MEGA 6.0 and CLUSTAL. W. Package.

Enzyme production: Production medium was consisting of MgSO₄ 0.2g, L-Cysteine HCl 0.5g, K₂HPO₄ 2g, (NH₄)₂SO₄ 3g, Soluble Starch 4.0g, Peptone 5g and trypticase 10g. Bacterial inoculum was added in production medium. The flasks were incubated at 37°C for 24 hrs followed by centrifugation at 10,000 xg for 10 min at 4°C to obtain the crude enzyme. The filtrate was then stored at -20°C to be used for enzyme purification.

Enzyme assay: The α-amylase (crude and purified) obtained from both bacterial strains was tested for their activity in accordance with 3,5-dinitrosalicylic acid (DNS) method. The media comprising fermented extract, soluble starch 1%, and phosphate buffer (20mM, pH=7) incubated at 37°C for 20 minutes, followed by addition of 2ml 3,5-dinitrosalicylic acid to observe the amount of reducing sugars liberated (Yang et al., 2003). The change in color due to reducing sugar was measured by UV-Vis spectrophotometer at 540 nm. The alpha amylase activity was calculated as the amount of enzyme releasing micromole of maltose per minute is equal to 1U of amylase activity under standard experimental conditions.

Purification of amylase enzyme: Enzyme precipitation was carried out by using 100 ml aliquot of bacterial suspension that was centrifuged at 6,000 xg 4°C for 15 minutes to obtain the cell free broth. At 4°C the supernatant was
brought to 70% saturation of ammonium sulphate and at 10,000 rpm the mixture was centrifuged for 15 min at 4°C. The obtained pellet was dissolved in phosphate buffer (0.1M: pH 6.8). The samples obtained after ammonium sulphate precipitation were subjected to sephadex G-75 column (0.9x60 cm) pre-equilibrated with 0.2 M phosphate buffer pH 6.8 and was eluted with the same buffer (Bozic et al., 2011). The fractions were collected at a flow rate of 24 ml/hr and assayed for amylase activity and protein estimation (280 nm). Fractions having maximum enzyme activity were collected, pooled and stored at 4°C for further studies.

**Enzyme molecular weight determination by SDS-PAGE:**
The fractions having high enzymatic activity were obtained from gel filtration chromatography were concentrated by freeze drying in lyophilizer (Labcono Freezono) and then used for the determination of relative molecular mass by the Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) on 12% gel (Laemmli., 1970). Coomassie brilliant blue R-250 was used for gel staining and was calibrated with marker of molecular mass of 10-100 kDa (Fermentos) and pH 7. The protein estimation was ascertained by using the Lowry method (Lowry et al., 1951) and a calibrated standard curve was made using bovine serum albumin to compare the results.

**Enzyme activity:**

a) **Effect of pH and temperature:** The effect of medium pH on the yield of α-Amylase was determined by growing the culture at different pH between 3.0 and 9.0 (Suresh and Chandrasekaran, 1999). The varying hydrogen ion concentrations were adjusted for the medium by molar solutions of NaOH and HCl. The effect of different temperatures ranging between 15 and 45°C was studied for optimum activity of enzymes.

b) **Effect of inhibitor and metal ions on the stability of purified α-Amylase:** The effect of enzyme inhibitor (EDTA) and metal ions (Ca²⁺, Mg²⁺, Co²⁺, Mn²⁺, Ni²⁺, Cu²⁺, Zn²⁺, Hg²⁺) were studied on the stability of α-Amylase. The enzyme activity was analyzed after pre incubation of the enzyme with the inhibitor for 2h at 4°C.

**RESULTS**

Isolation, screening and identification of bacterial strains:
Bacterial strains were isolated from anaerobic co-digestion reactor (CM+AR+FVW) and were tested for α-Amylase production by the starch hydrolysis test. Two out of six bacterial isolates were selected for further studies on the basis of their larger zone of hydrolysis on starch agar plates. Morphological and biochemical characterization of strains revealed Gram positive, road shape bacteria, H₂S +, TSI +, and Starch hydrolysis +ve, respectively (Table 1 and 2). On the basis of 16S rRNA (Fig. 1 and Fig. 2) the strains were identified as **Bacillus subtilis** RAS-1(KP799011) and **Bacillus licheniformis** strain x8 (GQ447911).

**Figure 1. Phylogenetic tree of Bacillus subtilis RAS-1.**
Clostridium perfringens RAS-4 (KP799010).

Partial purification of amylase enzyme by ammonium sulphate precipitation: The crude enzyme was precipitated with ammonium sulphate salt. α-Amylase enzyme from Bacillus subtilis (RAS-1) was partially purified with 70% of ammonium sulphate precipitation. It exhibited specific activity of 7.50 U/mg. The recovery and purification fold of amylase from Bacillus subtilis (RAS-1) were 37.5% and 2.60. While α-Amylase enzyme from Clostridium perfringens (RAS-4) was partially purified with 60% of ammonium sulphate precipitation. It exhibited specific activity of 6.01 U/mg, 2.29 purification fold with 32.38% recovery.

Purification of enzyme by using column chromatography: α-Amylase from Bacillus subtilis (RAS-1) was purified by gel chromatography (Fig. 3) showed that fraction 9 showed the highest specific activities of 39.63 and further characterized (Table 3). The specific activity of the enzyme after gel chromatography was about 39.81 U/mg, whereas the purification fold 13.82 and recovery percentage 30.97% for first peak, and with specific activity 39.44 U/mg, fold purity 11.80 and recovery percentage 29.35% for second peak.

Table 3. Purification profile of α-Amylase produced by Bacillus subtilis (RAS-1).

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total activity (U)</th>
<th>Total protein (mg)</th>
<th>Specific activity (U/mg)</th>
<th>Purification fold</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>720</td>
<td>250</td>
<td>2.88</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ precipitation (70%)</td>
<td>270</td>
<td>36</td>
<td>7.5</td>
<td>2.60</td>
<td>37.5</td>
</tr>
<tr>
<td>Column chromatography</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak-1</td>
<td>224</td>
<td>5.7</td>
<td>39.63</td>
<td>12.82</td>
<td>30.16</td>
</tr>
</tbody>
</table>

Table 4. Purification profile of α-Amylase produced by Clostridium perfringens (RAS-4).

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total activity (U)</th>
<th>Total protein (mg)</th>
<th>Specific activity (U/mg)</th>
<th>Purification fold</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>630</td>
<td>240</td>
<td>2.62</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ precipitation (70%)</td>
<td>204</td>
<td>34</td>
<td>6</td>
<td>2.29</td>
<td>32.38</td>
</tr>
<tr>
<td>Column chromatography</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak-2</td>
<td>205</td>
<td>6.08</td>
<td>33.74</td>
<td>13.2</td>
<td>31.3</td>
</tr>
</tbody>
</table>
Production of α-Amylase

α-Amylase from *Clostridium perfringens* (RAS-4) was purified by gel chromatography (Fig. 4) showed that fraction 12 have highest specific activity and further characterized and the detailed purification steps are summarized (Table 4). The specific activity of the enzyme after gel chromatographic step was shown 33.5 U/mg. Whereas, the purification fold 12.78 and recovery percentage 31.90%, for first peak and with specific activity 33.98 U/mg, purification fold 13.53 and recovery percentage 30.73 for second peak.

**Molecular weight estimation:** The apparent molecular weight of purified α-Amylase from *Bacillus subtilis* (RAS-1) and *Clostridium perfringens* (RAS-4) was characterized by using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The resulted single protein band on the 12% gel revealed the molecular weight of 52 kDa and 73 kDa respectively (Fig. 5). The molecular weight of purified enzyme was calculated according to the RF valves of the standards used from 100 to 10 kDa.

**Effects of the temperature on activity of purified α-amylase:** The effect of temperature on α-amylase was measured by incubating the enzyme at different temperature, ranges from (15-45°C). Enzyme activity of *Bacillus subtilis* (RAS-1) recorded at 15, 25, 30, 37,40 and 45°C were 5.0, 8.4, 12.43, 29.67, 33.89, 25.67 U/ml, respectively. While the maximum enzyme activity of *Bacillus subtilis* (RAS-1) was recorded at 40°C, above this temperature enzyme activity was declined. The optimum temperature for α-Amylase activity of *Clostridium perfringens* (RAS-4) was 37°C, above this temperature enzyme activity was decreased (Fig. 6). Optimal temperature was 40°C for *Bacillus subtilis* (RAS-1) with a maximum enzyme activity of 33.89 U/ml. It was also noted that 37°C was the optimum temperature for *Clostridium perfringens* (RAS-4). Moreover, the enzyme activity of *Clostridium perfringens* (RAS-4) was 28.43 U/ml at 37°C.

**Effect of the pH on activity of purified α-Amylase:** The enzyme activity of *Bacillus subtilis* (RAS-1) recorded at pH 3, 4, 5, 6, 7, 8 and 9. The optimum activity of α-Amylase by *Bacillus subtilis* (RAS-1) was observed at pH 8.0. The enzyme activity of *Clostridium perfringens* (RAS-4)
recorded at pH 3, 4, 5, 6, 7, 8 and 9. The maximum enzyme activity was observed in the medium of pH 7.0 in case of Clostridium perfringens (RAS-4). The α-Amylase enzyme was partially characterized under the effects of varying temperatures and pH values (Fig. 7).

**DISCUSSION**

During hydrolysis, obligatory and facultative anaerobic microorganisms such as Bacillus and Clostridium sp. secrete extracellular enzymes causing conversion of the complex macromolecules into monomers like sugars or alcohols, fatty acids, amino acids. The hydrolytic enzymes are mainly cellulases, lipases, proteases and amylases (Weiland, 2010; Parawira et al., 2005). Purified bacterial cultures were qualitatively screened on starch agar plates for the production of amylases and it was noted that the strains RAS-1 and RAS-4 shown comparatively larger zone of hydrolysis than other isolates. These bacterial isolates were identified on the basis of morphological and biochemical, and molecular techniques. The isolated strains were found to be rod shape and gram positive. The results of 16S rDNA analysis revealed that these bacteria (RAS-1 and RAS-4) were strains of Bacillus subtilis and Clostridium perfringens, respectively. It has been demonstrated by a number of reports that 16S rRNA based bacterial identification provides accurate and reliable results as compared to other methods.

The crude enzyme extract was partially purified using ammonium sulfate precipitation. Lowry’s method (Lowry et al., 1951) was used to measure the protein concentration by using bovine serum albumin as standard and the protein concentration of purified sample for Bacillus subtilis (RAS-1) was calculated out to be 0.56 mg/ml for first peak and 0.573 mg/ml for the second peak and protein concentration of purified sample of Clostridium perfringens (RAS-4) was recorded to be 0.6 mg/ml for first peak, 0.615 mg/ml for second peak and, previously Niaz et al. (2010) reported the protein concentration of Bacillus subtilis that was 10.13 mg/ml, and Swamy and Ray (1996) has reported the protein concentration as 0.0073. α-Amylase produced from Bacillus subtilis (RAS-1) was purified and showed that there were two peaks with specific activity of 39.81 U/mg, purification fold 13.82 and recovery percentage 30.97% for first peak, and with specific activity 39.44 U/mg, purification fold 11.80 and recovery percentage 29.35% for second peak. Amylase produced from Bacillus sp. was purified, a fold of purification was 2.93 with a yield of 84.3% (Indira et al., 2012).

α-Amylase produced from Clostridium perfringens (RAS-4) was purified by Sephadex G-75, showed there are two peaks, with specific activity 33.5 U/mg, purification fold 12.78 and recovery percentage 31.90 %, for first peak and with specific activity 33.98 U/mg, purification fold 13.53 and recovery percentage 30.73 for second peak. α-Amylase produced from Clostridium perfringens was purified by Sephacryl S-100 HR, a fold of purification was 20.4 with a yield 11.3% characterized and purified alpha amylase from Clostridium perfringens, observed three distinctive amylolytic peaks (Shih and Labbe, 1995).

SDS PAGE was used to determine the molecular weight of purified enzyme. The estimated molecular weight of α-Amylase from Bacillus subtilis (RAS-1) was to be 52 kDa. Haq et al. (2010) determined the molecular weight of purified enzyme of Bacillus subtilis which was 55 kDa. Molecular weight of purified enzyme of Clostridium perfringens (RAS-4) was 73 kDa. The molecular weight of purified enzyme was 76 kDa.

**Table 5. Effect of different divalent metal ions on the purified amylase enzyme activity produced by Bacillus subtilis and Clostridium perfringens.**

<table>
<thead>
<tr>
<th>Metal ions</th>
<th>1mM B. subtilis relative activity (%)</th>
<th>10mM B. subtilis relative activity (%)</th>
<th>1mM C. perfringens relative activity (%)</th>
<th>10mM C. perfringens relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100.00</td>
<td>100.00</td>
<td>100.00</td>
<td>100.00</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>115.23</td>
<td>113.73</td>
<td>116.54</td>
<td>114.65</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>101.85</td>
<td>101.25</td>
<td>99.61</td>
<td>98.52</td>
</tr>
<tr>
<td>Co²⁺</td>
<td>79.41</td>
<td>43.11</td>
<td>81.36</td>
<td>44.12</td>
</tr>
<tr>
<td>Mn²⁺</td>
<td>100.56</td>
<td>97.44</td>
<td>101.23</td>
<td>97.88</td>
</tr>
<tr>
<td>Ni²⁺</td>
<td>65.11</td>
<td>37.32</td>
<td>66.78</td>
<td>36.27</td>
</tr>
<tr>
<td>Cu²⁺</td>
<td>84.50</td>
<td>26.15</td>
<td>81.65</td>
<td>31.10</td>
</tr>
<tr>
<td>Zn²⁺</td>
<td>88.14</td>
<td>55.23</td>
<td>51.33</td>
<td>16.52</td>
</tr>
<tr>
<td>Hg²⁺</td>
<td>2.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
</tbody>
</table>

**Figure 7. Effect of pH on enzyme activity.**

**Effect of inhibitor and metal ions on the stability of purified α-Amylase:** The divalent metal ions Ca²⁺, Mg²⁺, Co²⁺, Mn²⁺, Cu²⁺, and Zn²⁺ were found to increase the activity of amylase at 1mM, and Ca²⁺ increase the activity by twofold. Co²⁺, Mn²⁺, and Ni²⁺ inhibited the enzyme activity to a lesser extent at 1mM. Where Hg²⁺, Ni²⁺and Cu²⁺ have a strong inhibitory effect on amylase activity as shown in Table 5, at 10 mM concentration.
Enzymes are very sensitive to the change in temperature. It has been suggested that temperature negatively influence supra molecular assembly pattern of proteins three dimensional structure of enzymes via breakdown of hydrogen bonds and disulfide bridges. Consequently, these factors render loss of the functional attribute of enzymes. In case of present research it could be suggested that at high temperatures, both the enzymes from Bacillus and Clostridium species possibly encountered a change in protein structures owing to breakdown of essential bonding between the monomers. These findings are consistent with some previous reports where it was found that enzyme activity as a function of temperature (Feller et al., 1998; Mabrouk et al., 2008).

The results demonstrated that both Bacillus subtilis (RAS-1) and Clostridium perfringens (RAS-4) were mesophilic bacteria as their optimum growth conditions were found to be falling between moderate temperature and pH. The enzyme stability under varying pH was also studied and results showed that maximum enzyme activity for Bacillus subtilis (RAS-1) was 25.13 U/ml at pH 8. It was noteworthy that increase from pH 8 caused significant activity loss for amylase isolated from B. subtilis (RAS-1). In case of Clostridium perfringens (RAS-4) the optimum hydrogen ion concentration pertaining to maximum enzyme activity (24.29 U/ml) was pH 7. Whereas, activity of the enzyme was markedly affected while moving from this optimum pH level, either decreased or increased. Generally, α-amylase are found to stable under varying pH between 3 to 9. However, maximum activity of this enzyme has been associated with neutral pH. The reason for this discrepancy is linked with the fact that the optimum pH of amylase activity and stability in the acidic to neutral range because large number of charged groups are present around active site of amylase (Uitdehaag et al., 1999; Bozic et al., 2011). Present study suggested that the amylases isolated from both bacterial strains could not withstand change from neutral pH owing to changes in protein structure. These findings are in reasonable agreement with the studies of (Swamy and Ray, 1996).

The availability or deficiency of metallic cofactors plays an important role to regulate and control enzyme actions. The presence of certain metallic ions with organic waste either enhances or inhibits the amylase activity and plays a vital role in substrate digestion. The key role of Ca²⁺ in regulating the stability, efficiency and configuration of the amylase are significantly reported (Goyal et al., 2005). EDTA is a metal chelating agent and inhibits the activity while making complex with inorganic part of enzyme (Asgher and Iqbal, 2011). The metal ions play a significant role to stimulate the enzymatic activity through binding between substrate and enzyme, act like a bridging agent (Afifi et al., 2008). To increase the activity of many enzymes metal ions required. The enzymes which require the metal ion and bind very tightly to sustain the activity are called metaloenzymes. A metaloenzymes perform like electrophilic catalysts in biochemical reactions to control the increase in number of electrons. Alpha-amylases are hydrolytic enzymes, glycoprotein, family GH-13 and are characterized as metaloenzymes (Bordbar et al., 2005).

**Conclusion**: Fruit and vegetable waste contains high content of polysaccharide, so it could be a good substrate for amylase production. Amylase enzyme could be used for the digestion of fruit and vegetable waste to increase the rate of biogas production. The isolated strains showed good potential for amylase production. The bacterial species Bacillus subtilis (RAS-1) and Clostridium perfringes (RAS-4) showed amylases production at pH up to 6–8 and temperature 40°C. The amylolytic activity was found to be increased by optimizing the conditions. α-Amylase produced by Bacillus subtilis (RAS-1) and Clostridium perfringes (RAS-4) was purified by precipitation and Gel chromatography. The SDS-PAGE is a reliable method for protein estimation, by which a band of 52 KDa and 73 KDa were estimated by Bacillus subtilis (RAS-1) and Clostridium perfringes, respectively.

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