Strategies to Improve Enzymes via Solid-State Fermentation

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Abstract Solid-state fermentation (SSF) is the fermentation process which occurs on a solid surface in the absence of "free" water, where the moisture is absorbed to the solid matrix. SSF is gaining an advantageous edge over other fermentation techniques due to its less complexity and more proximity to the normal environment of many microorganisms. On the other hand, a difficulty arises while estimating the biomass concentration in solid-state fermentation. Various factors like direct product application, the increased concentration of the product, less cost of production, and reduced energy requirement are responsible in making SSF as one of the potent technologies for various enzyme productions as seen in case of cellulase, tannase, and lipase. Improvisation of cellulase production in solid-state fermentation can be achieved to a greater extent by making use of varying degrees of substrates which are lignocellulosic in nature, the implicated microorganisms, culture, and process parameters like moisture content and water activity, nutrients diffusion, size of substrate particle, pH, temperature, surfactants, and bioreactor designs. Submerged fermentation whereas holds a different place in terms of various types of fermentations as it has only one major problem related to the oxygen transfer to microorganisms which in turn depends on the configuration, size, and the agitation/aeration system used in the reactor. In order to characterize oxygen transfer, a parameter is known as K La (oxygen transfer coefficient) whose value gives the estimation that how much of the oxygen is transferred by the equipment independent of the reactor volume and hence, for scale-up studies, it becomes an important parameter. In case of antioxidants production using SSF, it was observed that pomace tends to increase the antioxidant activity convergent with an increase in activity of β-glucosidase. Different studies tend to show that *P. floridensis* as an important organism used during the production of lingo cellulolytic enzyme and consecutive advancement in in vitro digestibility of wheat straw has been carried out to a larger extent.

Keywords Fermentation · Biomass cellulases · Lipases · Tannase · Phenolic oxidants

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1 Introduction

The fermentation process which takes place in such a situation where the free water is nearly or completely absent is called solid-state fermentation (SSF). These days definition of solid-state fermentation is taking a new turn as according to number of citations it has been defined as a process where we find microorganisms growing on damp particles of solid materials embedded in the form of beds, wherein there is a continuous flowing gas phase in the spaces between the particles (Behera and Ray 2016). In order to produce various chemicals used in industries, pharmaceutical products, feed, fuel, etc., SSF has come up as one of the prospective technologies. In SSF processes, natural raw materials are generally employed as carbon and energy source. Solid matrix in SSF is composed of chemically inactive material which requires a solution rich in nutrients. The solid material importantly should consist of sufficient amount of water in the form of moisture. Nature of the substrate has an indirect relation with the achievement of higher biochemical process rate because if the content of water absorbed is more than the required compared to dry weight of solid matrix, then it will result in high water activity (a_w) on the interface, leading to biochemical process increase (Mitchell 2011). In case of lower water activity, there is decrease in dispersion of nutrients through the solid matrix, whereas in case of higher water activity compaction in substrate particles takes place. Therefore, appropriate water activity and appropriate level of moisture in the solid substrate forms the essential elements for SSF processes. Along with other factors, surface area of solid substrate plays an important role. It should be generally large, in the range of 10^3 to 10^6 m2/cm^3 so that there is optimum growth on the interface (Manan and Webb 2018). Small size of substrate particles offers larger surface area for microbes to attack but at the same time pose hindrance in respiration and aeration due to interparticle space availability constraint. For the reason of cost-effectiveness in bioprocess optimization, sometimes it becomes necessary to compromise with the size of particles to be used in the process (Durand 2003), for instance, wheat bran, one of the substrates which is frequently used in fine and coarse forms for SSF. In order to achieve maximum production, most of SSF processes make use of mixture of these two forms. Substrates which are solid in nature provide a favorable environment to the bacteria and fungi. The characteristic hyphal growth pattern of filamentous fungi on the superficial surface of the substrate particles makes them as the best studied candidate for SSF. Different types of agriculture-based crop like barley and agro-industrial based leftover of rice and wheat bran, different types of oil cakes of coconut oil, palm kernel, soybean, sugarcane bagasse, cassava bagasse, fruit pulps, seeds like tamarind seeds, jackfruit seeds, corn cobs, etc. are the substrates which are frequently used for SSF processes (Mienda and Idi 2011). While growing on these substrates, microorganisms secrete various hydrolytic exoenzymes which help in breakdown of some complex carbon sources and nutrients which in turn promote biosynthesis and other microbial activities.

With the advent of technology and understanding of certain domains of biochemical engineering, more precisely mathematical modeling and fermenter design, it has

become possible to scale up various SSFs. Few fermenter designs and mathematical models have been adopted for commercial purposes. If these trends keep on flourishing at such a pace, a time will come when SSF technology would be developed quite well and would come in shoulder to shoulder with submerged fermentation technology (Viniegra-González et al. 2003).

There are numerous attractive advantages of SSF including the production of extracellular enzymes that are stable at various temperatures and pH ranges and high production volumes which is nearly 5.6 times larger than submerged fermentation. SSF is commonly used for the enzyme production because it encompasses the production of extremely concentrated crude enzymes that are associated with low costs for extraction and purification (Muthusamy and Ps 2013).

2 Improvement Strategies

2.1 Cellulase Production

Cellulases are enzymes that catalyze hydrolysis of α-1,4-D-glucan bonds in cellulose resulting in the formation of simpler products such as glucose, cellobiose, and cello-oligosaccharides. According to sequence analysis, from 82 families classified as glycoside hydrolase, 13 were identified as cellulases. Cellulases fall under the category of commonly studied enzymes such as cellobiohydrolases, glucosidases, and endoglucanases. Endoglucanases have a peculiar nature of producing nicks in the cellulose polymer due to which the reducing and nonreducing ends of the polymer are exposed to the environment. Cellobiohydrolases catalyze both reducing terminals and nonreducing terminals forming cello-oligosaccharides besides cellobiose products. Thereafter, glucosidases hydrolyze cellobiose liberating glucose. The action of cellulase complex consisting of cellobiohydrolases, glucosidases, and endoglucanases is synergistical in nature so that the crystalline cellulose gets converted to glucose. Due to wide range of applications, cellulases are the third largest industrial enzyme worldwide. These enzymes are also used as detergent enzymes and animal feed additives. In case the major transportation fuel was ethanol extracted from lignocellulosic biomass via enzymatic route, it will in turn make cellulase the largest volume industrial enzyme. There are a wide variety of cellulase-producing microorganisms comprising several anaerobic bacteria and fungi such as white-rot and soft-rot fungi. Cellulases derived from filamentous fungi for instant *Fusarium*, *Humicola*, *Penicillium*, *Phanerochaete*, *Trichoderma*, etc. are used for industrial applications since filamentous fungi and aerobic bacteria generally secrete free molecules of cellulases. As compared to yeast or bacteria, filamentous fungi cause difficulties in mass transfer and this is primarily due to its characteristic growth pattern. In order to overcome this problem, efficient technologies have been developed which in turn are leading to effective and high titer production of antibiotics, organic acid, and native enzymes. One of the most important cellulase-producing microorganisms which is studied in

detail is *Trichoderma reesei* that produces cellobiohydrolases of two types, CBH I and CBH II along with dual types of endoglucanases which consist of EG1 along with EG2. These enzymes are roughly in the proportion of 60:20:10:10, respectively, that collectively contribute about 90% of the enzymes. On contrary, less than 1% is contributed by seven glucosidases—BGLI to BGLVII (Singhania et al. 2010).

2.2 Cellulase Prerequisite Characteristics for Bioconversion

To achieve optimal biomass conversions, explicit features are required such as better thermotolerance, high enzyme activity, better tolerance to extreme pH, and decreased feedback inhibition. Cellulases which are secreted by various filamentous fungi such as *T. reesei* are acidic in nature. Acidic cellulases are preferred for those bioconversions where acidic pretreatment is given and while working with cocktail of acidic enzymes that require pH optima between 4 and 6. Accelerase®1500 is a trade name of cellulase that has an optimal pH range of 4.6 to 5.0 but below pH 4.0 or above pH 7.0 it becomes inactivated. Usually, celluloses work efficiently at 50 °C temperature and even Accelerase®1500 works efficiently in the temperature range of 50–65 °C. Use of single state fermentation to produce cellulase is quickly gaining attention as a technology which is very cost-effective especially due the use of microorganisms like fungi which produce reasonably large-scale cellulase due to the fermentation conditions that are quite similar to their natural conditions. Chahal had reported that *T. reesei* culture in SSF gives higher yield of cellulases as compared to the cultures in liquid. One of the important consequences of high production titer of cellulases by SSF is that it reduces downstream processing, thus decreasing the operation cost. Apart from various agro-based substrates, various wastes from agricultural domain can also be used as effective substrates for the process of enzyme production under SSF. This has even been validated through a review by Nigam and Singh. Pandey et al. described SSF technology for cellulase production and hence reported that SSF (a future technology) is useful for industrial enzyme production. For the very first time, Dutta et al. analyzed that when cellulase is produced by *Penicillium citrine* following SSF, it shows tolerance to alkali environment. The SSF is considered as a beneficial technology for the production of cellulase in bioconversion as purity is not considered important requirement for this application. SSF is an attractive technology wherein production conditions if optimized will result in better economical production of cellulase. SSF is better compared to SmF as it offers less catabolite repression, better productivity, increased product yield, and less generation of effluent. SSF with improved technology such as better operation control and enhanced bioreactor design can provide promising system for cellulase production (Singhania et al. 2010).

2.3 Tannase Production

Till date, many citations have shown interesting advantages of tannase production with the help of SSF (solid-state fermentation) as compared to submerged. Many scientists have studied the production of enzyme where they have observed solid-state fermentation with the help of various agro-wastes substrate rich in tannins. Leaves of Jamun are optimal source to produce the enzyme using SSF. Throughout SSF, maximum tannase production has been observed at 31.1 °C for incubating at about 96 long hours. However, it has been studied that carbon sources and other nitrogen sources when added to the medium do not affect tannase production. Influence of pH and temperature has been studied widely during the process of tannase activity and during the production of large amount of gallic acid from large amount of tannin-rich agro-waste by Reddy and Rathod. In order to produce tannase by the process of SSF, substrates with large amount of tannin content are used. The substrate is allowed to get completely moistened with large amount of minerals in the form of solution which is then inoculated with the selected organism. Sugarcane bagasse, creosote bush leaves (*Larrea tridentata*), oak galls (*Quercus infectoria*), large amount of sumac leaves (*Rhus coriaria*), myrobalan fruit (*Terminalia chebula*), sorghum leaves (*Sorghum vulgaris*), and Indian gooseberry leaves (*Phyllan thusemblica*) are considered to be the natural supports used to produce tannase on large scale. Studies have shown that the supports like polyurethane foam in combination with nutrient media are used on large scale. Modified solid-state fermentation (MSSF) was used for continuous gallic acid production and tannase production using *R. oryzae* rich strain. MSSF tends to increase tannase and gallic acid production yield by 1.6 and 4 times correspondingly with traditional SSF systems (Muthusamy and Ps 2013).

2.4 Lipase Production

Improvement in lipase production has taken place with the help of modifications that have been implemented in the nutrient source using *Rhizopus homothallicus* which is cultured with the help of process that involves SSF. *R. homothallicus* has been used for lipase production in solid-state fermentation (SSF) with the help of sugarcane bagasse as a support which is then impregnated with an adequate amount of medium containing liquid. It was observed that modification in nutrient present in the media was done for lipase production. Lipase production is largely affected due to nutrients that influence and affect the growth which mainly include urea, olive oil, and huge amount of oligo-elements. Previous studies reveal that improved and better medium provides good results for kinetic studies for growth and lipase production (Ramos-Sánchez 2015). An interdependence is observed to exist between the profiles in the presence of lipase and that of CO_2 production, pH changes, and O_2 consumption during lipase production where an incubation period of 12 h revealed a reading of 827 U/g DM. This production has been observed to show large increase in lipolytic activity in com-

parison to the results which were obtained using known medium to produce lipase. The results were then analyzed to be promising as this strain tends to produce high concentrations of lipase in an inexpensive and reliable medium, which contributes to its purification. In addition to this, the extraction of lipase from the solid medium was also studied to observe the effect, and hence efficiency in the recovery of the enzyme was attained with the help of Triton X-100 at 0.8% (w/v) (Rodríguez González 2006).

2.5 Phenolic Oxidants Production from Cranberry Pomace

Cranberry processing industry has reported production of by-product like Cranberry pomace which can be used extensively for the production of a large amount of phenolic ingredients that are value added. The process of pomace bioprocessing with the help of solid-state fermentation (SSF) and making use of food grade fungi has provided a distinctive and new strategy to enhance various properties especially those of nutraceutical and to produce a large amount of functional and other ingredients. Many functional phytochemicals occur as glycosides or their derivative forms which have comparatively reduced biological and physical activity to a large extent. Therefore, food grade fungus *Rhizopus oligosporus* has been used widely to develop this strategy (Vattem and Shetty 2002). One of the studies reveals that SSF of cranberry pomace has been carried out continuously for 16 days using oxygen sources, nitrogen sources such as ammonium nitrate (NH_4NO_3), and large amount of hydrolysate rich in fish protein (FPH). Nitrogen and oxygen treatments, however, tend to show an increase in water and phenolics which were extracted by 15–25% by day 10 in cranberry pomace. Also, it has been seen that antioxidant protection factor is maximum on 15th day in case of both nitrogen and oxygen treatments and was observed to be 22–27% advanced than that for water extracts and 16.7–19.7% for extracts of ethanol, respectively. The DPPH radical inhibition (DRI) capacity has been seen to increase by 6% for the NH_4NO_3 supplementation and steadily decreases for FPH treatment extracts with water. However, no variation is observed in case of ethanol extracts (White et al. 2010). Activity of β-glucosidase tends to increase by 65-fold in case of other treatment and by 90-fold in case of FPH treatment and with the increasing amount in phenolics which can be extracted and checked for antioxidant activity. HPLC indicates that ellagic acid tends to increase by 4–8-folds in extracts containing water for both oxygen and nitrogen treatments and therefore differences in diphenyl profiles throughout the SSC are examined with this technique. In case of ethanol extracts, this increase was observed to be between 15 and 25%. Hence, it was observed that pomace tends to increase the antioxidant activity convergent with an increase in activity of β-glucosidase. It has been observed that the ellagic acid was seen in HPLC profile, as a component having enriched anti-carcinogenic

properties. Function of antioxidant is, however, observed to show fluctuations for preventing major diseases linked with oxidation such as cancer and CVD. This innovative approach using SSF has been widely used to enhance and increase everyday phytochemicals for the sake of food and feed use to a great extent (Vattem and Shetty 2002).

2.6 Production of Lignocellulolytic Enzymes

It has been observed that increasing wheat straw digestibility degradation by wheat rot fungus has been done which has resulted in its improved value as animal feed. Also, the effect of large amount of moisture content, adequate amount of nitrogen sources inorganic in nature (NH_4Cl) and extracts containing malt sugar on lingo cellulolytic enzymes, and difference in other chemical components and amount of digestibility of wheat straw have been widely observed. Laccase production increases up to 36-fold with a wide increase in moisture content. However, enhancement in the production of CMCase and xylanase to a large extent was significant ($p < 0.05$) which was observed using these supplements. In vitro digestibility has been observed to upscale largely by almost 51% with a loss of 27.5% in lignin and 15.6% in overall organic matter. However, some of the findings tend to show that *P. floridensis* as an important organism used during the production of lingo cellulolytic enzyme and consecutive advancement in in vitro digestibility of wheat straw has been carried out to a larger extent (Sharma and Arora 2010).

2.7 Role of Temperature Control in SSF

It has been known that an important constituent in SSF is temperature control. Previous studies reveal that in continuous mixing, aseptic paddle mixing is done profitably for SSF with *Aspergillus oryzae* on a large amount of wheat kernels. It was observed that mixing continuously improves control in temperature and prevents homogeneities. However, it has been observed that rates of respiration that are observed in this organization can be compared to small and isothermally unmixed beds, showing that stirring continuously did not, however, cause extensive harm to the fungus/kernels entirely. However, it has been observed that increase in scale-up calculations for the paddle mixer is observed to show that cooling in walls becomes insufficient at the 4-m^3 scale for a fungus that grows abruptly like *Aspergillus oryzae*. In contrast to this evaporative cooling, temperature tends to be a very important aspect of systems with large-scale mixed constituents. Some experiments tend to show that addition of water is necessary when evaporative cooling is done to maintain sufficiently excess water activity of the solid matrix used as substrate. The process of mixing is, however, observed to be an important and necessary measure to make sure that addition of water is homogeneous in SSF. Also, process control by automation

can be achieved with the help of enthalpy balance. This was validated using paddle mixer through experiments. This has shown that mixing continuously provides promising possibilities for control of moisture, and therefore temperature control in solid-state fermentation becomes an integral factor for various productions (Nagel et al. 2001).

3 Conclusion

There is well-established fact that solid-state fermentation is one of the promising technologies to produce large number of industrially important enzymes. Tannase production initially started in early years of microbiology but still more research is needed in order to unravel many unknown facts about its efficient extraction and commercial production. Antioxidant production with the help of SSF has lead to opening of many paths including the one toward the prevention of some deadly oxidation-related diseases like cancer. SSF is preferred over various other fermentation technologies like SmF due to various reasons. One of them is the requirement of less amount of energy for the oxygen supply so that the system can cope up with the high oxygen demand.

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