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Microbial Isoamylases: An Overview

Rina Rani Ray
Microbiology Research Laboratory, Department of Zoology, Molecular Biology and Genetics, Presidency College, 86/1, College Street, Kolkata: 700 073, India

ABSTRACT

An overview presentation is made on current global status of microbial isoamylase, a potent enzyme used in food industries, their production, properties and applications. Although few microbial sources were reported to produce extra cellular isoamylase, the *Pseudomonas* isoamylases were mostly studied. Microbial isoamylases in general showed affinity mostly towards glycogen and amylopectin but not towards pullulan. The end products were maltotriose and malto oligosaccharides often with maltose and glucose. The purified enzymes showed the molecular weight 60-120 kDa and activity was shown only by the monomers. The isoamylases showed varied temperature and pH optima. Adsorbability onto raw starch by the enzyme made its purification more convenient. Saccharification potential of isoamylases was employed in food industries for preparation of high glucose syrup from starch. The isoamylase gene from *Pseudomonas amyloderamosa* and *Flavobacterium odoratum* KU was cloned into *Saccharomyces cerevisiae* and in *E. coli*, respectively. Further studies are going on gene sequencing and characterization of the isoamylase produced mainly from bacterial sources.

Key words: Isoamylase, glycogen debranching enzyme, microbial amylases, starch processing enzyme

INTRODUCTION

Starch is the principal (food) reserve polysaccharide in the plant kingdom and is one of the most abundant energy reserve materials in nature (Ray and Nanda, 1996). It forms an integral part of the multibillion food ingredients market and is indispensable in various food applications where it is used as precursor of sugars, as emulsion stabilizer, water binder, thickener and for production of bread and other bakery goods.

Application of starch in food, bakery, brewery and confectionaries requires starch modification, which is accomplished either by acid hydrolysis or by enzymatic treatment. The use of enzymes is preferred as it offers a number of advantages including improved yields and favourable economics (Satyanarayana *et al*., 2004) and therefore in modern food processing industries, starch breaking enzymes or amylases are widely used. Among the amylases, α amylase, β amylase, isoamylase, glucoamylase are note worthy, but in comparison to that of other amylases, report of isoamylases is significantly less.

Isoamylase (E.C.3.2.1.68, glycogen-6-glucanohydrolse) is a debranching amylase that hydrolyses 1, 6-α-D-glycosidic linkages (Fig. 1) of glycogen, amylopectin and α and β limit dextrins, producing linear malto oligosaccharides (Fang *et al*., 1994).

Although, isoamylases are found to be produced by both plant and microbial sources, for commercial purposes microbial strains are used due to their rapid producibility, easy handling and

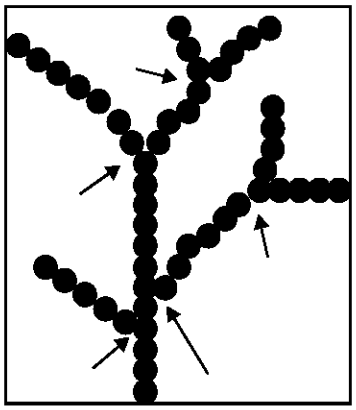

Fig. 1: Mechanism of starch hydrolysis by Isoamylase (Arrow indicates the cleavage point)

less expensiveness (Burhan *et al*., 2003). Amongst the microbial sources, maximum studies were carried on *Pseudomonas* sp. followed by other bacterial and fungal sources. But these isoamylases do differ from one another in their substrate affinity, major end product of hydrolysis and other characteristic features.

Although, a number of reviews are available on production and properties of various amylases (Regulapati *et al*., 2007; Ray, 2004), no comprehensive study is available on production, properties and other advanced research work done so far on microbial isoamylase,

The present review attempts to highlight production and properties of various microbial isoamylases and biotechnological approaches adopted to increase their production and ameliorate their industrial applications.

Structure of isoamylase: Katsuya and coworkers worked in detail in 1998 on the three dimensional structure of isoamylase from *Pseudomonas amyloderamosa* by X-ray structure analysis (Fig. 2) which revealed that the enzyme had 750 amino acid residues and a molecular mass of 80 kDa, The structure was elucidated by the multiple isomorphous replacement method and refined at 2.2 A resolution, resulting in a final R-factor of 0.161 for significant reflections with a root-mean-square deviation from ideality in bond lengths of 0.009 A. The analysis revealed that in the N-terminal region, isoamylase had a novel extra domain, the domain N. Katsuya *et al*. (1998) further detected that the N domain had an incomplete (beta/alpha) 8-barrel-type super secondary structure in the catalytic domain. A long excursed region was present between the third beta-strand and the third alpha-helix of the barrel but it could not be considered to be an independent domain, because this loop formed a globular cluster together with the loop between the fourth beta-strand and the fourth alpha-helix. Isoamylase was found to contain a bound calcium ion, but this was not in the same position as the conserved calcium ion reported in other alpha-amylase family enzymes.

Mode of action: Kjolberg and Manners (1963) described that isoamylase hydrolyses both the anomalous linkages in amylose and the inter chain linkages in amylopectin. It actually triggers at the carbohydrate-amino acid linkage of glycogen, that joins the terminal, reducing-end d-glucose unit of glycogen to the hydroxyl group of tyrosine in glycogenin, the primer protein for

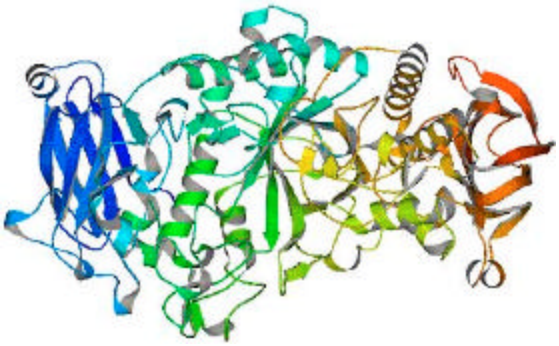

Fig. 2: Topology diagram of Isoamylase monomer (PDB code: 1bf2)

glycogen biogenesis (Lomako *et al*., 1992). The splitting of the glycogen-glycogenin bond by isoamylase indicates the α-anomeric configuration of the terminal d-glucose unit. For this specificity, 4-nitrophenyl α-maltotrioside and higher homologs also can act as substrates.

Application of isoamylases: Microbial isoamylase is found to have a number of applications both from research and industrial point of view. *Cytophaga* isoamylase was used for structural determination of glycogen and starch components (Lee and Whelan, 1972), isoamylase could also be used to examine the structure of the product and to produce branched cyclodextrins via reverse reaction (Kang *et al*., 2008). Isoamylase is used primarily in the production of food ingredients from starch, like glucose syrup, maltose, maltitol, trehalose, cyclodextrin and resistant starch. Glucose and maltose are widely used in food and pharmaceutical industries as sweetener, whereas maltitol is used as a sugar substitute in the production of non-calorigenic candies, chewing gum and other confectionary (Hirao *et al*., 1988) and trehalose is used in food as stabilizer, texturizer, humectant and sweetener (Olemposka-Beer, 2007). Cyclodextrins are used as encapsulating agents for food additives, flavours and vitamins, whereas the resistant starches are the non digestible starch used in medical purpose. Isoamylase is useful in the field of saccharification (Yamada *et al*., 1994). Even an isolated polypeptide with isoamylolytic activity could act on amylaceous substances to produce a high-maltose content product (Amemura and Futai, 1989) and therefore can be advantageously used for industrial purpose. This enzyme in combination with other amylolytic enzymes are used for the industrial production of amylose, maltose and glucose from starch (Sato and Park, 1980b; Norman, 1982; Stominska and Maczynski, 1985). Isoamylase can potentially be used in the elucidation of fine structures of polysaccharides and related α-glucans (Gunza-Smith *et al*., 1970; Akai *et al*., 1971; Fujita *et al*., 2003). It can also be used as effective additives in dishwashing and laundry detergents (Ara *et al*., 1993; Ito *et al*., 1998).

Production of microbial isoamylases

Sources of microbial isoamylases: Isoamylase may be produced intracellularly or extracellularly (Horwath and Rotheim, 1977) depending on the exact conditions of propagation. Intacellular isoamylase was first described in autolysed brewer's yeast (Maruo and Kobayashi, 1951) and in baker's yeast (Gunza *et al*., 1961), later an intracellular glycogen debranching enzyme complex was reported (Lee *et al*., 1970) from yeast. In 1982, an extracellular isoamylase was reported from yeast, *Lipomyces* sp. (Spancer Martins, 1982) and the next report came after a decade from *Hendersonula toruloidea* (Odibo *et al*., 1992).

First bacterial isoamylase was isolated, purified and characterized from *Pseudomonas amyloderamosa* (Harada *et al*., 1968). Since then production and further characterization of isoamylase was continued from a number of wild and mutant strains of *Pseudomonas* (Yokobayashi *et al*., 1970; Harada *et al*., 1972; Fujita *et al*., 1990; Katsuya *et al*., 1998) and other bacterial and fungal strains (Table 1).

Carbon source in fermentation media: In most of the cases, starch of variable concentration (0.25 to 3.0% (w/v) was used for the growth and isoamylase production (Spancer Martins, 1982; Takahashi *et al*., 1996) by various organisms. Dextrin was used as sole carbon source for isoamylase production in the fermentation medium of bacteria like *Escherichia intermedia* (Ueda and Nanri, 1967) and *Pseudomonas amyloderamosa* (Olemposka-Beer, 2007). Maltose was also used for the growth of these bacteria (Ueda and Nanri, 1967; Sugimoto *et al*., 1974; Fang *et al*., 1994). Production of isoamylase could be induced effectively by the maltose only if the glucose concentration is maintained below the inhibitory level in *Pseudomonas amyloderamosa* (Lai and Liu, 996).

Table 1: Various sources of microbial isoamylases

Microorganisms	References
Bacillus amyloliquefaciens	Urlaub and Wober (1975)
Bacillus sp.	Ara *et al*. (1993)
Bacillus circulans	Castro *et al*. (1992)
Bacillus stearothermophilus	Prayitno *et al*. (1996)
Flavobacterium sp.	Abe *et al*. (1999), Krohn *et al*. (1997), Sato and Park (1980a, b), Takahashi *et al*. (1996) and Horwath and Rotheim (1977)
Cytophaga sp.	Gunja-Smith *et al*. (1970), Lee *et al*. (1972), Gunja Smith (1974), Mitchell *et al*. (1969) and Evans *et al*. (1979)
Sulfolobus sp.	Fang *et al*. (2005), Park *et al*. (2007), Woo *et al*. (2008) and Kang *et al*. 2008
Pectobacterium sp.	Lim *et al*. (2001) and Cho *et al*. (2007)
Extremophiles	Ito *et al*. (1998)
Aerobacter aerogenous	Yusaku *et al*. (1970)
Xanthomonas maltophila	Yamada *et al*. (1994)
Arthrobacter ATCC 21920	Horwath, and Rotheim (1977)
Micrococcus ATCC 21919	Horwath and Rotheim (1977)
Rhodotherma marinus	Gomes *et al*. (2003)
Escherichia intermedia	Ueda and Nanri (1967)
Escherichia coli	Jeanningros *et al*. (1975)
Pseudomonas sp. and	Harada *et al*. (1968), Yokobayashi *et al*. (1970, 1973), Harada *et al*. (1972), Sugimoto *et al*. (1974), Kitagawa *et al*. (1975),
Pseudomonas amyloderamosa	Kato *et al*. (1977), Kainuma *et al*. (1978), Amemura *et al*. (1980), Cheng and Chang, (1986), Amemura *et al*. (1988), Tognoni *et al*. (1989), Houng *et al*. (1989), Chen *et al*. (1990) and Fujita *et al*. (1990)
Yeast	Maruo and Kobayashi (1951), Gunja *et al*. (1961), Sakano *et al*. (1969), Lee *et al*. (1970) and Kawai and Ishibashi (2009)
Lipomyces sp.	Spancer Martins (1982)
Hendersonula toruloidea	Odibo *et al*. (1992)
Saccharomyces sp.	Ma *et al*. (2000)
Rhizopus oryzae	Ghosh and Ray (2010)

Nitrogen source in fermentation media: Although, nitrogen source in a fermentation medium generally boost up the growth of the microorganism cultivated, it played some role in effecting the production of the enzyme. Hence, various workers optimized the culture media with suitable nitrogen source to get the highest yield of isoamylase from their working strains.

Peptone (Ueda and Nanri, 1967), $NaNO_3$ (Odibo *et al.*, 1992), yeast extract, ammonium sulfate (Ara *et al.*, 1993), peptone and yeast extract (Olemposka-Beer, 2007), Proteimax (Fang *et al.*, 1994), soybean protein (Takahashi *et al.*, 1996; Houng *et al.*, 1989), sodium glutamate and diammonium hydrogen phosphate (Harada *et al.*, 1968), yeast extract (Gomes *et al.*, 2003) were used as major nitrogen source in the culture media of different micro organisms. Yusaku *et al.* (1970) reported that their isolated strain of *Aerobacter aerogenous* showed two different isoamylase formation mechanisms representing either way according to the type of the nitrogen source used as the growth and enzyme production kinetics were changed remarkably with the change of nitrogen source from CH_3COONH_4 to $(NH_4)_2SO_4$.

Optimum temperature for enzyme production: Except extremophiles (Fang *et al.*, 2005) and *Bacillus stearothermophiles* (Prayitno *et al.*, 1996) growing at higher temperatures, the cultivation temperature for isoamylase producer ranged between 28-30°C (Ueda and Nanri, 1967; Ara *et al.*, 1993; Fang *et al.*, 1994; Yamada *et al.*, 1994; Takahashi *et al.*, 1996; Olemposka-Beer, 2007; Ghosh and Ray, 2010). *Lipomyces kononenkoae* showed a preference towards a lower temperature of 25°C (Spancer Martins, 1982).

Optimum pH for enzyme production: Isoamylase producers showed a broad range of pH preference for synthesizing the enzyme. *Pseudomonas amyloderamosa* showed optimum growth at pH 6.5-7.5 (Olemposka-Beer, 2007), whereas the mutant WU 2130 (Fang *et al.*, 1994) was cultivated at pH 5. *Flavobacterium* sp. showed highest isoamylase production at pH 6.8 (Horwath *et al.*, 1977) and 5.3 (Takahashi *et al.*, 1996). The mould *Hendersonula toruloidea* and mutant strain of bacteria *Pseudomonas amyloderamosa* MS1 were cultivated at pH 5.5 (Odibo *et al.*, 1992; Sugimoto *et al.*, 1974). *Xanthomonas maltophila* showed a broad range of pH preference 6-8 (Yamada *et al.*, 1994), whereas *Arthrobacter* sp and *Micrococcus* sp. were cultivated at pH 6.8 (Horwath *et al.*, 1977). Higher pH of 8 and 9.2 were preferred for isoamylase synthesis by *Rhizopus oryzae* (Ghosh and Ray, 2010) and *Bacillus* sp. (Ara *et al.*, 1993).

Cultivation time for enzyme production: Production of isoamylase took a longer time than other amylases like bacterial and fungal β amylases (Ray *et al.*, 1994; Ray and Chakraverty, 1998), α amylases (Aygan *et al.*, 2008; Ray, 2001). A minimum duration of 48 h were taken by *P.amyloderamosa* (Chen *et al.*, 1997; Fang *et al.*, 1994), *E. intermedia* (Ueda and Nanri, 1967). A period of 70-72 h were taken by *Bacillus* sp. (Ara *et al.*, 1993), *Rhizopus oryzae* (Ghosh and Ray, 2010), *Flavobacterium* sp. *Micrococcus* sp. and *Arthrobacter* sp. (Yamada *et al.*, 1994). Comparatively longer time of 96 and 120 h was taken by the mould *Hendersonula* (Odibo *et al.*, 1992) and yeast *Lipomyces* sp. (Spancer Martins, 1982). respectively.

Properties of microbial isoamylases: Isoamylase is considered as a direct debranching enzyme and is differentiated from other major starch debranching enzyme pullulanse by its ability to cleave all the α-1, 6 linkages of glycogen both inner and outer branching points of soluble amylopectin (Chen *et al.*, 1997) but it is unable to remove 2 and 3 glucose units of the side chain of β and α limit

dextrins of oligosaccharides. It has a higher activity than pullulanase and is not inhibited by maltose (Chen *et al*., 1997). Only the intra cellular isoamylase of *E. coli* (Jeanningros *et al*., 1975) was different from the other isoamylases with its inability to hydrolyze glycogen.

Substrate specificity: Kobayashi (1957a) studied the substrate specificity of the purified isoamylase. Being a debranching enzyme, isoamylase was found to cleave glycogen and thereafter glycogen was used for isoamylase assay. In yeasts, isoamylase was found to hydrolyze α-1.6 glucosidic linkage of both starch and glycogen (Kobayashi, 1955, 1957a, b). Glycogen also acted as substrate in the assay system of thermostable isoamylase from *Sufolobus* sp. (Park *et al*., 2007), *Bacillus* sp. (Ara *et al*., 1993), *Pectobacterium* (Lim *et al*., 2001), *Rhizopus oryzae* (Ghosh and Ray, 2010), *Pseudomomas amyloderamosa* (Amemura *et al*., 1980; Kato *et al*., 1977; Kitagawa *et al*., 1975; Yokobayashi *et al*., 1970). Isoamylase from *Xanthomonas maltophilia* (Yamada *et al*., 1994) most actively acted on glycogen, followed by amylopectin but hardly on pullulan, with an exception of bacterial isoamylase, described by Ueda and Nanri (1967) that could hydrolyze pullulan. Evans *et al*. (1979) reported that the isoamylase from a strain of *Cytophaga* had a very low but significant activity on pullulan and on alpha-dextrins having maltosyl side-chains. Amylopectin was also used as substrate for assaying the isoamylase of *Bacillus* sp. (Ara *et al*., 1993), *Pectobacterium* (Lim *et al*., 2001), *Sufolobus* sp. (Park *et al*., 2007), *Pseudomomas amyloderamosa* (Kato *et al*., 1977; Kitagawa *et al*., 1975; Yokobayashi *et al*., 1970; Katsuya *et al*., 1998) and in yeasts like *Lipomyces kononenkoae* (Spancer Martins, 1982) and in *Hendersonula toruloidea* (Odibo *et al*., 1992). Starch was used as substrate for determining the isoamylolytic activities of *Pseudomomas amyloderamosa* (Olemposka-Beer, 2007), *E. intermedia* (Ueda and Nanri, 1967) and *Lipomyces* sp. (Spancer Martins, 1982) According to Kainuma *et al*. (1978) as the *Pseudomonas* isoamylase could hydrolyze maltotriosyl branches more rapidly than those of the maltosyl branches, it required a minimum of three D-glucose residues in the B- or C-chain. Hence, the favored substrates for Ps. isoamylase were higher-molecular-weight polysaccharides like glycogen, amylopectin and starch.

Temperature optima: Four distinct ranges of temperature optima were found among the microbial amylases of which isoamylases extracted from yeasts generally showed low temperature optima and had poor heat stability. The yeast isoamylase showed optimum activity at 25°C (Gunja *et al*., 1961) 30°C (Kobayashi, 1957a) 30°C (Spancer Martins, 1982; Lee *et al*., 1970). A slightly higher range of temperature optima of 37-47°C was found in *Pectobacterium* sp. (Lim *et al*., 2001), *E. intermedia* (Ueda and Nanri, 1967), *P. amyloderamosa* (Olempsoka-Beer, 2007), *Hendersonula* sp. (Odibo *et al*.,1992). Most of the isoamylases reported, showed highest activity at a range of 50°-60°C, like 50°C in *Xanthomonas maltophilia* (Yamada *et al*., 1994), 52°C in *Pseudomonas* (Yokobayashi *et al*., 1970), 55°C in *Bacillus* (Ara *et al*., 1993), *Rhizopus oryzae* (Ghosh and Ray, 2010), 56°C in *B. stearothermophilus* (Prayitno *et al*., 1996). The thermophilic isoamylases showed high range of temperature optima of 75-80°C in *Sulfolobus* sp. (Fang *et al*., 2005; Park *et al*., 2007) and 75°C in *Rhodothermus marinus* (Gomes *et al*., 2003).

pH optima: In most of the reports, an optimum pH range was mentioned instead of a particular pH. Harada *et al*. (1968) reported optimum pH range of 5-6 in *Pseudomonas* sp. Yamada *et al*. (1994) reported it to be 3-5 for *Xanthomonas maltophila*, Olemposka-Beer (2007) reported 3-4 for *P. amyloderaosa*, Krohn *et al*. (1997) reported 5-8 for *Flavobacterium* sp. *Bacillus* isoamylases

showed a pH optima of 9 (Ara *et al.*, 1993), or 5 (Castro *et al.*, 1992). Other bacterial isoamylases showed best activities at various pHs like pH 7 for *Pectobacterium* sp. (Lim *et al.*, 2001), *Flavobacterium* sp. (Krohn *et al.*, 1997), 6.0 for *Flavobacterium* sp. (Hizukuri *et al.*, 1996). On the other hand, thermophilic bacteria like *Sulfolobus* sp. (Fang *et al.*, 2005; Park *et al.*, 2007; Woo *et al.*, 2008) were found to have activities towards acidic pH. Fungal isoamylases like yeast amylase (Kobayashi, 1955; Lee *et al.*, 1970) showed optimum pH at 6.2-6.4, whereas in *Lipomyces* (Spancer Martins, 1982), *Hendersonula* (Odibo *et al.*, 1992) and *Rhizopus oryzae* (Ghosh and Ray, 2010) the optima were 5.6, 7 and 5, respectively. The optimum pHs of baker's yeast isoamylase isozymes (three fractions) were 6.8, 5.6 and 5.6, respectively (Kawai and Ishibashi, 2009).

Themostability: According to Yamada *et al.* (1994), isoamylase of yeast and *Cytophaga* origin has poor heat stability, whereas the same enzyme of *Pseudomonas* origin does not have the problem of heat stability. *Pseudomonas* isoamylase reported by Yokobayashi *et al.* (1970) was stable at 45°C, but 95% activity was lost if exposed at 60°C. Isoamylase from *Xanthomonas maltophilia* was found to be stable at 45°C for 10 minutes and from *Bacillus* sp. (Ara *et al.*, 1993) at 30-40°C, but not above 65°C. Complete loss of activity after 10 min at 65°C was found in *Pseudomonas* isoamylase (Yokobayashi *et al.*, 1970). But the His tagged enzyme of *Sufolobus* (Fang *et al.*, 2005) was found to be stable at a high temperature of 80°C even after 2 h of exposure but both recombinant wild-type and His-tagged enzyme were more stable at room temperature than at 4°C. Even the isoamylase from *Hendersonula* (Odibo *et al.*, 1992) was found to be stable at 70°C for 30 min. It was found that *Pseudomonas* isoamylases were quite stable in maltose-containing buffer (Lin *et al.*, 1994), whereas isoamylase from yeast like *Lypomyces* sp. was very unstable, even at low temperatures (Spancer Martins, 1982). The thermostability at a pH range of 4-6 was increased after genetic engineering by Bisgard-Frantzen and Svendsen (2008).

pH stability: Isoamylase from *Pseudomonas amyloderamosa* (Yokobayashi *et al.*, 1973) showed a broad pH stability range of 2.5-7.5, but the same reported by Kato *et al.* (1977) was active between 3.5-5.5. Isoamylase from *Flavobacterium* sp. (Krohn *et al.*, 1997) was assayed at 22°C that displayed activity and stability optima of pH 5.0-7.5, whereas, the *Bacillus* (Ara *et al.*, 1993) isoamylase was active between pH range 5-11.5 and more than 50% of the original activity remained detectable between pH 6.6-10.5. *Xanthomonas maltophilia* (Yamada *et al.*, 1994) isoamylase was stable at 3.5-6.0. The isoamylase activity of *Pseudomonas amyloderamosa* WU 5315 was stable over the pH range from 5.5 to 6.25 while only about 30% of the activity remained at pH 6.5 (Wu *et al.*, 1994).

Incubation time: A reaction time of 10 to 15 min was required for assaying the activities of isoamylase from *Hendersonula toruloidea*, (Odibo *et al.*, 1992), *Xanthomonas maltophila* (Yamada *et al.*, 1994), *Rhizopus oryzae* (Ghosh and Ray, 2010) and *Bacillus* sp. (Ara *et al.*, 1993), but a longer duration of 30 and 60 min was needed for determining the activity of isoamylase from *Pseudomonas amyloderamosa* (Olemposka-Beer, 2007) and *E.intermedia* (Ueda and Nanri, 1967), respectively.

Inhibition of enzyme: The enzyme could be inhibited by a number of inhibitors that included metal ions, thiol inhibitors, saccharides etc. Treatment of isoamylase of *Pectobacterium crysanthemi* with metal ions Zn^{2+}, Mg^{2+}, Mn^{2+} at a concentration of 5 mM resulted in 20, 80, 70% residual

activities respectively (Lim *et al*., 2001). Hg^{2+} caused a drastic reduction in the isoamylase activities of *Bacillus* sp. (Ara *et al*., 1993) and *Pseudomonas amyloderamosa* (Kitagawa *et al*., 1975). Other metal ions acting as potent inhibitors of isoamylase activities were Ag^{2+} and Cu^{2+} presumably due to their interference with the active site of the enzyme. Activities were completely lost after treatment with detergents like SDS in *Pectobactrium* sp. (Lim *et al*., 2001), *Pseudomonas* sp. (Kitagawa *et al*., 1975) Thiol inhibitors like pCMB although inhibited the isoamylase activity in *Pseudomonas* (Yokobayashi *et al*., 1970) and *Xanthomonas maltophilia* (Yamada *et al*., 1994) but did not affect the samein *Hendersonula* (Odibo *et al*., 1992). EDTA, NaF, N-Bromosuccinimide, 2,4-dinitrofluorobenzene, 2-hydroxy-5-nitrobenzyl bromide, guanidine hydrochloride, β-mercaptoethanol, were found to act as potent inhibitors of *Pseudomonas* isoamylase (Yokobayashi *et al*., 1970). Some poly and oligosaccharides like cyclomaltoheptaose, glucose, xylose, maltose, isomaltose, maltotriose also reduced the enzyme activity of isoamylase from *Pseudomomas* sp. (Kitagawa *et al*., 1975).

Assay method: A method for determining the isoamylase activity was devised by Kobayashi (1955). A properly diluted enzyme solution was allowed to act at 20°C on a 1% solution of glutinous-rice starch buffered to pH 6.2. After 24 h, 0.2 mL of then reaction mixture was mixed with 2 mL of 0.01 N I_2 solutions and diluted to 25 mL. Optical density (E) of the solution at 620 μm was read in a Pulfrich photometer, using a 1 cm cell. The increment of E at 620 μm was proportional to enzyme activity within certain ranges. The amount of enzyme was expressed in the isoamylase unit, i.e., an increase of E 0.200 being taken as 10 units. Isoamylase activity was also assayed with amylopectin as substrate, by determining the increase in iodine staining power (Maruo and Kobayashi, 1951). Ghosh and Ray (2010) measured isoamylase activity by incubating the assay mixture (1 mL) containing an equal volume of enzyme and 1% (w/v) Oyster glycogen in 0.1 M phosphate buffer (pH-5) at 55°C for 5 min. The reducing sugar released was measured by the dinitrosalicylic acid method (Bernfeld, 1955) taking glucose as standard. Blanks were prepared with inactivated enzymes. One unit of isoamylase was defined as that amount of enzyme that liberated 1 μmole of glucose/mL/min of reaction (Ara *et al*., 1993). A method for detection of isoamylase in polyacrylamide gel by using a two step replica gel revealing assay was devised by Gonzalez (1994).

End product analysis: Maltose, maltotriose and maltotetraose were the main hydrolysis product of *Hendersonula* isoamylase (Odibo *et al*., 1992), maltose along with maltooligosaccharides were detected as the major end product of isoamylase extracted from *Bacillus* sp. (Ara *et al*., 1993) and from *Lipomyces* (Spancer Martin, 1982). Degradation product of glycogen by isoamylase of *Bacillus stearothermophilus* was detected as a single spot of glucose on thin layer chromatogram (Prayitno *et al*., 1996). Only maltotriose, but no maltose or glucose could be detected as the end product of isoamylolysis in *Flavobacterium* sp. (Sato and Park, 1980a). In *Sulfolobus solfataricus* linear maltooligosaccharides (Park *et al*., 2007) and maltose and beta-cyclodextrinin (Kang *et al*., 2008) were found to be the end products of isoamylase action. Isoamylase from various strains of *Pseudomonas amyloderamosa* were reported to cleave the branching points completely in glycogen or amylopectin to produce maltose + maltooligosaccharides (Yokobayashi *et al*., 1970; Kitagawa *et al*., 1975; Kato *et al*., 1977; Amemura *et al*., 1980, 1988; Katsuya *et al*., 1998; Fujita *et al*., 1990). Yeast isoamylase acted on α, β-limit dextrin (DP 9.6) to liberate glucose as well as maltose and higher oligosaccharides (Sakano *et al*., 1969).

Enzyme activity: Isoamylase from *Bacillus* (Ara *et al.*, 1993) showed a specific activity of 470 U mg^{-1} after reacting with oyster glycogen, which was increased respectively to 522 and 667 U mg^{-1} when bovine muscle glycogen and corn amylopectin were used as respective substrates. From shake culture of a strain of *Aerobacter aerogenous* Yusaku *et al.* (1970) obtained isoamylase with activity of 500 U mL^{-1}. Kato *et al.* (1977) reported the isoamylase activity in *Pseudomonas* sp was 190 U mg^{-1}. Krohn *et al.* (1997) detected the variations of activity of isoamylase from *Flavobacterium* with the change in substrates, as in presence of oyster glycogen, recombinant isoamylase showed a specific activity of 182 U mg^{-1}, which was changed to 120, 154 and to 174 U mg^{-1} in presence of starch, amylopectin and rabbit muscle glycogen respectively. A specific activity of about 59 U mg^{-1} was reported by Yokobayashi *et al.* (1970) and Fujita *et al.* (1990) from *Pseudomonas amyloderamosa*. Horwath and Rotheim (1977) reported the isoamylase activities of the strains of *Flavobacterium* sp. ATCC 21918, *Micrococcus* so ATCC 21919 and *Arthrobacter* sp. ATCC 21920 showed activities of 28.2, 12.9 and 6.3 U mL^{-1}, respectively. Yamada *et al.* (1994) opined that isoamylases of *Pseudomonas* had a low productivity and researchers were trying to remove this disadvantage by adopting various biotechnological approaches and finding new strains with high isoamylase activities. Wu *et al.* (1993) was able to increase the yield of his working strain of *P. amyloderamosa* by classical mutagenesis upto a titer of 5100 U mL^{-1}. The extracellular isoamylase activity of transformed *Saccharomyces cerevisiae* could reach 86 U mL^{-1} after 4 days cultivation (Chen *et al.*, 1998).

Molecular weight of purified enzyme: Purified isoamylases from various microbes showed the molecular weight of 60-120 kDa and in most of the cases these enzymes were a monomer or a dimer. It may be mention worthy that the rice isoamylase is a homo tetramer to homo hexamer (Fujita *et al.*, 1999). Isoamylase from *Pseudomonas amyloderamosa* purified by raw starch adsorption-desorption (Fang *et al.*, 1994) showed four protein bands on SDS PAGE corresponding to monomer, dimer, trimer and tetramer of the enzyme, where enzyme activity was shown by the monomer (M.W 78,000) only. The molecular weight of *Pseudomonas amyloderamosa* was determined by Yokobayashi *et al.* (1970) by gel electrophoresis and low speed sedimentation was 95,000. Amemura *et al.* (1980) detected it as monomer, having a molecular weight of 94,000. Kitagawa *et al.* (1975) found it to be a composition of two subunits with mol wt 105,000 and 50,000, not linked covalently with each other. The same enzyme studied by Katsuya *et al.* (1998), was found to have the mol wt of 80, 000. Sato and Park (1980a) purified isoamylase from *Flavobacterium* sp. by fractionation with ammonium sulfate, chromatography with DEAE cellulose, DEAE sephadex and CM-cellulose and obtained a single band in SDS PAGE. With the help of SDS PAGE, Chang and Chang (1986) demonstrated that the isoamylase was a polymorph of protein aggregates of 82, 160, 250, 340, 420 and 537 kDa size species on basic PAGE. Krohn *et al.* (1997) detected it's molecular weight 83,000. The same molecular weight was found by Park *et al.* (2007) in *Sulfolobus* isoamylase, which was a dimmer or a tetramer. According to Woo *et al.* (2008) in *Sulfolobus* solfataricus, the enzyme existed in two oligomeric states in solution, as a dimer and tetramer. *Bacillus* isoamylase (Ara *et al.*, 1993) purified by gel filtration technique indicated that it was a monomer of mol wt 65,000. In *Xanthomonas maltophila* (Yamada *et al.*, 1994) the mol wt of the isoamylase was 105,000. The yeast isoamylase was purified by Sakano *et al.* (1969) by DEAE cellulose and gel filtration on sephadex G 100 and CM-cellulose chromatography. Lee *et al.* (1970) detected that the purified yeast isoamylase was made up of at least two subunits. Isoamylase from another yeast strain *Lipomyces* sp. had a molecular weight of around 65,000. Odibo *et al.* (1992)

found the purified isoamylase of *Hendrsonula toruloidea* had a molecular weight of 83,000. *Cytophaga* sp. Lee *et al.* (1972) was found to secrete the isoamylase with molecular weight of 120,000. The molecular weight of the enzyme from Pectobacterium *carotovorum* subsp. *carotovorum* (Cho *et al.*, 2007) was also estimated to be 74 kDa by activity staining of a SDS-PA gel.

Genetic analysis: Since, cloning of isoamylase gene and its expression in vector is a prior requirement to detect the sequence of the enzyme, the *P.amyloderamosa* gene *pmi* encoding isoamylase was cloned and sequenced (Amemura *et al.*, 1988). The Isoamylase Gene (ISO) of *Pseudomonas amyloderamosa* JD210, an isoamylase-hyperproducing mutant, was cloned in an isoamylase-deficient and transformable mutant strain K31 (Chen *et al.*, 1990). Its nucleotide sequence contained an open reading frame of 2328 nucleotides (776 amino acids) encoding a secreted isoamylase precursor. Isoamylase gene (iso) of *Pseudomonas amyloderamosa* was amplified by polymerase chain reaction and cloned into *Saccharomyces cerevisiae* vectors under the control of alcohol dehydrogenase gene and glyceraldehyde-3-phosphate dehydrogenase gene promoters (Chen *et al.*, 1998). On the other hand, two plasmids, designated pRTI and pTI, were constructed to allow the integration of a bacterial isoamylase gene (iso) into *Saccharomyces cerevisiae* G23-8 chromosome by Ma *et al.* (2000). Transcription of iam gene from *P. amyloderamosa* was studied by Fujita *et al.* (1989). Cloning and nucleotide sequence of the isoamylase gene from a strain of *Pseudomonas* sp. was done by Tognoni *et al.* (1989). Isoamylase gene of *Flavobacterium odoratum* KU was expressed in *E. coli* (Abe *et al.*, 1999). Isoamylase gene from *Flavobacterium* sp. was extensively studied by Barry *et al.* (1996). In further study, the sequence analysis of iam from *Flavobacterium* sp. suggested that transcriptional control of this gene was mediated through the product of another gene, malt regulatory gene (Krohn *et al.*, 1997). In *P. amyloderamosa*, Fujita *et al.* (1990) found that after introducing the plasmid pIAM275, pUC9 carrying iam gene in *Escherichia coli*, recombinant plasmid did not direct the synthesis of isoamylase in it. An intracellular isoamylase gene from *Pectobacterium chrysanthemi* P35 was cloned and characterized by Lim *et al.* (2001). The gene encoding for isoamylase of the *Pectobacterium carotovorum* subsp. *carotovorum* (Pcc) LY34 was cloned and expressed into *Escherichia coli* DH5α by Cho *et al.* (2007). The isoamylase gene (glgX) had an open reading frame of 1,977 bp encoding 658 amino acid residues. According to them isoamylase from Pcc LY34 had 70% amino acid identity with isoamylase from *Pectobacterium chrysanthemi* and the sequences around those residues were highly conserved in isoamylase of different origins and GlgX of the glg operon in glycongen biosynthesis. A treX in the trehalose biosynthesis gene cluster of *Sulfolobus solfataricus* ATCC 35092 was reported to produce TreX, which hydrolyzed the alpha-1, 6-branch portion of amylopectin and glycogen (Park *et al.*, 2007). In the subsequent experiment, Park *et al.* (2008) found the TreX existed as a tetramer in the presence of DMSO at pH 5.5-6.5 which showed a 4-fold higher catalytic efficiency than the dimmer and they presumed that TreX might remain associated with glycogen metabolism by selective cleavage of the outer side chain. The isoamylase gene was cloned to an expression vector with a T7lac promoter. Both wild-type and His-tagged isoamylases were expressed in *Escherichia coli* (Fang *et al.*, 2005). Although generally similar to the monomeric structure of isoamylase, TreX exhibited two different active-site configurations depending on its oligomeric state. The N terminus of one subunit was located at the active site of the other molecule, resulting in a reshaping of the active site in the tetramer. This was accompanied by a large shift in the flexible loop (amino acids 399-416), creating connected holes inside the tetramer. Mutations in the N-terminal region resulted in a sharp increase in alpha-1,4-transferase activity and a reduced level

of alpha-1,6-glucosidase activity. On the basis of geometrical analysis of the active site and mutational study, Woo *et al.* (2008) suggested that the structural lid (acids 99-97) at the active site generated by the tetramerization was closely associated with the bifunctionality and in particular with the alpha-1,4-transferase activity. Few information regarding isoamylase gene isolated from different microbes are given herewith:

form *Sulfolobus solfatricus* P2 (Uniport AAK42273.1 CAA69504.1, CAC23738.1, NP_343483.1 andPBD/3D P95868) from *Escherichia coli* K-12 MG1655 (Uniport:AAC76456.1, CAP11380.1, NP_417889.1 and PBD/3D P15067.3) and *Pseudomonas amyloderamosa* SB-15 (Uniport AAA25854.1, CAA31754.1 and PBD/3DP10342.3).

Biotechnological approaches

Mutagenesis: In order to increase the enzyme production hyperproducing strains of *Pseudomonas amyloderamosa* were bred by stepwise classical mutagenesis using UV and MNNG as mutagens whereby a 22 fold increase in enzyme production could be achieved (Wu *et al.*, 1993). To identify the essential residues of isoamylase in *Flavobacterium odoratum* KU, site directed mutagenesis was done by Abe *et al.* (1999).

Raw starch adsorption: One of the characteristic features of isoamylase is its adsorbability on raw starch, which was effectively utilized for various purposes including purification. Kato *et al.* (1977) showed that isoamylase can be effectively adsorbed onto cross linked amylase gel and eluted by maltose containing buffer. Fang *et al.* (1994) described the recovery of isoamylase by adsorption on raw starch and desorption thereof. Later the method of purification by affinity separation followed by raw starch adsorption and desorption was patented by Fang *et al.* (1988). Lai *et al.* (1998) found that *Pseudomonas* isoamylase immobilized physically by raw starch adsorption resulted in an increase in pH and temperature stability and a retention of about 75% of activity even after 75 days. Chou *et al.* (1999) showed that *P. amyloderamosa* isoamylase could be preserved in adsorbed form at room temperature for eight months with high stability. According to Lin *et al.* (1994), the raw starch-enzyme suspension of *Pseudomonas amyloderamosa* when packed into a funnel-type glass filter instead of conventional flask to elute the raw starch adsorbed isoamylase, the recovery was increased from 53.6% to about 81% and the concentration of isoamylase was also increased 42-fold. Further, the addition of 1% potassium sorbate into it increased the self life of the enzyme.

Immobilization: For judicious exploitation of enzymes and further characterization, it may be immobilized onto various matrices. Isoamylase recovered from the fermentation broth of *Pseudomonas amyloderamosa* was immobilized by Chen *et al.* (19970 onto water-insoluble carriers like chitin, CM-cellulose and a temperature-sensitive reversibly soluble copolymer (N-isopropylacrylamide-co-N-acryloxysuccinimide). The debranching actions of immobilized and free isoamylase, was checked by Sunarti *et al.* (2001). Waxy maize amylopectin was treated with *Pseudomonas* isoamylase immobilized on magnetic supports in order to prepare various kinds of partial hydrolysates to check the arrangement and size of the amylose chains (Hisamatsu *et al.*, 1995).

Saccharification: As isoamylase ameliorates sugar production, to find out new microbial isoamylases with high saccharifying potential and ability to convert native, non expensive starch

residues to sugar become the need of the hour. Extra cellular isoamylase from *Rhizopus oryzae* PR7 MTCC 9642 was found to saccharify soluble potato starch and various native raw starches collected from domestic effluents, of which arrow root, tamarind kernel, tapioca and oat were noteworthy (Ghosh and Ray, 2010). Bioconversion of starch hydrolysate to a high DX glucose syrup at pH 3-5 by the enzyme mixture of a glucoamylase and an acidophilic *Pseudomonas* isoamylase (Hayashibara, Japan) was successfully done by Norman (1982). According to Castro *et al.* (1992) the properties of extracellular isoamylase of *Bacillus circulans* would allow its use in normal saccharification processes in the starch industries. Thermophilic enzyme of *Sulfolobus* sp. showed a potential to be used in industry to degrade the debranching points of starch at a high temperature (Fang *et al.*, 2005) and could be employed for the production of high-maltose syrups and highly purified maltose by a combination of debranching enzymes (Spancer Martins, 1982). According to Krohn *et al.* (1997), isoamylase could be used for industrial production of various syrups from starch. Amemura *et al.* (1980) and Kato *et al.* (1977) opined this enzyme was applicable for industrial production of amylose, maltose and D-glucose from starch, alone or in combination with beta-amylase and for glucoamylase production.

Analysis of toxicity: Checking of toxicity is a must prior to be used in food industry and the isoamylase enzyme preparation was tested to confirm compliance with the General Specifications and considerations for Enzyme Preparations used in Food Processing by Hayashibara, Japan (Olemposka-Beer, 2007). The isoamylase preparation from *P. amyloderamosa* has been used in food industry in countries such as Japan for more than two decades. No acute toxicity, no toxicity after short term (13 weeks) toxicity study, no genotoxicity and pathogenecity were reported. The recommended use levels range from 50-500I AU g^{-1} starch for the production of food ingredients.

Other sources of isoamylases: Besides microbial sources other isoamylase producing sources are plant like Arabidopsis (Zeeman *et al.*, 1998), rice endosperm (Fujita *et al.*, 2003), *Chlamydomonas reinhardtii* (Dauvillee *et al.*, 2001), *Phaseolus vulgaris* (Takashima *et al.*, 2007), rye (Xu *et al.*, 2009), potato tuber ((Bustos *et al.*, 2004); maize (Rahman *et al.*, 1998) *Homo sapiens* (Clave *et al.*, 1995) and *Rhizopthera* (Cinco-Moroyoqui *et al.*, 2008.) In plant, the enzyme is required for normal synthesis of amylopectin (Hussain *et al.*, 2003), whereas it is a normal secretion from human pancreas.

Concluding remark: Enzymes have become an integral part of human need in day to day life, particularly in the modern food, pharmaceutical, detergent and textile industries. In food processing industry, amylase predominantly are applied during processing of raw and gelatinized starch. In order to accelerate the splitting of starch, various industrially prepared microbial amylase are being added. This requires extensive researches on microbial amylases. Despite so many commercial values, research on microbial isoamylase is found to remain restricted within few papers and patents and in comparison to that of α-, β- and glucoamylases and also plant isoamylases, research on microbial isoamylase remain somewhat neglected. Hence a comparative account of isoamylases reported from various microbial sources, difference in their characteristic features and recent biotechnological advancement for the enhancement of productivity and industrial applicability of microbial isoamylases becomes extremely necessary. Although isoamylase related sequences of only few micro organisms are available and x-ray crystallographic structure of the enzyme is revealed (Katsuya *et al.*, 1998), still a number of behavioral contradictions exist among these

isoamylases, like affinity towards pullulan (Ueda and Nanri, 1967), pH and temperature optima, chemostability etc, which needs further clarification. Future work should focus on deduction of the amino acid sequences of these isoamylases that may be helpful in identifying the protein motifs (Ara *et al.*, 1993) which will further determine the characteristic features of these enzymes. Moreover, from theoretical point of view various types of BLAST searching may be employed to identify the relationships of homology, or through searching the dictionary of protein motifs of the active sites, the characteristic feature of a newly isolated isoamylase may be predicted. But above all, isoamylase an enzyme of immense practical importance must be made industrially applicable for production of various sugars and allied products.

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REFERENCES

Abe, J., C. Ushijima and S. Hizukuri, 1999. Expression of the isoamylase gene of *Flavobacterium odoratum* KU in *Escherichia coli* and identification of essential residues of the enzyme by site directed mutagenesis. Applied Environ. Microbiol., 65: 4263-4270.

Akai, H., K. Yokobayashi, A. Misaki and Y. Harada, 1971. Complete hydrolysis of branching linkages in glycogen by pseudomonas isoamylase: Distribution of linear chains. Biochim. Biophys. Acta, 237: 422-429.

Amemura, A., Y. Konishi and T. Harada, 1980. Molecular weight of the undegraded polypeptide chain of *Pseudomonas amyloderamosa* isoamylaase. Biochim. Biophys. Acta, 611: 390-393.

Amemura, A., R. Chakraborty, M. Fujita, T. Noumi and F. Masamitsu, 1988. Cloning and nucleotide sequence of the isoamylase gene from *Pseudomonas amyloderamosa* SB-15. J. Biol. Chem., 263: 9271-9275.

Amemura, A. and M. Futai, 1989. Polypeptide possessing isoamylase activity and its uses. Patent CA 1336509. http://brevets-patents.ic.gc.ca/opic-cipo/cpd/eng/ patent/1336509/summary.html.

Ara, K., K. Saeki and S, Ito, 1993. Purification and characterization of an alkaline isoamylase from an alkalophilic strain of *Bacillus*. J. General Microbiol., 139: 781-786.

Aygan, B., H. Arikan, S. Korkmaz, S. Dincer and O. Colak, 2008. Highly thermostable and alkaline α-amylase from a halotolerant *alkaliphilic Bacillus* sp. AB 68. Brazilian J. Microbiol., 39: 547-553.

Barry, G.F., G.M. Kishore and B.M. Krohn, 1996. Isoamylase gene from *Flavobacterium* sp. compositions containing it and methods using it. Patent CA 2195786 WO1996/003513. http://www.wipo.int/pctdb/en/wo.jsp?WO=1996003513.

Bernfeld, P., 1955. Amylases α and β. Method Enzymol., 1: 149-158.

Bisgard-Frantzen, H. and A. Svendsen, 2008. Starch debranching enzyme. US Patent 7374922. http://www.wikipatents.com/US-Patent-7374922/ starch-debranching-enzymes.

Burhan, A., U. Nisa, C. Gokhan, C. Omer, A. Ashabil and G. Osman, 2003. Enzymatic properties of a novel thermostable, thermophilic, alkaline and chelator resistant amylase from an alkaliphilic *Bacillus* sp. Isolate ANT-6. Process Biochem., 38: 1397-1403.

Bustos, R., B. Fahy, C.M. Hylton, R. Seale and N.M. Nebane *et al.*, 2004. Starch granule initiation is controlled by a heteromultimeric isoamylase in potato tubers. Proc. Natl. Acad. Sci. USA., 101: 2215-2220.

Castro, G.R., G.F. Garcia and F. Sineriz, 1992. Extracellular isoamylase produced by *Bacillus circulans* MIR-137. J. Applied Microbiol., 73: 520-523.

Chen, J.H., Z.Y. Chen, T.Y. Chow, J.C. Chen, S.T. Tan and W.H. Hsu, 1990. Nucleotide sequence and expression of the isoamylase gene from an isoamylase-hyperproducing mutant, *Pseudomonas amyloderamosa* JD210. BBA. Gene Struct. Expression, 1087: 309-315.

Chen, J.P., J.J. Lee and H.S. Liu, 1997. Comparison of isoamylase immobilization to insoluble and temperature sensitive reversibly soluble carriers. Biotechnol. Techn., 11: 109-112.

Chen, P.H., L.L. Lin and W.H. Hsu, 1998. Expression of Pseudomonas amyloderamosa isoamylase gene in Saccharomyces cerevisiae. Biotechnol. Lett., 20: 735-739.

Cheng, S.M. and T.C. Chang, 1986. Multiple forms of Pseudomonas isoamylase. J. Chinese Agric. Chem. Soc, 24: 265-271.

Cho, K.M., E.J. Kim, R.K. Math, Shah Md.A. Islam and S.J. Hong *et al.*, 2007. Cloning of isoamylase gene of *Pectobacterium carotovorum* subspecies carotovorum LY 34 and identification of essential residues of enzyme. J. Life Sci., 17: 1182-1190.

Chou, W.P., P.M. Wang and W.S. Chu, 1999. Preservation of isoamylase adsorbed onto raw corn starch. Biotechnol. Techn., 13: 259-261.

Cinco-Moroyoqui, F.J., F.I. Diaz-Malvaez, A. Alanis-Villa, J.M. Barron-Hoyos, J.L. Cardenas-Lopez, M.O. Cortez-Rocha and F.J. Wong-Corral, 2008. Isolation and partial characterization of three isoamylases of Rhyzopertha dominica F. (Coleoptera: Bostrichidae). Comp. Biochem. Physiol. B Biochem. Mol. Biol., 150: 153-160.

Clave, P., S. Guillaumes, I, Blanco, N. Nabau and J. Merce *et al.*, 1995. Amylase, lipase, pancreatic isoamylase and phospholipase a in diagnosis of acute pancreatitis. Clin. Chem., 41: 1129-1134.

Dauvillee, D., C. Colleoni, G. Mouille, M.K. Morell and C. Hulst *et al.*, 2001. Biochemical characterization of wild-type and mutant isoamylases of *Chlamydomonas reinhardtii* supports a function of the multimeric enzyme organization in amylopectin maturation. Plant Physiol., 125: 1723-1731.

Evans, R.M., D.J. Manners and J.R. Stark, 1979. Partial purification and properties of a bacterial isoamylase. Carbohydr. Res., 76: 203-213.

Fang, T.Y., L.L. Lin, W.H. Hsu and H. Wen, 1988. Method for recovery and purification of isoamylase by adsorption on raw starch. United States Patent 5811277. http://www.freepatentsonline.com/5811277.html.

Fang, T.Y., L.L. Lin and W.H. Hsu, 1994. Recovery of isoamylase from *Pseudomonas amyloderamosa* by adsorption-elution on raw starch. Enzyme Microbiol. Technol., 16: 247-252.

Fang, T.Y., W.C. Tseng, C.J. Yu and T.Y. Shih, 2005. Characterization of the thermophilic isoamylase from the thermophilic archeon *Sulfobolobus solfataricus* ATCC 35092. J. Mol. Catalysis B: Enzymatic, 33: 99-107.

Fujita, M., A. Amemura and M. Futai, 1989. Transcription of the isoamylase gene (iam) in *Pseudomonas amyloderamosa* SB-15. J. Bacteriol., 171: 4320-4325.

Fujita, M., S. Sakai, M. Futai and A. Amemura, 1990. Characterization of an Isoamylase-hyperproducing Mutant of Pseudomonas amyloderamosa. Agric. Biol. Chem., 54: 2315-2321.

Fujita, N., A. Kubo, Jr. P.B. Frasisco, M. Nakakita, K. Harada, N. Minaka and Y. Nakamura, 1999. Purification, characterization and cDNA structure of isoamylase from developing endosperm rice. Planta, 208: 283-293.

Fujita, N., K. Akiko, S. Dong-Soon, W. Kit-Sum and J. Jay-Lin *et al.*, 2003. Antisense inhibition of isoamylase alters the structure of amylopectin and the physicochemical properties of starch

in rice endosperm. Plant Cell Physiol., 44: 607-618.

Ghosh, B. and R.R. Ray, 2010. Saccharification of raw native starches by extracellular isoamylase of *Rhizopus oryzae*. Biotechnology, 9: 224-228.

Gomes, I., J. Gomes and W. Steiner, 2003. Highly thermostable amylase and pullulanase of the extreme thermophilic eubacterium *Rhodothermus marinus*: Production and partial characterization. Bioresour. Technol., 90: 207-214.

Gonzalez, R.D., 1994. A simple and sensitive method for detectin of pullulanase and isoamylase activities in polyacrylamide gels. Biotechnol. Techniques, 8: 659-662.

Gunja, Z.H., D.J. Manners and K. Maung, 1961. Studies on carbohydrate-metabolizing enzymes. 7. Yeast isoamylase. Biochem. J., 81: 392-398.

Gunja-Smith, Z., J.J. Marshall, C. Mercier, E.E. Smith and W.J. Whelan, 1970. A glycogen debranching enzyme from Cytophage. FEBS Lett., 12: 96-100.

Gunza Smith, Z., 1974. Cytophaga isoamylase. United State Patent 3790446. http://www.google.com.pk/patents?hl=en&lr=&vid=USPAT3790446&id=wFU7AAAAEBAJ&oi=fnd&dq=Cytophaga+isoamylase.&printsec=abstract#v=onepage&q&f=false.

Harada, T., K. Yokobayashi and A. Misaki, 1968. Formation of isoamylase by Pseudomonas. Applied Microbiol., 16: 1439-1444.

Harada, T., A, Misaki, H. Akai, K. Yokobayashi and K. Sugimoto, 1972. Characterization of Pseudomonas isoamylase by its actions on amylopectin and glycogen: comparison with Aerobacter pullulanase. Biochimica et Biophysica Acta (BBA)-Enzymol., 268: 497-505.

Hirao, M., H. Hijiya and T. Miyaka, 1988. Anhydrous crystals of maltitol and whole crystalline hydrogenated starch hydrolyzate mixture solid containing the crystala and process for the production and uses thereof. United States Patent 4717765. http://www.freepatentsonline.com/4717765.html.

Hisamatsu, M., M. Hirata, A. Sakamoto, K. Teranishi and T. Yamada, 1995. Partial hydrolysis of waxy maize amylopectin by isoamylase immobilized on magnetic support. Starch-Starke, 48: 6-9.

Hizukuri, S., T. Kozuma, H. Yoshida, J. Abe, K. Takahashi, M. Yamamoto and N. Nakamura, 1996. Properties of Flavobacterium odoratum KU Isoamylase. Starch-Starke, 48: 295-300.

Horwath, R.O. and P. Rotheim, 1977. Process for producing isoamylase. United States Patent 4001083. http://www.wikipatents.com/US-Patent-4001083/ process-for-producing-isoamylase.

Horwath, R.O., G.W. Cole and J.A. Lally, 1977. Process for producing isoamylase. United States Patent 4001084. http://www.wikipatents.com/US-Patent-4001084/ process-for-producing-isoamylase.

Houng, J.Y., K.C. Chen and W.H. Hsu, 1989. Production of isoamylase by Pseudomonas amyloderamosa mutant strain JD210. Proc. Natl. Sci. Counc. Repub. China B, 13: 9-14.

Hussain, H., A. Mant, R. Seale, S. Zeeman and E. Hinchliffe *et al*., 2003. Three isoforms of isoamylase contribute different catalytic properties for the debranching of potato glucans. Plant Cell, 15: 133-149.

Ito, S., T. Kobayashi, K. Ara, K. Ozaki, S. Kawai and Y. Hatada, 1998. Alkaline detergent enzymes from alkaliphiles: Enzymatic properties, genetics and structures. Extremophiles, 2: 185-190.

Jeanningros, R., N. Creuzet, C. Frixon and J. Cattaneo, 1975. A debranching enzyme in *Escherichia coli*. Biochem. Soc. Trans., 3: 336-337.

Kainuma, K., S. Kobayashi and T. Harada, 1978. Action of Pseudomonas isoamylase on various branched oligo and poly-saccharides. Carbohydr Res., 61: 345-357.

Kang, H.K., H. Cha, T.J. Yang, J.T. Park and S. Lee *et al.*, 2008. Enzymatic synthesis of dimaltosyl-beta-cyclodextrin via a transglycosylation reaction using TreX, a Sulfolobus solfataricus P2 debranching enzyme. Biochem. Biophys. Res. Commun., 366: 98-103.

Kato, K., Y. Konishi, A. Amemura and T. Harada, 1977. Affinity chromatography of pseudomonas isoamylase on cross-linked amylose gel. Agric. Biol. Chem., 10: 2077-2080.

Katsuya, Y., Y. Mezaki, M. Kubota and Y. Matsuura, 1998. Three-dimensional structure of *Pseudomonas* isoamylase at 2.2 A resolution. J. Mol. Biol., 281: 885-897.

Kawai, K. and F. Ishibashi, 2009. Studies on isoamylase isozymes (I): An improved method of polyacrylamide-gel disc electrophoresis on fractionation of isozymes of bakers yeast isoamylase. Memoirs Osaka Kyoiku Univ. Nat. Sci. Applied Sci., 17: 89-102.

Kitagawa, H., A. Amemura and T. Harada, 1975. Studies on the inhibition and molecular properties of crystalline Pseudomonas isoamylase. Agric. Biol. Chem., 39: 989-994.

Kjolberg, O. and D.J. Manners, 1963. Studies on carbohydrate-metabolizing enzymes. 9. The action of isoamylase on amylose. Biochem. J. 86: 258-262.

Kobayashi, T., 1955. Studies on isoamylase Part VIII. On the determination of isoamylase activity. Bull. Agric. Chem. Soc. Japan, 19: 163-166.

Kobayashi, T., 1957a. Studies on isoamylase IX. Purification of isoamylase. Nippon. Nogeikagaku. Kaishi, 31: 865-867.

Kobayashi, T., 1957b. Studies on substrate specificity of the isoamylase X. Nippon. Nogeikagaku. Kaishi, 31: 866-872.

Krohn, M., G.F. Barry and G.M. Kishore, 1997. An isoamylase with neutral pH optimum from a Flavobacterium species: Cloning, characterization and expression of the iam gene. Mol. Gen. Genet., 254: 469-478.

Lai, J.T. and H.S. Liu, 1996. Production enhancement of Pseudomonas amyloderamosa isoamylase. Bioproc. Biosys. Eng., 15: 139-144.

Lai, J.T., S.C. Wu and H.S. Liu, 1998. Investigation on the immobilization of pseudomonas isoamylase onto polysaccharide matrices. Bioproc. Biosys. Eng., 18: 155-161.

Lee ,E.Y.C. , J.H. Carter, L.D. Nielsen and E.H. Fischer, 1970. Purification and properties of yeast amylo-1,6-glucosidase-oligo-1,4.far. 1,4-glucantransferase. Biochemistry, 9: 2347-2355.

Lee, E.Y.C. and W.J. Whelan, 1972. Glycogen and Starch Debranching Enzymes. In: The Enzymes, Boyer, P.D. (Eds.). 3rd Edn., Vol. 5. Academic Press, New York, pp: 191-234.

Lim, W.J., S.R. Park, S.J. Cho, M.K. Kim and S.K. Ryu *et al.*, 2001. Cloning and characterization of an intracellular isoamylase gene from Pectobacterium chrysanthemi PY35. Biochem. Biophys. Res. Commun., 287: 348-354.

Lin, L.L., T.Y. Fang, W.S. Chu and W.H. Hsu, 1994. Improved elution of isoamylase adsorbed on raw starch and the preservation of purified enzyme. Lett. Applied Microbiol., 9: 383-385.

Lomako, J., W.M. Lomako and W.J. Whelan, 1992. The substrate specificity of isoamylase and the preparation of apo-glycogenin. Carbo. Res., 227: 331-338.

Ma, Y.J., L.L. Lin, H.R. Chien and W.H. Hsu, 2000. Efficient utilization of starch by a recombinant strainof Saccharomyces cerevisae producing glucoamylase and isoamylase. Biotechnol. Applied Biochem., 31: 55-59.

Maruo, B. and T. Kobayashi, 1951. Enzymic scission of the branch links in amylopectin. Nature, 167: 606-607.

Mitchell, T.G., M.S. Hendrie and J.M. Shewan, 1969. The taxonomy, differentiation and identification of *Cytophaga* sp. J. Applied Bact, 32: 40-50.

Norman, B.E., 1982. Saccharification of starch hydrolysates. United States Patent 4335208. http://www.freepatentsonline.com/4335208.html.

Odibo, F.J.C., N. Okafor, M.U. Tom and C.A. Oyeka, 1992. Purification and some properties of a starch debranching enzyme of Hendersonula toruloidea. World J. Microbiol. Biotechnol., 8: 102-105.

Olemposka-Beer, Z., 2007. Isoamylase from Pseudomonas amyloderamosa. Chem. Tech. Assessment pp:1- 6.

Park, H.S., J.T. Park, H.K. Kang, H. Cha, D.S. Kim, J.W. Kim and K.H. Park, 2007. TreX from Sulfolobus solfataricus ATCC 35092 displays isoamylase and 4-alpha-glucanotransferase activities. Biosci. Biotechnol. Biochem., 71: 1348-1352.

Park, J.T., H.S. Park, H.K. Kang, J.S. Hong and H. Cha *et al*., 2008. Oligomeric and functional properties of a debranching enzyme (TreX) from the archaeon Sulfolobus solfataricus P2. Biocatal. Biotransform, 26: 76-85.

Prayitno, N.R., R. Melliawati and E. Sukara, 1996. Screening of thermostable inulinase and isoamylase producing microbes from several geothermal hot springs in Indonesia. Ann. Rep. ICBIOTECH Japan, 19: 753-770.

Rahman. A., K. Wong, J.J. Jane, A.M. Myers and M.G. James, 1998. Characterization of SU1 isoamylase, a determinant of storage starch structure in maize. Plant Physiol., 117: 425-435.

Ray, R.R., S.C. Jana and G. Nanda, 1994. β-amylase from Bacillus megaterium B6. Folia Micribiologica, 39: 567-570.

Ray, R.R. and G. Nanda, 1996. Microbial β-amylases: Biosynthesis, characteristics and industrial applications. CRC Critical Rev. Microbiol., 22: 181-199.

Ray, R.R. and R. Chakraverty, 1998. Extra cellular β-amylase from *Syncephalastrum racemosum*. Mycol. Res., 102: 1563-1567.

Ray, R.R., 2001. Production of α -amylase and xylanase by an alkalophilic strain of Penicillium griseoroseum RR 99. Acta Microbiol. Polon., 50: 305-309.

Ray R.R., 2004. Beta-amylases from various fungal strains. A review. Acta Microbiol. Immunol. Hung., 51: 85-95.

Regulapati, R., N. Prem Malav and N. Sathyanarayana Gummadi, 2007. Production of Thermostable α-amylases by solid state fermentation-A review. Am. J. Food Technol., 2: 1-11.

Sakano, Y., T. Kobayashi and Y. Kosugi, 1969. Purification and substrate specificity of Yeast isoamylase. Agric. Biol. Chem., 33: 1535-1540.

Sato, H.H. and Y.K. Park, 1980a. Purification and characterization of extracellular isoamylase from *Flavobacterium* sp. Starch - Starke, 32: 132-136.

Sato, H.H and Y.K. Park, 1980b. Production of maltose from starch by simultaneous action of beta-amylase and *Flavobacterium Isoamylase*. Starch-Starke, 32: 352-355.

Satyanarayana, T., S.M. Noorwez, S. Kumar, J.L.U.M. Rao, M. Ezhilvannan and P. Kaur, 2004. Development of an ideal starch saccharification process using amylolytic enzymes from thermophiles. Biochem. Soc. Trans., 32: 276-278.

Spancer Martins, I., 1982. Extracellular isoamylase produced by the yeast lipomyces kononenkoae. Applied Environ. Microbiol., 44: 1253-1257.

Stominska, L. and M. Maczynski, 1985. Studies in the application of pullulanase in starch saccharification process. Starch - Starke, 37: 386-390.

Sugimoto, T., A. Amemura and T. Harada, 1974. Formations of extracellular isoamylase and intracellular alpha-glucosidase and amylase(s) by Pseudomonas SB15 and a mutant strain. Applied Microbiol., 28: 336-339.

Sunarti, T.C., T. Nunome, N. Yoshio, M. Hisamatsu, 2001. Study on outer chains released from amylopectin between immobilized and free debranching enzymes. J. Applied Glycosci., 48: 1-10.

Takahashi, K., J. Abe, T. Kojuma, M. Yoshida, N. Nakamura and S. Hizukuri, 1996. Production and application of an isosmylase from Flavobacterium odoratum. Enz. Microb. Technol., 19: 456-461.

Takashima, Y., T. Senoura, T. Yoshizaki, S. Hamada, H. Ito and H. Matsui, 2007. Differential chain-length specificities of two isoamylase-type starch-debranching enzymes from developing seeds of kidney bean. Biosci. Biotechnol. Biochem., 71: 2308-2312.

Tognoni, A., P. Carrera, G. Galli, G. Lucchese, B. Camerini and G. Grandi, 1989. Cloning and nucleotide sequenceof the isoamylase gene from a strain of *Pseudomonas* sp. J. Gen. Microbiol., 135: 37-45.

Ueda, S. and N. Nanri, 1967. Production of isoamylase by *Escherichia intermedia*. Applied Microbiol., 15: 492-496.

Urlaub, H. and G. Wober, 1975. Identification of isoamylase, a glycogen debranching enzyme, from Bacillus amyloliquifaciens. FEBS Lett., 57: 1-4.

Woo, E.J., S. Lee, H. Cha, J.T. Park, S.M. Yoon, H.N. Song and K.H. Park, 2008. Structural insight into the bifunctional mechanism of the glycogen-debranching enzyme trex from the archaeon sulfolobus solfataricus. J. Biol. Chem., 283: 28641-28648.

Wu, D.H., C.Y. Wen, W.S. Chen, L.L. Lin and W.H. Hsu, 1993. Selection of antibiotic resistant mutants with enhanced isoamylase activity in *Pseudomonas amyloderamosa*. Biotech. Lett., 15: 883-888.

Wu, D.H., C.Y. Wen, L.L. Lin, W.S. Chu and W.H. Hsu, 1994. Effect of pH on isoamylase production by *Pseudomonas amyloderamosa* WU 5315. Lett. Applied Microbiol., 19: 67-69.

Xu, J., M. Frick, A. Laroche, Z.F. Ni, B.Y. Li and X.Z. Lu, 2009. Isolation and characterization of isoamylase gene from rye waxy gene. Genome, 52: 658-664.

Yamada, Y., T. Sato and T. Ohya, 1994. Isoamylase and process for producing the same. United States Patent 5352602. http://www.freepatentsonline.com/ 5352602.html.

Yokobayashi, K., A. Misaki and T. Harada, 1970. Purification and properties of pseudomonas isoamylase. Biochim. Biophys. Acta, 212: 458-469.

Yokobayashi, K., H. Akai, T. Sugimoto, M. Hirao, K. Sugimoto and T. Harada, 1973. Comparison of the kinetic parameters of Pseudomonas amyloderamosa and Aerobacter pullulanase. Biochim. Biophys. Acta, 293: 197-202.

Yusaku, F., S.M. Shiosaka and S. Ueda, 1970. Isoamylase production by aerobacter aerogenes: (I) Effect of nitrogen source. Soc. Biosci. Bioeng. Japan, 48: 8-13.

Zeeman, S.C., T. Umemoto, W.L. Lue, P. Au-Yeung, C. Martin, A.M. Smith and J. Chen, 1998. A mutant of Arabidopsis lacking a chloroplastic isoamylase accumulates both starch and phytoglycogen. Plant Cell., 10: 1699-1712.