Discussion
5. DISCUSSION

In the present work, the isolation of fungi from Western Ghats soils, screening of the isolates for amylase production, characterization of selected fungal isolates by morphological, microscopic and molecular methods, optimization of the physiological and environmental conditions for the maximum production of amylases, partial purification, optimization of amylase activity and characterization of amylases of two fungal isolates have been attempted.

5.1. Isolation and characterization of soil fungi:

Isolation and screening of fungal strains utilizing complex substrates like carbohydrates and proteins exploring them as sources of industrially useful enzymes are major initiatives in biotechnological research. Several attempts, in this regard, to isolate fungi from different sources are made. Innovative techniques such as different media formulations, enrichment techniques or other approaches are required to be applied in order to permit the slow growing and rare species to grow and overcome the competition from the fast growing and abundant species (Srinivasan, 2004). Plating soil samples on agar media is a routine procedure followed to isolate fungi from soil. Other than this routine procedure, Warcup method, soil direct plating technique, baiting technique and stress techniques have been described and used by several workers to explore microbial biodiversity.

Formulation of selective media are generally based on the use of chemicals and/or antibiotics at concentrations which inhibit some fungi but are not inhibitory to others. This provides unlimited opportunities for manipulations of the culture medium employed for isolation purposes, while incorporation of one or more antibacterial antibiotics prevents competition from bacterial colonies. Suppression of
fast growing fungi to enable isolation of the relatively slower growing species has been achieved by incorporating growth retardants in the medium. Rose Bengal has been used by several investigators (Srinivasan, 2004).

In the present work, more than 200 fungal isolates belonging to 15 different genera were isolated from the soils of Western Ghats by using some usual and innovative isolation techniques such as spread plate technique and Warcup method, soil direct plating technique and stress techniques. More number of isolates was obtained by spread plate technique and soil direct plating technique compared to other techniques. Less number of isolates was obtained by stress techniques. Isolation was carried out by using different media such as PDA, SDA, PCA and CZA amended with Chloramphenicol. Rose Bengal was also used to check the growth of some common fast growing fungi.

Generally filamentous fungi are identified mainly using morphological characteristics (Bakri et al., 2009). The spore morphology and the manner in which the spores are produced have been the essential criteria for classification as well as generic and specific identification (Srinivasan, 2004). But the fungal identification based only on the macroscopic and microscopic features are not dependable as there can be different morpho/biotypes within a single species, they are also time consuming and require a great deal of skill and expertise (Bakri et al., 2009). On the contrary, molecular methods are more rapid and more specific. In ribosomal DNA sequencing, intragenic transcribed spacers (ITS) and intergenic regions (IGR) are more variable than the coding regions and these variable rDNA regions could offer valuable guidelines for characterizing and differentiating between two closely related species. There is considerable consensus regarding the
use of ITS sequencing as the initial step in mold identification. An international
*Aspergillus* working group recently recommended the use of the ITS region for
subgenus/section-level identification for the genus *Aspergillus*. According to Geiser
*et al.* (2007) there is no one method namely, morphological, physiological or
molecular that works flawlessly in recognizing species. Therefore wherever
possible, the use of morphological, physiological and molecular data in
circumscribing *Aspergillus* species is advocated.

Therefore in the present study, the selected fungal isolates were
characterized and identified based on the morphology of the colony, shape and
arrangements of spore bearing structures and shape and arrangements of spores and
also by molecular characterization.

5.2. Screening of fungal isolates for Amylase production:

All the isolates were screened for starch hydrolyzing ability on starch agar
medium containing 1% soluble starch as carbon source. Starch being a polymer
macromolecule, cannot pass through the cell wall and hence the fungi need to break
the starch in to small molecules which can later be incorporated in to the cell. For
breaking the starch, the organisms need to produce extracellular amylolytic enzymes
which will diffuse through the media and hydrolyze starch surrounding the colony.
Hence if the isolate is able to produce amylase, the starch content surrounding the
media will be hydrolysed. When iodine solution is flooded over the media, iodine
forms a reversible purple colored complex with only the starch present in the media.
As the starch content of the surrounding media has been hydrolysed, a clear zone of
discoloration surrounding the colony indicates the amylase production by a fungal
isolate. This starch plate assay is a primary screening technique that gives an idea of
the production of amylase by the isolates. Usually it is considered as greater the 
zone of hydrolysis, greater will be the amylolytic potential of the isolates. However 
zonation can not in any way be correlated quantitatively with the amount of α-
amylase produced because the hydrolytic activity of other amylolytic enzymes such 
as glucoamylase. Therefore isolation of improved producers of α-amylase using 
starch plate can only be partially selective (Kuek and Kidby, 1984). Hence the 
secondary screening is done in which the amount of extracellular protein produced 
and amylase activities of the culture filtrate are estimated. The fungal isolates that 
produce more extracellular proteins and show more amylase activity were selected. 
Hence, secondary screening of fungal isolates is the true indication of extent of 
amylase production by the isolates.

In the present study also, all the isolates were primarily screened by the 
starch plate assay. The isolates that produced maximum zone of hydrolysis were 
selected for secondary screening by surface fermentation using modified Czapek 
Dox broth. After incubation period, the cultures were filtered and total protein 
produced by the isolates and amylase activity was assayed by Lowry’s method and 
DNS method respectively. After primary screening, 14 isolates were selected for 
secondary screening by submerged fermentation based on the zone of hydrolysis 
formed. Based on the result obtained by secondary screening, two fungal isolates 
were selected for the further studies concerning the characterization of the isolates 
by molecular technique, production optimization of amylase, partial purification, 
catalytic properties and characterization of amylases.

5.3. Optimization of amylase production: Although amylases can be produced 
by several microorganisms, it remains a challenging task to obtain a strain capable
of producing commercially acceptable yields. Selection of a suitable strain is the most significant factor in the amylase production process. Commercial production of amylases is carried out in various steps, essentially because the environmental factors required for the optimum growth of the microorganism being employed for production may differ from those required for the production of enzymes. These parameters include nutrient supplementation, pH of the medium, osmotic relationship, and degree of aeration, temperature and the control of contamination during fermentation. Increased production of the enzyme could be obtained by manipulating the growth conditions and medium composition (Pandey et al., 2000). By optimizing the cultural conditions such as inoculum size, nutritional requirements, temperature, pH, agitation, aeration, and dissolved oxygen etc., the enzyme production can be enhanced by many fold (Gigras et al., 2002). Enzyme production commences at a low rate during the logarithmic-growth phase but reaches its maximum value during the stationary phase towards onset of sporulation. Time course study and agitation determines the efficacy of the batch process and subsequent product formation. The pattern of accumulated reducing sugar after specific incubation time is characteristic to the species (Matrai et al., 2000).

Optimization of production of amylase by the two isolates namely, *Aspergillus sydowii* MO 43 and *Aspergillus candidus* MO 199 was done in triplicates varying one parameter at a time on a completely randomized design.

The optimized conditions for amylase production from *Aspergillus sydowii* MO 43 are as follows; wheat bran as solid substrate material, maltose and the combination of maltose and starch as carbon source, ammonium chloride as nitrogen source, initial pH of the production medium of 6, an incubation temperature of 37°C,
an inoculum load of 20% w/v, an incubation period of 120h, additional starch concentration of 1.5%, a salinity concentration of 4%, presence of light.

The optimized conditions for amylase production from *Aspergillus candidus* MO 199 are as follows; wheat bran as solid substrate material, glucose and the combination of glucose and starch as carbon source, yeast extract as nitrogen source, initial pH of the production medium of 5, an incubation temperature of 25°C, an inoculum load of 20% w/v, an incubation period of 168h, additional starch concentration of 2%, a salinity concentration of 6%, incubation in the presence of light.

Among the ten different materials tested for them to be used as ideal solid substrate materials for *A. sydowii* MO 43, wheat bran showed maximum amylase activity and was found to be the most ideal solid substrate material followed by cassava powder, rice bran, sweet potato powder and powdered groundnut cake. Cotton seed cake showed least amylase activity. The amylase production pattern of *A. candidus* MO 199 also showed the same trend with wheat bran showing the maximum amylase production. Surprisingly for unknown reason, Amorphophallus powder showed least activity.

Similar results were reported by several workers. According to Gandhi *et al.* (1974), among Wheat bhusa, Wheat bran, Rice bran, Rice husk, Tamarind seedmeal, Jack fruit seedmeal, Accacia seedmeal, Peanut shell husk and Maize cobs, Wheat bran and Accacia seed meal appeared to be the best supporting media followed by Jack fruit seedmeal for the production of amylase by *Aspergillus oryzae* BI 8. Balkan and Ertan (2007) reported wheat bran to be an ideal solid substrate material for the production of α-amylase by *Penicillium chrysogenum* by SSF. The
production of extracellular amylases was investigated by Chimata et al. (2010). Various agricultural residual substrates like wheat bran, rice bran and green gram husk were studied for enzyme production by *Aspergillus* sp.MK07 by solid state fermentation (SSF) and it was found that amylase production was highest when wheat bran was used as substrate. Xu et al. (2008) experimented on the optimization of nutrient levels for the production of α-amylase by *Aspergillus oryzae* As 3951 in Spent Brewing Grains as solid state material and reported to have attained maximum production of amylase. Singh et al., (2010); Balkan et al., (2011); and Negi and Banerjee (2010) also got the same results. Negi and Banerjee, (2010) attributed production of highest amylase by wheat bran on account of its composition: 15.5% protein; 64.51% carbohydrates; 4.2% fat and other nutrients also in desirable amount, probably provides most of the essential nutrients. There are some contrasting reports of suitable substrate materials. Silva et al. (2005) noticed that of the different substrate materials such as soluble starch, cassava pulp, cassava processing wastes (solid and liquid wastes from the starch-extraction process), corn bran and corn processing waste, Cassava pulp was a good alternative substrate for amylase production of saccharogenic and dextrinogenic amylases by *Rhizomucor pusillus* A 13.36. Alva et al. (2007) showed that of the different substrate materials such as wheat bran, black gram bran, rice bran, coconut oil cake, groundnut oil cake, gingelly oil cake and corn cob, *Aspergillus sp.* on rice bran produced maximum amount of amylase. Bhattacharya et al. (2011) reported the production of maximum amylase using sugar cane bagasse as solid substrate material.

Wheat bran was amended with different carbon sources and their effect on amylase production was studied. Maltose and starch in case of *A. sydowii* MO 43
were found to be most appropriate carbon sources for scaling up the production of amylases. Glucose reduced the production of amylase probably by catabolite repression. The result obtained for *A. sydowii* MO43 is in accordance with some earlier reports. Kuo and Hartman (1966) reported in case of *Thermoactinomyces vulgaris* that maltose and soluble starch were the only two carbohydrates that induced substantial amylase production. The production of α-amylase by the ruminal anaerobic fungus *Neocallimastix frontalis* on cellulose, cellobiose, glucose, xylan, and xylose as growth substrates found to be less effective than starch, but maltose was almost as effective. Addition of glucose to cultures grown on low levels of starch, in which little glucose accumulated, suppressed α-amylase production, and in bisubstrate growth studies, active production of the enzyme only occurred during growth on starch after glucose had been preferentially utilized (Mountfort and Asher, 1988). Nguyen *et al.* (2000) reported more α-amylase production when maltodextrin and maltose were used as carbon sources than starch. Chavez *et al.* (2004) reported maximum α-amylase production by *Trichoderma* sp. by using maltose. Kathiresan and Manivannan (2006) noticed that maltose at a concentration of 1% was optimum for the production of α-amylase by *Penicillium fellutanum* isolated from coastal mangrove soil. Sindhu *et al.* (2009) reported that maltose enhanced amylase production by *Penicillium janthinellum*. Identical observations were earlier reported by Lachmund *et al.* (1993) and Morkeberg *et al.* (1995). Nahas and Waldemarin (2002) optimized the carbon sources and reported lactose followed by maltose was suitable for maximum production of amylase by *A. ochraceous*. Fructose, sucrose and glucose inhibited growth and amylase production. Identical observations were earlier recorded in *A. oryzae* (Yabuki *et al.*, 1997). α-amylase
production is also subjected to catabolite repression by glucose and other sugars, like most other inducible enzymes (Morkeberg et al., 1995; Bhella and Altosaar, 2004).

On the contrary, glucose and starch in case of *A. candidus* MO 199 were ideal carbon sources for amylase production. Alva et al. (2007) found that *Aspergillus* sp. produced maximum amylase when glucose was used as a supplementary carbon source. Singh et al., (2010) found glucose as the best supplementary carbon source.

For both the organisms, starch acted as a good carbon source for maximum production of amylase. Olama and Sabry (1989) reported starch and glycogen to be the best sources of carbon for α-amylase production by *A. flavus* and *Penicillium purpureascence*. Chimata et al. (2010) and Bhattacharya et al. (2011) also found starch to be an ideal carbon sources for maximum amylase production; α-Amylase is an inducible enzyme and is generally induced in the presence of starch or its hydrolytic product, maltose (Sindhu et al., 2009).

Among the nitrogen sources tested, *A. sydowii* MO43 was found to prefer inorganic nitrogen sources, ammonium chloride and ammonium nitrate, whereas *A. candidus* preferred organic sources, yeast extract followed by peptone in particular. Urea was found to inhibit amylase production by *A. candidus*. The studies carried out by Kuo and Hartman (1966) indicated that the inorganic nitrogen sources did not support substantial amylase production. Nahas and Waldemarin (2002) reported casamino acids, ammonium and sodium sulfate to be the optimal nitrogen source for maximum production of amylase by *A. ochraceous*. Kathiresan and Manivannan (2006) noticed that peptone at a concentration of 0.5% was optimum for the
production of α-amylase by *Penicillium fellutanum* isolated from coastal mangrove soil. Xu *et al.* (2008) reported the use of Corn steep liquor, which provides elemental nitrogen, vitamins and other nutrients to the medium as an important nitrogen source for α-amylase production by *A. oryzae*. Rose’s and Guerra (2009) worked on the optimization of synthesis of amylase by *Aspergillus niger* strain UO-01 under solid-state fermentation with sugarcane bagasse by using response surface methodology and reported yeast extract at 11.59 mg/g concentration of dry support. Chimata *et al.* (2010); Negi and Banerjee (2010) reported peptone (organic nitrogen source) and NaNO₃ (inorganic nitrogen source) (NH₄)₂SO₄ and NH₄Cl inhibited amylase production; Balkan *et al.* (2011) found urea (1% w/w) to be the best nitrogen source; Yeast extract was reported as the best nitrogen source by Bhattacharya *et al.* (2011).

A pH of 6 was found to be ideal for maximum amylase production by *A. sydowii* and *A. candidus* produced more amylase at pH 5. Amylase production was affected badly at alkaline pHs. Kundu and Das (1970) worked on the effect of different media and pH on the production of amylase by *A. oryzae* and reported that the fungus was found to produce the α or β amylase depending upon the composition of the medium and growth conditions. The optimum pH of the medium was found to be dependent on the composition of the media. Some media, however, favored the stationary liquid culture fermentation at pH 7.5 and submerged culture fermentation at pH 7.0 in presence of 0.5% CaCO₃. Ohara’s medium containing NaNO₃ (0.3%) and urea (0.3%) as nitrogen sources was found to be suitable at pH 7.5 in submerged fermentation but Feniksova and Dvadsatova’s medium, containing NaNO₃ (0.9%) and malt (1%) as nitrogen sources, gave highest amylase yields.
Nahas and Waldemarin (2002) reported a pH of 5.0 to be optimum for maximum production of amylase by *A. ochraceous*.

Kathiresan and Manivannan (2006) noticed that a pH of 6.5 was optimum for the production of α-amylase by *Penicillium fellutanum* isolated from coastal mangrove soil. Prakasham *et al.* (2006) found that pH 4 was optimum for amylase production by *A. awamori*. Alva *et al.* (2007) reported the optimum pH for the production of amylase by *Aspergillus sp.* to be 5.8. Rose’s and Guerra (2009) reported the optimum pH for the of synthesis of amylase by *A. niger* strain UO-01 under solid-state fermentation with sugarcane bagasse by using response surface methodology was pH 6.

Amylase production was observed to occur at 25°C, 32°C and 37°C and maximum production by *A. sydowii* was observed at 37°C. The further increase in incubation temperature (45°C and 52°C) drastically affected amylase production. *A. candidus* was found to show maximum amylase production at 25°C compared to all other incubation temperatures. At temperatures of 32°C and 37°C, amylase production was found to be decreased to a marginal extent and at incubation temperature of 52°C, the growth and the amylase production was completely inhibited probably because of the evaporation of water content from the production media. In the earlier studies concerning temperature optimization, Nahas and Waldemarin (2002) reported 30°C to be the optimum incubation temperature for maximum production of amylase by *A. ochraceous*. Kathiresan and Manivannan (2006) noticed that incubation temperature of 30°C was optimum for the production of α-amylase by *Penicillium fellutanum* isolated from coastal mangrove soil. Prakasham *et al.* (2006) found that *A. awamori* could produce maximum amylase at
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an incubation temperature of 31°C. Alva et al. (2007) noticed that Aspergillus sp. produced maximum amylase at 30°C. Similar observation was made by Xu et al. (2008) and they reported the optimum incubation temperature α-amylase production by A. oryzae to be 30°C. In another study, Rose’s and Guerra (2009) reported the optimum temperature for the synthesis of amylase by Aspergillus niger strain UO-01 under solid-state fermentation with sugarcane bagasse by using response surface methodology was 30.2°C.

In the study concerning the optimization of inoculum load the organisms, A. sydowii and A. candidus were able to produce maximum amylase at 20%. The further increase in inoculum load lowered amylase production where it became nearly stagnant. A study by Balkan and Ertan (2007) showed that the optimum inoculum load in case of SSF is dependent on the type of solid substrates used and the particle sizes of the substrate materials. It was concluded that optimal inoculum concentration for the production of α-amylase by Penicillium chrysogenum by SSF were 20%; 20%; 20% and 30% for corncob leaf (CL), rye straw (RS), wheat straw (WS) and wheat bran (WB) respectively. WB showed the highest enzyme production with 160 U/mL under optimum conditions.

The study regarding the optimization of incubation period, amylase production gradually started from first day and in case of A. sydowii, it reached maximum by 120 hours. With the further incubation up to 192hrs, the amylase production got lowered and seemed to be reached the stagnancy. The incubation beyond 192hrs reduced amylase production drastically. In case of A. candidus the production of amylase reached maximum by 168 hrs. With the further increase in incubation period, the amylase production got in to stagnancy. Our observation is
supported by most some of the studies concerning the optimization of incubation period for amylase production. Most of the studies report that an incubation period of 96 h (4 days) to 168 h (7 days) is required for more amylase production and beyond this incubation period amylase production is reduced. Kathiresan and Manivannan (2006) noticed the production of $\alpha$-amylase by *Penicillium fellutanum* was highest at an incubation period of 96h. According to Alva *et al.* (2007) the optimum incubation period required for the maximum production of amylase by SSF by *Aspergillus sp.* isolated from seeds was 6 days. Xu *et al.* (2008) reported the optimum incubation period for the production of $\alpha$-amylase by *Aspergillus oryzae* As 3951 was 96h.

Some other studies contradict our observation. Nahas and Waldemarin (2002) reported 2 days incubation period was optimum for maximum production of amylase by *A. ochraceous*. According to Singh *et al.* (2010) the optimum incubation period to be 80h. The reduction of amylase production beyond the optimum incubation period might have been due to the denaturation of the enzyme caused by the interaction with other components in medium. It could have been also be due to the fact that the microorganism was on its exponential phase during the 8 days of fermentation and resulted in the maximum production of enzyme. At the later stage, when nutrients were depleted, it reached its stationary phase and could have started producing secondary metabolites, resulting in a lower yield of enzyme (Balkan *et al.*, 2011).

The trials of optimization of starch concentration for optimal amylase production by *A. sydowii* showed maximum enzyme production was observed at an additional starch concentration of 1.5%. The starch concentration beyond 1.5%
anyhow did not increase the amylase production. In case of *A. candidus*, maximum amylase production was observed at 2% initial concentration. The starch concentrations below and above 2% showed decreased amylase activity. Similarly Prakasham *et al.* (2006) found that *A. awamori* could produce maximum amylase at an initial starch concentration of 3%.

*A. sydowii* showed maximum amylase activity at 4% NaCl concentration. At 8% and 10% NaCl concentrations, the growth as well as the amylase production found to be reduced drastically. *A. candidus* showed maximum amylase activity at 6% NaCl concentration. At 8% NaCl concentration, the amylase production reduced to a little extent and 10% NaCl concentration, the growth as well as the amylase production found to be reduced drastically.

Amino acids in conjunction with vitamins have also been reported to affect a-amylase production. However, no conclusion can be drawn about the role of amino acids and vitamins in enhancing the α-amylase production in different microorganisms as the reports are highly variable (Gupta *et al.*, 2003). α-Amylase production by *Bacillus amyloliquefaciens* ATCC 23350 increased by a factor of 300 in the presence of glycine (Zhang *et al.*, 1983). The effect of glycine was not only as a nitrogen source rather it affected α-amylase production by controlling pH and subsequently amylase production increased. Alanine, DL-nor valine and D-methionine were effective for the production of alkaline amylase by *Bacillus* sp. A-40-2. However, the role of amino compounds was considered to be neither as nitrogen nor as a carbon source, but as stimulators of amylase synthesis and excretion (Ikura and Horikoshi, 1987). It has been reported that only asparagaine gave good enzyme yields (Kundu *et al.*, 1973) while the importance of arginine for -
amylase production from B. subtilis has also been well documented (Lee and Parulekar, 1993).

In an interesting study, the effect of light on the production of amylase was conducted by incubating the inoculated media at three different incubation conditions such as incubation in the presence of light, in dark and usual incubation condition. A. sydowii produced amylase to the greater extent in light, followed by incubation in the complete absence of light than the usual incubation conditions of light and dark. In case of A. candidus, amylase production was found to be inhibited in the absence of light. Amylase production was highest when incubated in the presence of light and usual incubation condition. The effect of light on fungal sporulation and release is an established fact. In some fungi, the formation of spores takes place only when they are exposed to light, while in some the release of spores takes place only towards the light source. In Pilobolus, the sporangiospores are always bent towards the incident light and the sporangia shoot out from the sporangiophores in the direction of light. In members of Discomycetes producing deeply capsulate apothecia, the asci are positively phototrophic and discharge their spores towards the light source. Some fungi sporulate abundantly in darkness. Germination and sporulation of fungi may be influenced by exposure to light or alternate light and darkness, short exposure to ultraviolet radiation and variation in the intensity and wave length of light (Aneja, 1996). But the effect of light and darkness during the incubation on amylase production has never been studied before. Further experimentation in this regard is required.

**5.4. Purification and properties of amylases:** Enzyme application in pharmaceutical and clinical sectors requires high-purity amylases. Thus, it is
significant to develop economic processes for their purification to obtain chemically pure enzymes with maximum specific activity (Pandey et al., 2000).

Traditionally the purification of amylases from fermentation media has been done in several steps, which include centrifugation of the culture (a step of extraction may be required for solid media), selective concentration of the supernatant, usually by ultrafiltration, and selective precipitation of the enzyme by ammonium sulphate or organic solvents such as ethanol in the cold. Then the crude enzyme is subjected to chromatography (usually affinity or ionexchange chromatography) and gel filtration (Pandey et al., 2000).

The partial purification and characterization of amylases was attempted. First the culture filtrates were fractionated by ammonium sulphate, dialyzed to remove the salt and then the fractionated samples were subjected to ion exchange chromatography. Similar protocol was followed by Sindhu et al. (2011). After analyzing the active fractions from ion exchange chromatography, it was found that 2.33 and 2.13 fold purification was achieved and 232.8% and 213.5% enhancement in the amylase activities were observed in A. sydowii MO 43 and A. candidus MO 199 respectively.

The catalytic properties such as the effect of temperature, pH, thermostability, pH stability, effect of incubation time, enzyme concentration and substrate concentration of amylases of A. sydowii and A. candidus were studied and optimized.

Amylase of A. sydowii showed maximum activity at 40°C. The increase in incubation temperature to 50°C slightly decreased the amylase activity and at 60°C, the amylase activity decreased drastically because of the denaturation of the enzyme.
At 70°C, the enzyme activity reached to almost nil. Amylase of *A. candidus* showed maximum activity at 50°C, reaching least activity at 70°C. This is essentially because of the denaturation of the enzyme. Similar findings were reported by Kundu and Das (1970) for amylase of *Aspergillus oryzae* El 212. They observed that at higher temperatures, enzyme activity decreased sharply and a complete inactivation occurred at 65°C. The optimal activity of the enzyme in citrate phosphate buffer was at 50°C. Yabuki *et al.* (1977); Wilson and Ingledew (1982); Wanderley *et al.* (2004); Ramachandran *et al.* (2004) and Patel *et al.* (2005) also reported the optimum temperatures for the activity of amylases of *Aspergillus oryzae* M-13, *Schwanniomyces alluvis*, *Cryptococcus flavus* and *Aspergillus oryzae* respectively. Alva *et al.* (2007) reported the optimum temperature of amylase of *Aspergillus* JGI 12 to be 30°C. Najafi *et al.* (2005) reported the optimized temperature for amylase of *Bacillus subtilis* AX20 to be 55°C; Omemu *et al.* (2005) reported that the crude amylase preparation of *A. niger* AM07 had temperature optima at 60°C; Messaoud *et al.* (2004) found the optimum temperature for the activity of amylase of *Bacillus cereus* UN 116 to be at pH 6 and 65°C; Doss and Anand (2012) and Kumar and Duhan (2011) reported the optimum temperature as 60°C and 60-70°C respectively. Silva, *et al.* (2005) found that dextrinogenic and saccharogenic amylases of *Rhizomucor pusillus* A 13 exhibited optimum activities at 75 °C; Krishnan and Chandra (1983) reported the optimum temperature of the purified amylase enzyme of *Bacillus licheniformis CUMC 305* at 90°C. Generally from the literatures it is observed that the optimum temperature for fungal amylases lies between 30 °C to 65 °C and the optimum temperature for the activity of bacterial amylase lies between 50-80 °C. The optimum temperature for amylases from fungal and yeast sources has
generally been found to be between 30 and 70°C (Gupta et al., 2003; Sun et al., 2010). Optimum temperatures for amylase in the earlier studies on the *Penicillium* species were reported between 30°C and 60°C (Balkan and Ertan, 2005; Sindhu, 2005; Ertan et al., 2006). These results are consistent with our findings.

The temperature stability profiles were studied by incubating the partially purified enzyme at respective temperatures for 1hr. Amylase of *A. sydowii* was more stable at 40°C and the stability went on decreasing with the increase in incubation temperature. At 70°C, the enzyme was completely denatured and the activity was zero. *A. candidus* amylase was stable even at 50°C where it showed maximum activity. At 60°C and 70°C also the enzyme retained its activity. Kundu and Das (1970) found the amylase of *Aspergillus oryzae* El 212 was stable when kept at 30°C for 60 min.; Yabuki *et al.* (1977) reported 75% loss of activity after heat treatment (50°C, pH 5.4, for 30 min); the amylolytic enzymes of *Schwanniomyces alluvius* was quickly inactivated at temperatures above 40°C (Wilson and Ingledew, 1977). A striking temperature stability was reported by Krishnan and Chandra (1983) where 91% of the activity of *Bacillus licheniformis* CUMC305 amylase was remained at 100°C even after four hour incubation in the presence of substrate. In the absence of substrate the enzyme was found to be a bit less stable. The enzyme retained 91, 79, and 71% maximal activity after 3 h of treatment at 60°C, 3 h at 70°C, and 90 min at 80°C, respectively. Peixoto *et al.* (2003) reported that the amylase from *Rhizopus microsporus* var. *rhizopodiformis* was thermotolerant, maintaining activity even at 60°C. Similarly in another study, Wanderley *et al.* (2004) found that the amylase of *Cryptococcus flavus* was stable at 50°C to 60°C. The amylase of *A. oryzae* was stable above 50°C also in the presence of Ca²⁺ ions (Patel *et al.*, 2005). According to
Silva et al. (2005), the saccharogenic or dextrinogenic enzymes of *Rhizomucor pusillus* A 13 were highly thermostable, with no detectable loss of saccharogenic or dextrinogenic activity after 1 hour and 6 hours at 60°C, respectively. Alva et al. (2007) observed the retention of 60% activity even on incubation at 60°C 1 hour.

The enzymes are very much sensitive to pH. *A. sydowii* amylase showed maximum activity at alkaline pH of 8; the enzyme showed activity at acidic pH (pH 5 and 6), neutral pH (pH 7) and alkaline ranges (pH 9 and 10) also. This finding is in accordance with the report of Krishnan and Chandra (1983) who reported the optimum pH of amylase of *B. licheniformis* was pH 9.0. Alva et al. (2007) reported that the amylase of *Aspergillus sp. JGI 12* showed two peaks each at acidic and alkaline regions. *A. candidus* amylase showed maximum activity at pH 5 and pH 6 respectively. The amylase also showed activity at neutral and basic pH ranges at pH 7, 8 and 9. Messaoud et al. (2004) found the optimum pH for the activity of amylase of *Bacillus cereus* UN 116 to be at pH, 6; Wanderley et al. (2004) optimized *Cryptococcus flavus* amylase activity and reported the optimum pH 4-6. Wilson and Ingledew (1982) observed an optimum pH of 6.3. Patel et al. (2005) found optimal activity of amylase of *A. oryzae* at pH 5. Omemu et al. (2005) reported that the crude amylase preparation of *A. niger* AM07 had a pH optima at 4.0; Doss and Anand (2012) reported a optimum pH of 7.0.

The pH stability profile was studied by incubating the partially purified amylases in buffers of different pH for 1 hr and estimating the residual activity. Both the amylases were stable at wide pH ranges. Even after 1 hr, the normal activity was retained at pH ranges 5 to 10. *A. sydowii* amylase was found to be more stable at pH 6, 7, 8, and 9. *A. candidus* amylase was also found to be stable
in these pH ranges only. pH stability from 5-11 (Kundu and Das, 1970); pH 5-9, (Yabuki et al, 1977); pH 4.5 to 7.5 (Wilson and Ingledew, 1977) were reported. Krishnan and Chandra (1983) reported differential stability pattern of amylase of \textit{B. licheniformis}. The enzyme showed 100% stability in the pH range 7 to 9; 95% stability at pH 10; and 84, 74, 68, and 50% stability at pH values of 6, 5, 4, and 3, respectively, after 18 h of treatment. Peixoto et al. (2003) reported that the amylase from \textit{Rhizopus microsporus var. rhizopodiformis} enzymes acid alkali tolerant maintaining stability and wide range of pH respectively.

The effect of incubation period on amylase activity was studied by incubating the enzyme and substrates for varied periods. Both the amylases of \textit{A. sydowii} and \textit{A. candidus} showed maximum activities at 50 minutes of incubation. With the further incubation, the rate of amylase activity did not increase. Therefore the optimum incubation period for amylase activity is 50 minutes.

In the study of effect of concentration of amylase on amylase activities of both \textit{A. sydowii} and \textit{A. candidus} increased gradually from 0.1 ml till 0.4 ml enzyme concentration. The amylase activity reached maximum at 0.5 ml enzyme and reached to 5.0 and 5.2 respectively.

The effect of substrate (starch) concentration on amylase activity was studied by varying the substrate concentration keeping the enzyme concentration constant. The activity of amylases increased gradually with the increase in starch concentration. The activity of amylase of \textit{A. sydowii} reached maximum at a starch concentration of 4 mg and the activity of amylase of \textit{A. candidus} reached maximum at a starch concentration of 3 mg. Omemiu et al (2005) reported the optimum
substrate concentration of 3%; Doss and Anand (2012) reported the substrate concentration 1.5 to 2.0% under standard assay conditions.

5.5. Characterization of Amylases: The amylases of Aspergillus sydowii MO 43 and Aspergillus candidus MO 199 were characterized by SDS PAGE for the determination of approximate molecular weight. The approximate molecular weights of the amylases were found to be 40kDa and 50kDa respectively. Molecular weights of α-amylases vary from about 10 to 210kDa. The lowest value, 10kDa for Bacillus caldolyticus and the highest of 210 kDa for Chloroflexus aurantiacus has been reported (Gupta et al., 2003) Molecular weights of microbial α-amylases are usually 50-60 kDa as shown directly by analysis of cloned α-amylase. Sindhu et al. (2011) reported the approximate molecular weight of amylase of Penicillium janthinellum NCIM 4960 to be 42.7 kDa. Vardhini et al. (2013) reported the approximate molecular weight of amylase of A. niger to be 43 kDa. A raw starch digesting α-amylase from Cryptococcus sp. S-2 was found to be 66 kDa (Iefuji et al., 1996); Campos and Felix (1995) purified glucoamylase from the thermophilic fungus Humicola grisea to homogeneity and reported its molecular mass and isoelectric point were 74 kDa; Abdelmalek-Khedher et al. (2008) determined the molecular weight of a novel α amylase from Sclerotinia sclerotiorum as 43 kDa; Metin et al. (2010) determined the molecular weight of Penicillium citrenum as 65 kDa. Nouadri et al. (2011) reported the approximate molecular weight of amylase of Penicillium camemberti PL.21 as 60.5 kDa.

Amino acid sequencing:

After Ingel digestion, the peptides subjected to LC MS/MS and peptides were identified. The peptides obtained from Ingel digestion of amylase of A. sydowii
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MO 43 resulted in to 356 residue sequence starting "GDVIYQIIID. The calculated molecular weight of the sequence was 40.19kDa. Glycine was the N-terminal amino acid and Lysine was the C-terminal amino acid. The atomic composition of the deduced sequence was Carbon (C)- 1801, Hydrogen (H) 2705, Nitrogen (N) 483, Oxygen (O) 550 and Sulfur (S) 8 with total number of 5547 atoms. The deduced molecular formula was C$_{1801}$H$_{2705}$N$_{483}$O$_{550}$S$_{8}$.

The peptides obtained from Ingel digestion of amylase of A. candidus MO 199 resulted in to 455 residue sequence starting “EIAGMDNKSOQ”. The calculated molecular weight of the sequence was 49423.3kDa. Glutamic acid was the N-terminal amino acid and Cysteine was the C-terminal amino acid. The atomic composition of the deduced sequence was Carbon (C) 2303, Hydrogen (H) 3583, Nitrogen (N) 543, Oxygen (O) 615 and Sulfur (S) 22 with total number of 5547 atoms. The deduced molecular formula was C$_{2303}$H$_{3583}$N$_{543}$O$_{615}$S$_{22}$.

The 3 dimensional structure of both the amylases reveal the parallel ($\beta/\alpha$) 8-barrel. The $\alpha$-amylase family members are multidomain proteins containing the main catalytic domain in the form of a parallel ($\beta/\alpha$) 8-barrel (domain A) that is interrupted by a usually small domain in the place of the loop 3 connecting the strand $\beta$3 with the helix $\alpha$3 (domain B) and succeeded by the antiparallel $\beta$-sandwich domain (domain C). The $\alpha$-amylase-type of the barrel was confirmed in all members of the $\alpha$-amylase family whose three-dimensional structure has already been determined. The ($\beta/\alpha$) 8-barrel of $\alpha$-amylases was first revealed in the structure of Taka-amylase A (Matsuura et al., 1984), i.e. in the structure of the $\alpha$-amylase from Aspergillus oryzae. Since this type of fold was first identified in triose-phosphate isomerase (TIM), the ($\beta/\alpha$) 8-barrel is often simply called TIM-barrel (Farber and
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Petsko, 1990). It is a barrel of eight inner parallel β-strands surrounded outside by eight α-helices.

They possess four conserved regions in their primary sequence. Some of these conserved amino acids form the catalytic site and some are involved in the stability of the conserved TIM barrel topology (Karuki and Imanaka, 1999; van der Maarel et al., 2002).

The studies conducted by Janecek (2009) reported that among the GH13 α-amylases, those produced by plants and archaebacteria exhibit common sequence similarities that distinguish them from the α-amylases of the remaining taxonomic sources. Despite the close evolutionary relatedness between the plant and archaenal α-amylases, there are also specific differences that discriminate them from each other.

Comparison of known tertiary structures of various α-amylase family members with sequence alignments have shown that differences in specificity result from different variation of substrate binding at the β->α loops (Svensson, 1994; Janecek, 1997). Also the active-site cleft is not of the same shape in each case (Kamitori et al., 1999; Przylas et al., 2000), despite the fact it always contains the same catalytic triad accompanied, however, by several additional residues depending on a given enzyme specificity (Matsuura, 2002). Differences especially in the length, sequence and secondary structure have also been seen within the domain B protruding out of the catalytic TIM-barrel in the place of the loop 3 (Jespersen et al., 1991, 1993). It was pointed out that these differences may be directly related to enzyme specificity (Janecek et al., 1997). With regard to domain C succeeding the catalytic TIM-barrel, this domain could contribute to the overall catalytic domain stability by shielding the hydrophobic residues of the barrel (Katsuya et al., 1998).
The most known amyloytic enzymes are α-amylase (EC 3.2.1.1), β-amylase (EC 3.2.1.2) and glucoamylase (EC 3.2.1.3) that are, however, quite different from each other. They differ not only in their primary and tertiary structures, but also in their catalytic machineries and reaction mechanisms employed (Janecek, 1994a; Janecek, 2009). From the structural point of view both α-amylase and β-amylase rank among the TIM-barrel enzymes, i.e. they possess the (β/α) 8-barrel catalytic domain, while glucoamylase adopts a helical version of catalytic TIM-barrel, the so-called (α/α) 6-barrel (Janecek, 2009).

Abdelmalek-Khedher et al. (2008) found a novel α-amylase (α-1,4-α-D-glucan glucanohydrolase, E.C. 3.2.1.1), ScAmy43, in the culture medium of the phytopathogenic fungus Sclerotinia sclerotiorum grown on oats flour. It was purified to homogeneity, ScAmy43 appeared as a 43 kDa monomeric enzyme, as estimated by SDS-PAGE and Superdex 75 gel filtration. The MALDI peptide mass fingerprint of ScAmy43 tryptic digest as well as internal sequence analyses indicate that the enzyme has an original primary structure when compared with other fungal α-amylases. However, the sequence of the 12 N-terminal residues is homologous with those of Aspergillus awamori and Aspergillus kawachii amylases, suggesting that the new enzyme belongs to the same GH13 glycosyl hydrolase family.