9 Production of Enzymes by Solid-state Fermentation

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9.1 INTRODUCTION

The process of solid-state fermentation for food applications is one of the oldest knowledges available to humans. To many communities it is a part of their traditional knowledge to prepare the fermented food (Hesseltine, 1977). The expanded form of Japanese word for Koji relates to growth of mold. The traditional fermentations such as Koji, Tofu, miso, sauceges, pickles, ensilling are the extension of the traditional fermentation knowledge exploiting the GRAS strains of fungi and bacteria to carry out the fermentation. The processes provide extracellular fungal enzymes and have been the basis to initiate the microbial enzymes production by SSF in industrial environment. The koji process undoubtedly influenced the first production of microbial enzymes by SSF on industrial scale. The emergence of modern SSF based enzyme industry may be attributed to the entrepreneurship of Jokichi Takamine and later by Underkofler's efforts in producing mold bran enzymes for saccharification of grain (Bennett, 2001). The discovery of Penicillin in early thirtee's and streptomycin, chloramphenicol and tetracyclin's in early fifty's took the sheen of the emerging SSF process and emphasized on submerged fermentation. The SSF process development was still in its infancy and was considered not suitable for many of the potential large-scale applications.

However, recently with lot of effort being put into value addition of agro-industrial residues and publications emphasizing the possibilities in SSF (Pandey, 1992, 1994, 1999, 2000, 2001; Pandey and Soccol, 2000; Lonsane & Ghildyal, 1992; Babu & Satyanarayana, 1996; Haltrich et al., 1996; Mitra et al., 1996; Selvakumar et al., 1996; Tengerdy, 1998) commercial operations using SSF process were developed in countries such as Japan, India, USA, and France. The successful operation of the companies utilizing the SSF process ensured that the process gets its due attention and apart from enzymes the pharmaceutical products and other secondary metabolites were also manufactured. The major

applications of solid-state fermentation involve processes exploiting fungal systems and to a lesser extent bacterial systems, however, recently some actinomycetes were also reported especially for production of bioactive compounds using SSF (Kota and Sridhar, 1998; Sircar et al., 1998; Saudagar et al., 2006). The fungal systems are an obvious choice, as these systems require solid support matrices for their growth under natural circumstances. In natural habitats fungi grow on wood, bark, leaves of plant, decaying organic matter and other moist surfaces. Given this tendency for adhered growth under natural environments fungi have been exploited for maximum extent. The enzymes being produced by SSF include cellulases, hemicellulases, pectinase, amylases, α- and β-galactosidases, caffeinase, tannase, proteases etc. The major support matrices include bran's (wheat, rice, barly), oil cakes (sesame, soy, olive, coconut, mustard), bagasse (sugarcane, cassava, orange), coffee pulp and husk (Pandey and Soccol, 2000) and also substrates such as Jackfruit seed powder, tamarind seed powder. In the present chapter the authors are presenting a brief account of developments in SSF process during last five years as the earlier developments had already been discussed in various reviews and book chapters.

9.2 MICRO-ORGANISMS AND METHODS OF IMPROVING SSF PRODUCTIVITY

The potential candidates for enzyme production using SSF process are bacteria and fungi. The bacteria from diverse habitat have been isolated and studied for fermentative applications however mesophilic and themophilic fungi have been more successfully utilized. A number of commercial enzymes such as alpha-amylase and glucoamylases, pectinases, hemicellulases, phytases, xylanases and proteases are produced by fungi. The fungal systems used for koji preparations such as *A. sojae*, *A. oryzae* and other like *A. niger* are considered as GRAS strains (Generally Regarded as Safe). The fungal strains such as *A. terreus, A. tamarii, A. ustus, Rhizopus oryzae, Rhizopus oligosporus* and *Penicillium* sp. strains are also being frequently explored for production of enzymes. Apart from the commonly used fungal strains, bacterial strains such as *Bacillus* spp. are being increasingly exploited (Table 1).

The yield enhancement of the SSF process has been mostly studied by traditional approach of screening for new culture with improved production and their process optimisation. However, now recombinant DNA approach using genetically engineered systems is also being explored. The approach is of potential importance given the ability to modify the host genome by introducing novel genes responsible for improved traits. The recombinant DNA approach has been successfully applied for studying the phytase, amylase, protease and biochemicals such as diacetal, folate, riboflavin, mannitol and lactic acid. The recombinant DNA approach

has been successfully exploited for improvement of lactic acid bacteria especialy for applications in dairy industry. The applications included flavour enhancement, resistance to bacteriophages, addition of nutritional components and stability and structure of end products. However, the recombinant approach requires the cultural conditions of the modified organisms to be examined to provide conditions that would fully exploit the increased potential of the culture. Also, the process of strain improvement involves the continual genetic modification of the culture, followed by reappraisals of its cultural requirements (Stanbury et al., 1995) and needs special training to cater to these requirements. The initial efforts in genetic modifications were concentrated using traditional approaches such as exposing to mutagenic agents such as ethyl methyl sulphonate, NNG, NTG and UV radiations. However, to exploit enzyme production tight metabolic regulatory elements need to be modified using recombinant DNA approaches. This approach of modifying the entire production pathways is called as metabolic pathway engineering. This requires complete knowledge of the pathway and various control elements working in it.

9.3 SUBSTRATES USED FOR THE PRODUCTION OF ENZYMES IN SSF

The recent SSF process studies have explored a variety of substrates varying from agro-residues to wastes of industries such as potato chips, spent brewing grain, paper and wood processing industries. The wastes such as saw dust and wood chippings have a huge potential for cellulases and hemicellulases production. Substrates such as sugar cane bagasse, wheat bran, rice bran, maize bran, gram bran, wheat straw, rice straw, rice husk, soyhull, sago hampas, grapevine trimmings dust, saw dust, corncobs, coconut coir pith, banana waste, tea waste, cassava waste, palm oil mill waste, aspen pulp, sugar beet pulp, sweet sorghum pulp, apple pomace, peanut meal, rapeseed cake, coconut oil cake, mustard oil cake, cassava flour, wheat flour, corn flour, steamed rice, steam pre-treated willow, starch, etc. have been studied for SSF applications. Recently green gram husk was used as a good support for alkaline protease production (Prakasham et al., 2006). Also oil cakes and their combinations have been explored recently with much success for phytase, amylase and protease production. The other developments include the solid-state fermentation process using inert support material supplemented with chemically defined liquid media for production of enzymes, metabolites and biological control agents (Ooijkaas et al., 2000). The SSF process may have a major application in production of bio-control agents for large-scale field applications given the requirement of biocontrol agents at an economical price and ease of directly using the fermentation media. The several studies have already reported effect of substrate, its particle size and moisture level/water activity in the media.

Table 1. Microorganisms for enzyme production using SSF

S. No.	*Organism*	*Substrate*	*Enzyme*	*Reference*
***Aspergillus* spp.**	*A. oryzae*	Eucalyptus pulp Bagasse pulp	Xylanase	Szendefy et al., 2006
	Aspergillus foetidus	Corn cobs	Xylanase	Shah and Madamwar, 2005
	Aspergillus versicolor	Wheat bran	Xylanase	Jeya et al., 2005
	Aspergillus niger	Rice straw	Xylanase	Park et al., 2002
	A. niger KK2	Rice straw+ Wheat bran	Xylanase	Kang et al., 2004
	Aspergillus niger KK2	Rice straw and wheat bran	Cellulase	Kang et al., 2004
	A. oryzae NRRL 2217	Coconut oil cake+ wheat bran (1:3)	Neutral protease	Sumantha et al., 2005
	A. oryzae NRRL 1808	Wheat bran	Neutral protease	Sandhya et al., 2005
	Aspergillus niger	Deseeded sunflower head	Pectinase	Patil and Dayanand 2006a, b, c
	Aspergillus awamori	Grape pomace	Pectinase	Botella et al., 2005
	Aspergillus sp. HA-2	WB+sucrose	gluco-amylase	Anto et al., 2006
	A. oryzae	COC+starch+peptone WB+GOC	α-amylase	Ramchandran et al., 2004a, b
	Aspergillus sp.	Wheat bran	α-amylase	Ellaiah et al., 2002
	Aspergillus niger	Wheat bran+olive oil	lipase	Mahadik et al., 2002
	A. oryzae AK9	Koji meal media	Phytase	Chantasartrasamee, 2005
	Aspergillus niger	Wheat bran+soy meal	Phytase	Krishna & Nokes, 2001
	Aspergillus niger Aa-20	*Larrea tridentata* Cov. (gobernadora) powder	Tannase	Trevino-Cueto et al., 2007
	Aspergillus foetidus	myrobolan (*T. chebula*) fruit+gallo seed pod cover	Tannase	Purohit et al., 2006
	Aspergillus niger ATCC 16620	Palm kernel cake Tamarind seed powder	Tannase	Sabu et al., 2005
	Aspergillus sp. S1-13	Crab shells	Chitinase	Rattanakit et al., 2003
***Tricho-derma* spp.**	*Trichoderma harzianum*	Sorghum flour	Xylanase	Fadel et al., 2001

	T. longibrachiatum ATCC 36838	Wheat bran+crude chitin	Chitinase	Kovacs et al. , 2004
	Trichoderma harzianum	Wheat bran+ chitin+ yeast extract	Chitinase	Nampoothiri et al., 2004
***Penicillium* spp.**	*Penicillium canescens*	Wheat straw	Xylanase	Bakri et al., 2003
	Penicillium sp.	wheat bran+ soy protein	Alkaline protease	Agarwal et al., 2004
	Penicillium decumbens	Wheat straw + wheat bran	Cellulase	Mo et al., 2004
	Penicillium viridicatum RFC3	Wheat bran+orange bagasse (1:1)	Pectinase	Silva et al., 2005
	P. chrysogenum	Wheat bran+chitin	Chitinase	Patidar et al., 2005
	P. aculeatum NRRL 2129	Wheat bran	Chitinase	Binod et al., 2005
	P. simplicissimum	Babassu oil cake	Lipase	Gutarra et al., 2005
	P. simplicissimum	Soy cake	Lipase	Di Luccio et al., 2004
***Rhizopus* spp.**	*R. homothallicus* IRD-13a	sugarcane baggase	Lipase	Rodiguez et al., 2006
	R. oligosporous	Almond meal	Lipase	Haq et al., 2002
	R. oligosporus	Coconut oil cake	Phytase	Sabu et al., 2002
	Rhizopus sp.	Coconut oil cake+sesame oil cake	Phytase	Ramchandran et al., 2005
***Mucor* spp.**	*Mucor racemosus*	Coconut oil cake	Phytase	Bogar et al., 2003
	Mucor racemosus NRRL 1994	Wheat bran+sesame oil cake	Phytase	Roopesh et al., 2006
***Bacillus* spp.**	*Bacillus* sp. JB-99	Rice bran	Xylanase	Virupakshi et al., 2005
	Bacillus sp.	Green gram husk	Alkaline protease	Prakasham et al., 2006
	Bacillus sp.P-2	Wheat bran	Alkaline protease	Kaur et al., 2001
	Bacillus sp.	Wheat bran+ polygalacturonic acid	Pectinase	Kashyap et al., 2003
	Bacillus sp. PS-7	WB+glycerol+ soybean meal	α-amylase	Sodhi et al., 2005
	Bacillus subtilis	Wheat bran	α-amylase	Baysal et al., 2003
	Bacillus sp. AS-1	Wheat bran	α-amylase	Soni et al., 2003

9.4 SSF REACTOR DEVELOPMENT

The delay in SSF being the major mode of fermentation can be partly attributed to the bioreactors initially available. During the initial phases mostly tray type of fermenters were in use with poor instrumentation support and also, heat generated during the process was poorly dissipated. Although recently research was directed on SSF reactor development however, simultaneous instrumentation development has so far been lagging. Initially, automated koji (tray) cultivation was most frequently applied (Pandey et al., 1999). Later on different bioreactor configurations such as periodic pressure solid state fermenter (Tao et al., 1999), immersion, expanded bed and tray type reactor (Couto et al., 2002), intermittent agitation rotating drum type (Kalogeris et al., 2003) and a new bioreactor "PlaFractor" (Suryanarayan, 2003) were developed for the production of enzymes, biocontrol agents and pharmaceuticals.

The traditional tray type reactors consist stacked trays contained in a container with sterile humidified air being circulated for aeration and moisture maintenence. Although, the column bioreactors have been used for enzyme production studies (Pandey et al., 1996; Mitchell et al., 1999) however, these reactors suffering from differences in growth, enzyme synthesis and sporulation pattern in different segments of the column (Pandey et al., 1996) could not be used for industrial scale fermentation. Recently some efforts were made to improve upon the tray bioreactors by using spouted bed bioreactor. The studies suggested that intermittent spouting of tray reactor with air achieved high production levels of α-amylase with yields comparable to packed bed bioreactor (Silva and Yang, 1998). Also, periodically dynamic changes of air have been found effective in controlling temperature instead of agitation and rotation that damage or disrupt fungal mycelia and reduce the porosity of the substrates (Chen et al., 2005).

9.5 FACTORS AFFECTING ENZYME PRODUCTION IN SSF SYSTEMS

The various factor which need to be studied for successful SSF process development include: a suitable substrate and microorganism, the treatment requirement for easy substrate availability to micro-organism, substrate particle size, water content and water activity of the substrate, relative humidity, nature of inoculum, temperature stability, heat dissipation and maintenance of uniformity, duration of fermentation (Pandey et al., 1999). Although, the SSF systems have been known for their simplicity and relatively low level of instrumentation requirements but for process automation purposes more instrumentation support is necessary. Although, the PlaFractor fermenter developed at Biocon India, has been developed keeping the process automation requirements in mind. However, various aspects of SSF processes such as development of probes mainly for moisture measurement, control and regulation of process variables,

analytical procedures to specific media and measurement of the gaseous environment still need to be explored (Durand, 2003). Also, various problems of scale like mass compaction, shrinkage, and reduction of the heat transfer (Durand, 2003) etc. require careful studies. The factors affecting the fermentation scale-up in SSF are mainly poor heat removal (Mitchell et al., 2003) leading to problems in temperature control and oxygen limitation (dos Santos et al., 2004). The air being poor conductor of heat is not suitable for conducting the heat and also it requires maintaining certain level of moisture, which can only be done by providing humidified air. However, the disadvantage of poor heat removal can be used advantageously using thermophilic organisms which require elevated temperatures (Kalogeris et al., 2003).

9.6 ENZYMES PRODUCED BY SSF

Although much work has been carried out on SSF for the production of enzymes of industrial importance such as proteases, cellulases, ligninases, xylanase, pectinase, amylase, glucoamylase, etc., attempts are also being made to study SSF processes for the production of inulinases, phytases, tannase, phenolic acid esterase, microbial rennet, aryl-alcohol oxidase, oligosaccharide oxidase, tannin acyl hydrolase, a-L arabinofuranosidase, etc. using SSF systems. In the following sections, a brief account on production of selected enzymes in SSF systems would be discussed.

9.6.1 Cellulases and hemicellulases

Cellulose is an insoluble molecule consisting of glucose units ranging from 2000-10000 residues while some chains may be still bigger in size. The glucose units are arranged linearly as β-(1-4)-D-glucopyranose and form crystals. The intra-molecular and intra-strand hydrogen bonds hold the network. The cellulase has many uses as an anticake agent, emulsifier, stabilizer, dispersing agent, thickener, and gelling agent and also has water-holding capacity. The cellulases represent a group of enzymes capable in breaking cellulose. They comprise of endoglucanases (1,4-β-D-glucan glucanohydrolases), exoglucanases or cellobiohydrolases (1,4-β-D-glucan cellobiohydrolases) and β-glucosidases or cellobiases (β-D-glucoside glucohydrolases). The cellulases have various applications ranging from extraction and/or clarification of fruit and vegetable juices, to baking, brewing, biostoning of denim and deinking of the recycled pulp and paper.

Trichoderma reesei is the most studied mesophilic fungus and five endoglucanases, two cellobiohydrolases and one β-glucosidase have been already identified from this source. The other organisms studied include *Aspergillus niger, Melanocarpus* sp., *Scytalidium thermophilum and Thermoascus aurantiacus*. The cellulase

production studies in *Melanocarpus* sp. and *Scytalidium thermophilum* suggested that expression profile of different components of cellulase complex in thermophilic fungi, *Melanocarpus* sp. MTCC 3922 is independently regulated while in *Scytalidium thermophilum* MTCC 4520 it is co-regulated (Kaur et al., 2006). The cellulose rich agricultural crop wastes such as wheat straw, rice straw, corncob, corn stover, wheat bran, etc. have been used for cellulase production.

Similarly, the SSF has been extensively utilized for production of hemicellulases. The hemicelluloses are a group of complex carbohydrates containing xylans, uronic acid and arabinose. The main chain of xylans is built from β-1, 4-linked xylopyranosyl residues. The backbone is usually substituted to various degrees by residues of 4-O-methyl-D-glucuronic acid, D-glucuronic acid, or L-arabinofuranose, and in some cases is also esterified by acetyl groups (Biely, 2003). Due to the structural heterogeneity of the xylans, xylan-degrading enzyme systems include several hydrolytic enzymes. The best known of these are endo-β-1,4-xylanases, which attack the main chain of xylans, and β-xylosidases which hydrolyze xylo-oligosaccharides to D-xylose (Haltrich et al., 1996). Xylanases have various applications including production of oligosaccharides, baking, starch recovery from wheat flours and aid in the extraction and clarification of fruit juices. The hemicellulases also find application in feed industry to improve the digestibility of feed and in paper and pulp industry for biopulping and help in reducing the consumption of chlorides for developing an ecofriendly process

The xylanase production has recently been reported from lot of fungal systems such as *Thermomyces lanuginosus*, *Thermoascus aurantiacus, Aspergillus awamori*, *A. niger*, *A. oryzae, Penicillium canescens*, *Ceriporiopsis subvermispora*, *Melanocarpus albomyces*, *P. thermophila* J18 and *Trichoderma reesei.* The substrates used mostly for xylanase production include wheat bran, corn cobs, sugarcane bagasse, bagasse pulp, spent sulphite liquor, rice straw, wheat straw, sorghum flour and eucalyptus pulp. However, lignocellulosic materials especially wheat bran has been more successful in production with higher titers being attributed to its hemicellulose nature, favorable degradability and the presence of some nutrients in the carbon source (Sonia et al., 2005). Also, wheat straw is reported to be ideally suitable for xylanase production in *T. aurantiacus* and *Penicillium canescens* cultures (Kalogeris et al., 1999; Bakri et al., 2003).

9.6.2 Ligninases

Lignins are three-dimensional phenylpropanoid polymers, considerably resistant to microbial degradation in comparison with polysaccharides and other naturally occurring biopolymers. Ligninases have applications in delignification of lignocellulosic materials, which can be used as the feedstock for the production of biofuels, paper pulp and animal feedstuff. These may also be used in pulp

bleaching, paper mill wastewater detoxification, pollutant degradation, or conversion of lignin into valuable chemicals. Lignin itself is good asphalt binder with applications in road constructions and also has fuel applications.

The Ligninases comprise a group of enzymes represented by Lignin peroxidase (LiP, EC- 1.11.1.7), manganese peroxidase (MnP, EC- 1.11.1.13) and laccase (EC-1.10.3.2). LiP and MnP are heme-containing glycoprotein which require hydrogen peroxide as an oxidant. LiP oxidizes non-phenolic lignin structures by abstracting one electron and generating cation radicals, while, MnP oxidizes Mn(II) to Mn(III), which then oxidizes phenolic compounds to phenoxy radicals. This leads to the decomposition of lignin substructure. Laccases are multi-copper phenol oxidases, which reduces oxygen to water and simultaneously catalyze the oxidation of aromatic pollutants like anilines and phenols. Several methods using laccase, immobilized laccase and laccase/mediator system have been developed for the treatment of the textile effluents. This enzyme decolorizes some azo dyes without direct cleavage of the azo bond through a highly non-specific free radical mechanism, thereby avoiding the formation of toxic aromatic amines. Recently, laccases produced from various organisms such as white-rot fungus *Daedalea quercina*, *Stereum hirsutum* and *Peniophora sp* and *Streptomyces cyaneus* were studied for decolorization of synthetic dyes (Baldrian, 2004) and biopulping of softwood chips in SSF (Wolfaardt et al., 2004; Berrocal et al., 2004). The biological pretreatment studies of wheat straw resulted in better quality cellulose pulps.

9.6.3 Proteases

Proteases constitute a very large and complex group of enzymes, which differ in properties such as substrate specificity, active site and catalytic mechanism, pH and temperature activity and stability profiles. Milk clotting enzymes have been in use to transform milk into products such as cheese. Use of proteases in baking is another important food application. The inability of the plant and animal proteases to meet the demands led to an increased interest in microbial proteases. Proteases from microbial sources are preferred to the enzymes from plant and animal sources because of their ease of manipulation for biotechnological applications. Microbial proteases are classified based on the pH range of their activity as acidic, neutral and alkaline proteases and based on the functional group at their active site as serine proteases, aspartic proteases, cysteine proteases, and metalloproteases.

Neutral proteases are active within a narrow pH range, have pH optima near neutral, have relatively low thermal tolerance and have applications in preparation of food hydrolysates, baking, protein modification, in leather, animal feeds and pharmaceutical industries. *Aspergillus oryzae* is the predominant source of the

enzyme. Its affinity for hydrophobic amino acids is an advantage in minimizing the bitterness in protein hydrolysates. While, acid proteases may be classified into two groups based on weather their catalytic activity resembles rennin or pepsin. Rennins like enzymes are characterized by their ability to clot milk and have major commercial importance in cheese manufacture. The group of pepsin like acid proteases includes bovine and porcine pepsin and microbial acid proteases mainly from *Aspergilli*. The microbial acid proteases have applications in the hydrolysis of soybean protein in soy sauce manufacture, improvement of baking properties and digestive aids. Microbial pepsin-like acid proteases mainly produced by strains of *Aspergillus* and *Rhizopus* spp. are active in acidic pH and have moderate temperature tolerance.

Solid-state fermentation for proteases has been a preferred mode of fermentation with fungal systems, because of the presence of extracellular enzymes making their recovery easier. The SSF processes usually being simpler can use cheaper agro-industrial residues such as wheat bran, rice bran, sesame oil cake, soybean oil cake, coconut oil cake etc. for production. The recovery also is easier because, the enzyme is produced in concentrated form. The neutral and acidic proteases are widely used in food industry for cheese ripening, meat tenderization, in brewing industry, and for production of protein hydrolysates, as a digestive aid and in bread making.

9.6.4 Lipases

Lipases catalyse the hydrolysis of ester bonds at the interface between water insoluble fatty acid ester or glyceride phase and the enzyme-containing aqueous phase. Lipases find their application in hydrolysis of fats and oils, in transesterification, production of biosurfactants, digestive aid formulation, detergents, enantiomeric separations and steriospecific transformations. Lipases are being increasingly explored for tolerance to organic solvents with applications in organic synthesis for chiral selective reactions The chirality is a key factor in the efficacy of many drug products and agrochemicals, and thus the production of single enantiomers of chiral intermediates is becoming increasingly important in the pharmaceutical industry.

Although, lipases from various microorganisms have been produced but commercially available lipases are mostly from *Aspergillus niger* and *Rhizopus oryzae. The R. oryzae* lipase has found application in bio-diesel production (Ban et al., 2001). Various agro-industrial residues such as coconut oil cake extract, cotton cake, soy cake, gingelly oil cake, olive oil cake, sugarcane bagasse, barley bran, wheat bran, babassu oil cake have been evaluated singly as well as in various combinations, for lipase production. Apart from fungal systems such as *Aspergillus niger*, *Rhizopus oryzae, Penicillium restrictum*

Penicillium simplicissimum, *Trichoderma versicolour*, and *T. Hirsuta,* bacterial systems such as *Bacillus megaterium* have also been studied recently for lipase production under SSF conditions.

9.6.5 Galactosidases and pectinases

There has been considerable interest to produce α- galactosidase (EC- 3.2.1.22), β-galactosidase (EC- 3.2.1.23) and polygalacturoanase (PG, EC- 3.2.1.15) in SSF processes. These enzymes have application in the pharmaceutical and food industries. α-Galactosidase (α-D-galactopyranoside galactohydrolase EC 3.2.1.22) finds applications in industries ranging from beet sugar production to hydrolysis of raffinose and stachyose present in soybean, cowpea and other leguminous crops. The presence of these oligosaccharides is the reason of diarrhoea and flatulence caused by consumption of soy products and pre-treatment with α- galactosidase may help their enhanced applications in food and feed industries. *Aspergillus oryzae* has been mostly used as a source of α-galactosidase. Although, recently actinomycetes have also been studied for α- galactosidase production but the relative advantage of *Aspergillus oryzae* as a GRAS strain makes its enzyme acceptable for food and feed applications.

β-Galactosidase (or lactase) hydrolyzes the milk sugar, lactose, to its components glucose and galactose and thus finds applications in milk and milk products meant for lactose intolerant peoples, for prevention of lactose crystallization in frozen and condensed milk products, for the reduction of water pollution caused by whey and also for increasing the sweetening properties of lactose. Although, production of β-galactosidase has been studied with various orgaisms such as *Trichoderma reesei*, *Aspergillus niger*, *Bifidobacteria*, *Lactobacillus acidophilus*, *Kluyveromyces fragilis*, *kluyveromyces lactis*, *Kluyveromyces marxianus*. However, only *Trichoderma reesei* and *Aspergillus niger* have been studied for SSF production. The various substrates used for fermentation include arabinoxylan, wheat bran and lactose etc. and further exploitation using SSF is desired.

The pectinases, a complex of pectin-degrading enzymes, consist mainly of pectin methylesterases, endo- and exo-polygalacturonases, pectin lyases and causes deesterification, chain splitting and glycoside-bond cleavage. They have been used extensively by fruit, wine and vegetable industries for various functions such as maceration, extraction, liquefaction, clarification and valorization. *Aspergillus niger*, *A. carbonarius*, *A. sojae* and *Rhizopus sp.* are the most frequently used fungi for pectinase production in commercial scale. A variety of substrates such as soy and wheat flour, sugar beet pulp, deseeded sunflower head, orange pomace, lemon pulp, orange bagasse, sugarcane bagasse have been studied singly and in combination for pectinase production.

9.6.6 Glutaminase

L- glutaminase (L-glutamine amidohydrolase - E.C. 3.5.1.2) is the enzyme deamidating L-glutamine and plays a major role in the cellular nitrogen metabolism of both prokaryotes and eukaryotes. L-glutaminase is useful in the food industry as it increases the glutamic acid content of the fermented food thereby imparting a unique flavour. Besides its food value, ability of this enzyme to bring about degradation of glutamine poses it as a possible candidate for enzyme therapy, which may replace or combine with L-asparaginase in the treatment of acute lymphocytic leukaemia. However, not much is reported recently on L-glutaminase production. The recent reports on L-glutaminase production reported the exploitation of *Zygosaccharomyces rouxii* NRRL-Y 2547, *Cryptococcus nodaensis*, *Beauveria* sp and *Streptomyces rimosus* but only *Zygosaccharomyces rouxii* NRRL-Y 2547 and *Beauveria* sp. were studied for SSF applications. The substrates such as wheat bran and sesamum oil cake have been studied apart from inert supports such as polystyrene supplemented with glucose and L-glutamine.

9.6.7 Amylases

The amylases can be broadly classified into two major classes of alpha-amylase and glucoamylase. The α-amylase (endo-1, 4-α-D-glucan glucohydrolase, EC-3.2.1.1) randomly cleaves the 1,4-α-D- glucosidic linkages between adjacent glucose units in linear amylose chain, and glucoamylase or amyloglucosidase (exo-1, 4-α-D-glucan glucanohydrolase, EC- 3.2.1.3) hydrolyses single glucose units from the non-reducing ends of amylose and amylopectin in a stepwise manner. The glucoamylases are capable of hydrolysing both α-1,4 and α-1,6 linkages. The enzymes have applications in the starch processing, food, fermentation, textile, detergent and paper industries. The amylases are used in baking industry to improve the quality of dough, in fermentation industry for glucose production for fermentative applications, in pharmaceutical for preparation of glucose syrups and in feed industry to act as a digestive aid. The *Aspergillus oryzae* has been a preferred organism for fungal alpha-amylases for food and pharmaceutical applications while both *Apergillus* and *Rhizopus* spp. have been used for glucoamylase production. The recent studies suggested that oil cakes/ meal such as coconut oil cake, soy meal may also be used efficiently in mix substrate fermentation for amylase production. The other in expensive substrates such as spent brewing grain, rice husk, rice flakes, cassava starch, sugar cane bagasse, orange bagasse, molasses, rice bran, maize meal, millet cereal, wheat flakes, barley bran, crushed maize, corncobs and crushed wheat have also been studied in solid state fermentation (SSF). However, in most of the studies wheat bran has been found to be the best substrate for amylase production in SSF. Given the requirement of starch processing industry to have thermotolerant amylase, the efforts were directed in identifying thermostable amylases. The bacterial and actinomycetes amylases were found to be having thermostability

properties. However, for food related applications amylases from actinomycetes may not found much favour. The bacterial alpha-amylase from *B. stearothermophilus* GRE1, *Bacillus subtilis*, *Bacillus licheniformis*, *Geobacillus thermoleovorans* have been found to be moderate to highly thermostable while alpha-amylase from other sources such as *Bacillus cereus* MTCC 1305, *Bacillus amyloliquefaciens* and fungal systems such as *Thermomyces lanuginosus* have also been studied.

The fungal glucoamylase production has already been reported using inexpensive nutrient sources such as wheat bran, tea waste and coconut oil cake. However, recently rice-processing waste (coarse, medium and fine waste) along with rice powder was also studied for glucoamylase production. The various isolates studied include *Aspergillus* sp. HA-2, *Aspergillus niger*, *Scytalidium thermophilum*, *Aspergillus awamori*, *Rhizopus oligosporus* and *Thermomucor indicae-seudaticae*. The thermophilic mold *Thermomucor indicae-seudaticae* was reported for production of a thermostable and neutral glucoamylase optimally active at 40°C and pH=7, when grown in wheat bran moistened with a salt solution in 1:2.5 (w/v) ratio supplemented with 2% cotton oil seed cake (Kumar and Satyanarayana, 2004).

9.6.8 Phytase

Phytases (myo-inositol hexakisphosphate phosphohydrolase, EC 3.1.3.8) catalyses the release of phosphate from phytate (myso-inositol hexaksiphosphate). Several cereal grains, legumes and oilseeds, etc. have their phosphorus stored in the form of phytate. The phytate phosphorus is not easily accessible for monogastric animals due to low inherent phytase activity and passes in manure. The areas with high density of cattle and swine farming suffer from ground water contamination due to the high-phytate phosphorus manure. The pollution of water bodies due to high phosphate manure has raised serious problems of water blooms and growth of toxin producers. Phytases appear of significant value in effectively controlling phosphate pollution. Although, phytase can be produced from a host of sources including plants, animals and micro-organisms. Microbial sources, however, are promising for their commercial exploitations. Strains of *Aspergillus* spp., chiefly *A. ficuum* and *A. niger* have most commonly been employed for industrial purposes. Apart from them *Rhizopus oligosporus, Rhizopus oryzae, Mucor racemosus and Aspergillus ficuum* have also been studied for phytase production. Given the desirability of thermostable phytase to survive the feed pelleting process search has now began for thermotolerant phytase and attempts have already been made to use genetic engineering to manipulate the phytase for thermostability. The traditional approach using thermophilic molds such as *Sporotrichum thermophile* has also been evaluated for thermostable phytase production. Recent studies suggest agro-industrial residues such as

sesame oil cake, coconut oil cake, groundnut oil cake and soy oil cake are suitable as a single substrate as well as in combination for phytase production under SSF conditions.

9.6.9 Inulinase

Inulin is a naturally occurring polyfructan in plants consisting of linear chains of β (2,1)-linked fructose residues attached to a terminal sucrose molecule. The polysaccharide is widely distributed in plants such as Jerusalem artichoke, Chicory, Dahlia and Asparagus. Microbial inulinases (2,1-β-D fructan fructanohydrolase (EC 3.2.1.7) are usually inducible and exo-acting enzymes, which catalyse the hydrolysis of inulin by releasing the end fructose molecule. Different *Aspergillus, Staphylococcus* sp. and *Kluyveromyces* strains have been studied for the production of inulinases. The solid-state fermentation for the production of inulinase using coconut oil cake and ssugarcane bagasse has been studied as support and carbon source for production of inulinase. The nitrogen supplementation in the form of corn steep liquor has also been studied. The optimum fermentation conditions for inulinase production were found to be: 36°C and 20 wt.% of corn steep liquor using *Kluyveromyces marxiana* (Mazutti et al., 2006). However not much work has been reported of late in inulinase production using SSF and most of the work still remains in SmF conditions. Although, studies were conducted by immobilizing the conidia of *Aspergillus niger* 20 Osm producing extracellular inulinase on pumice stones and polyurethane sponge for use in repeated-batch processes, only some factors affecting inulinase biosynthesis by the mycelium were studied. It was observed that immobilization enabled repeated-batch enzyme production and as many as six subsequent 24 h batches could be fermented by using the same carrier (Skowronek and Fiedurek, 2006). However given the importance of inulinase in production of mannitol, inulo-oligosaccharides—low caloric oligosaccharides, bio-ethanol and also high fructose syrup for sweetening applications, improved inulinase production remains to be explored by SSF.

9.6.10. Miscellaneous enzymes

The various other enzymes reportedly being explored using SSF process include tannase, chitinase, invertase, and alpha L-arabinofuranosidase etc.

9.7. RECOVERY OF THE ENZYMES

Recovery process for enzymes depends upon the nature of product and its intended application. The higher end applications may require more purified enzymes while the industries such as textile, paper and pulp and feed industry may require the crude enzyme preparations. While the enzyme required for

pharmaceutical and organic synthesis may be the purified one to avoid any side reactions leading to undesired by products. Since most of the enzymes are secreted in the medium hence, they may be simply extracted with water or buffer solutions after mixing for 30 min- 1 hr and removing the mycelia by centrifugation. Since most of the industries desire to have a cost effective downstream process and most of the enzyme preparations have been found useful even in the crude form, hence the enzyme extraction is the preferred mode of recovery. Extraction temperature and pH of the extractant may be sometimes important for enzyme stability. Further purification of enzyme preparations may be brought by ammonium sulphate or acid precipitation followed by ultrafiltration and column chromatography. However, this may lead to overall cost increase of final product.

9.8 CONCLUSIONS

The SSF process has been found to be cost effective for many enzyme production processes vis a vis SmF. The increasing cost of feed for SmF, value addition to agro-industrial residues and ease of operation makes the SSF process more favourable. Also, SSF process requires less initial capital and incurs low operating cost and is suitable for agriculture-based economies. The production of industrial enzymes by SSF will have an important role in future biotechnologies. The focus in SSF application is required to study for enzyme specific SSF process development, development of host specific environments, SSF targeted fungi and bacteria and on their genetic improvement for desired tasks. The emerging trends also suggest development of IPR for SSF related processes. The SSF offers suitable conditions for microorganisms capable of growing at elevated temperatures and future developments may lead to their better exploitation of thermotolerant organisms and products by SSF.

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