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SOLID STATE FERMENTATION FOR ENZYME PRODUCTION FOR FOOD INDUSTRY

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ABSTRACT

The use of enzymes for improved production and enhanced quality of industrial products in particular fermented foods, received considerable attention of both academic studies as well as industries. This in turn, triggered interest in re-evaluating alternative new or existing technologies in the field of enzyme production. Solid State Fermentation (SSF) is briefly defined as the fermentation with far less (to almost no) free water when compared to the submerged fermentation. The sufficient amount of the moisture is needed to maintain cellular growth. SSF exhibits interesting features, e.g. considerably less water use, therefore more environmentally friendly, easier to downstream, decreased overall process cost, improved product stability, larger portfolio of enzymes or locally very specific productivity rates owing to the heterogeneity of the substrate of generation of sub-populations.
This review lists and discusses the merits of the recent studies on the production of some of the well-studied enzymes in various industries, namely amylase, proteinase and pectinase via solid-state fermentation, vis-à-vis to various factors affecting the process e.g. moisture content, aeration rate, substrate particle size, temperature, and inoculum size. A separate focus is dedicated on the differences between submerged and solid state fermentation, not only from a fermentation unit operation but also from overall process design point-of-view. Lastly, we discuss the challenges in scale-up of SSF process and various reactor types.

Keywords: Solid-state fermentation, Amylase, Protease, Pectinase, Food industry, Industrial Enzymes

INTRODUCTION

Fermentation is defined as a process of breakdown of organic substances by microorganisms which results in a release of the desired products and heat **(Stanbury *et al.*, 2003**). This is undoubtedly a crucial process in food industry for several final products to the end user, but also for production of several intermediate products or ingredients. Considering the free water content, there are two main groups, namely Submerged Fermentation (SmF) and Solid state fermentation (SSF). Owing to the abundant water content, the continuous phase (filling the space between nutrients) is liquid in SmF, while in SSF the continuous phase consists of gas (H. **Chen, 2013).** The solid material in SmF ranges between 1-7% (up to 15% for High Cell Density Fermentations), which mostly are the nutrients (carbon and nitrogen sources) typically homogeneously distributed in the liquid **(Chen & Zhu, 2013)**. In contrast, SSF is characterized with low water content near the absence of the free water **(Arora *et al,* 2017**), or generally with necessary amount of moisture to maintain the microbial growth **(Pandey, 2001; Steudler *et al.*, 2015**). The typical appearance of SSF is dry or mash-like Figure 1, and the content is rarely homogeneous, which is one of the challenges in solid state fermentation. During fermentation, the substrate typically becomes contagious in texture shown in Figure 2.

Figure 1 Solid state material before fermentation and its mash appearance

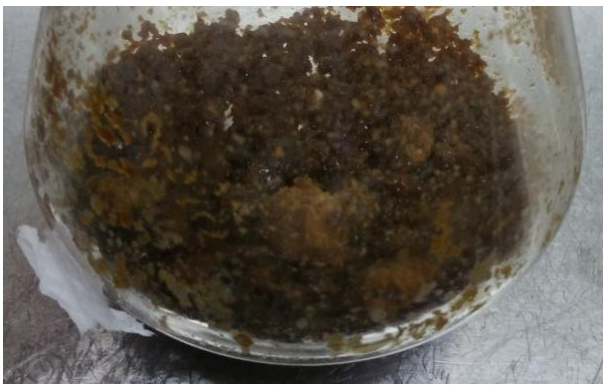

Figure 2 Contagious texture of the solid material after fermentation

Besides the water content, SSF is additionally characterized based on aeration and agitation applied, which also have an impact on the texture of the substrate. From process point of view, this classification yields four different subgroups in SSF:

a) unforced aeration, without mixing;
b) forced aerated, without mixing;
c) unforced aeration, forced mixing;
d) forced aeration, forced mixing **(Durand, 2003)**

Solid-state fermentation recently has received great attention in scientific research and in application in numerous industries, owing its advantages compared to submerged fermentation e.g. simple process, concentrated fermented material translated as smaller equipment and space requirements, high product yield, easy and cheap downstream process, lack of foaming during fermentation **(Roy *et al.*, 2013).** Additionally, SSF is conceived to be more natural for several microorganisms, in particular for fungi growing naturally in low moisture media, than SmF.

Advantages and disadvantages of solid state fermentation

The advantages and disadvantages of the solid state fermentations are going to be shown in the Table 1 below in the short outline. Metabolic overheating is described

as one of the disadvantages of SSF when compared to submerged fermentation **(Chen & Zhu, 2013)**. The increase in temperature of the solid substrate during fermentation may damage the cells and decrease the production rate **(Du *et al*., 2014).** Ashley *et al*., reported using a mathematical model for packed bed bioreactor that periodic air reversal is not a good technique for preventing overheating, however, periodical stirring during a certain time was found to be useful to decrease the metabolic overheating temperature in solid matter **(Ashley *et al*., 1999)**.

Another important factor is the water content since all living systems need water for growth. Compared to fungi, bacteria needs higher moisture content for optimal growth, which in turn, is used as selection factor for fermentation, in favor of fungi **(Ramesh & Lonsane, 1990)**. Interestingly, high levels of water is also unfavorable for enzyme production using SSF, since it decreases the oxygen transfer, as reported by **(Ramesh & Lonsane, 1990)**, where α-amylase is produced by *Bacillus licheniformis* M27 using wheat bran as solid substrate. The authors concluded that optimal levels of water is needed for production of enzyme with highest activity.

Table 1 Advantages and disadvantages of solid state fermentation

Solid state fermentation			
Factors	Advantages	Disadvantages	Ref.
Water content	Water content in the medium is low	Evaporation water during the fermentation process	**(Ozdemir *et al*., 2012; Torrado *et al*., 2011)**
Concentrated product	Produced products are more concentrated, production rate is high, high stability of the product	Hard to adjust the pH inside the solid matter	**(Karp *et al*., 2015; Ortiz *et al.*, 2016)**
Downstream process	Easy and cheap downstream process, no need for complicated reactor	Hard to get rid of the solid material after the downstream process	**(Ertan Inceoglu *et al.*, 2014b; Zaslona & Trusek-Holownia, 2015)**
Process	Simple fermentation process, no foam formation	Problems with scaling up agitation and aeration	**(El-Shishtawy *et al*., 2015; Samarntarn, *et al*., 1999)**
Environmental concern	Environmentally friendly, low output waste	Realize of methane (which if it is used for energy can cause no harm)	**(Chen *et al*., 2014; De Azeredo, *et al*, 2006)**
Suitability for micro.	Suitable for work with filamentous fungi	Problem with bacteria as a reason of low water activity	**(Du *et al*., 2014; Mukhtar & Ikram., 2012)**
Media and inoculum	Nutrients of the medium are more concentrated, Minimize catabolite repression	High amount of inoculum more than 10% hard to homogenize the content	**(Ertan *et al*., 2014a; Holker *et al*., 2004; Pham, *et al*., 2010)**
Structure	Can be realized in open environment, Gas phase as continuous phase	Problems with substrate shrinkage, Sampling is more complicated than in submerged fermentation	**(H. Kh. *et al*., 2011) (J. Chen & Zhu, 2013)**
Contamination, Indigenous culture	Easy bacterial contamination control, fermentation with indigenous culture, small work space	Hard in controlling metabolite temperature realized during the process	**(El-Shishtawy *et al*., 2015) (Karp *et al*., 2015; Tabassum, *et al*., 2014)**
Cost	Cheap agro industrial, material cheap overall process		**(Castilho *et al*., 2000; Ferracini-Santos & Sato, 2009; Shabbiri *et al*., 2012)**

PRODUCTS OF SOLID STATE FERMENTATION

Several food products are traditionally produced via SSF. The well-known red rice, produced by fermentation of rice grains using the fungi *Monascus purpureus*, known as Koji process, is an example of SSF. Currently this is typically performed in tray reactors stacked in a temperature and moisture controlled fermentation rooms, used to produce various products in so-called "Koji bed"**(Karthikeyan & Sivakumar, 2010**). Beyond Koji, SSF has drawn increasing amount of attention and has been used for the manufacture of commercially important products in food, pharmaceutical, medical, agro, and biofuel industry. Production of aroma components via SSF for food applications have been reported. An example is the Chinese light-style liquor, for which the specific aroma was attributed to *Pichia anomaly* during SSF **(Kong *et al*., 2014)**. Biofuel production from agricultural and food residues using bacteria or fungi via SSF have been reported **(Chen *et al*., 2014; Zhou *et al*., 2015)**. On medical applications, SSF has been reported to be used for antibiotics production, in particular Penicillin using *Penicillium chrysogenum* which increased the production **(Barrios-Gonzalez *et al*., 1988)**. About ten years later, the use of a cocktail of *Streptomyces* have been reported to be used for tetracycline production **(Asagbra *et al.*, 2005)**, focusing further in environmental parameters like temperature, pH and moisture content. The authors reported 4.3 fold higher production rate compared with submerged fermentation, indicating SSF as promising alternative for tetracycline production. Other studies focusing on other antibiotics via SSF, meroparamycin, are also reported **(El-Naggar, 2009)**. Despite the large portfolio of products via SSF is available, enzymes are the most important one, since they have wide area of application, making them commercially highly demanded. This review is therefore going to explore the production of various enzymes in solid state fermentation, their characteristics and new improvements of the process. In particular we focus on proteases, amylases and pectinases and their application in food industry.

PROTEASE PRODUCTION IN SOLID STATE FERMENTATION

Proteases (proteinases) are a group of enzymes capable to hydrolyze the protein bonds resulting in the production of their constituents amino acids according to this, proteases are considered the most important industrial enzyme gaining around 60% of the total enzymes in the market **(Pokora *et al*., 2017)**. There are known three types of proteases based on the optimum pH: acidic, neutral, and alkaline proteases. Acidic proteases have an irreplaceable impact in industries like animal feed, food and cheese processing *etc.* **(Siala *et al.*, 2012).** Neutral proteases are so important for the food industry for the reason of fewer bitterness byproducts realized after reaction due to intermediate rate reaction **(Shabbiri *et al*., 2012)**. Meanwhile, alkaline proteases are widely used in industries such detergent, leather, textile, photographic, food and feed, and chemical industry (**Pokora *et al*., 2017**). Protease production for dehairing in leather industry was reported **(Vijayaraghavan *et al.*, 2012)** by using solid state fermentation. They used livestock waste as solid material supplemented with organic carbon and nitrogen sources and 140% (v/w) moisture content using *Bacillus subtilis* W. They reported a highly active enzyme and with a good potential in leather and detergent industry. As a follow up, concomitant enzyme production of cellulase and protease using separately *Bacillus cereus* AT and *Bacillus subtilis* IND19 and reported significant improvement in protease production **(Vijayaraghavan *et al*., 2016; Vijayaraghavan *et al*., 2014)**. In addition, for multi-enzyme production using cow dung, additional nutritional supplements are needed.

One of the most important advantages of SSF for enzyme production is the potentiality for using industrial residues with low price. In this context, an article using feather residues from poultry industry to produce protease was reported. Both SmF and SSF were used as fermentation process to make a collation between their indications in protease production by *Streptomyces sp. 594.* The protease produced by SmF resulted highly thermophilic and more active compared to protease produced in SSF. For the first time in this article *Streptomyces* are used for protease production by SSF **(De Azeredo *et al*., 2006)**. Moreover, residues like *Saccharomyces cerevisiae* dried cells from fermentation process were used for alkaline protease production by using *Cellulosimicrobium cellulans*, this medium showed to highly support protease production even though salt enrichment were necessary **(Ferracini-Santos & Satos, 2009)**.

The substrate components of a solid medium were screened to improve protease production by *Bervibacterium linens* DSM 20158, substrate components which were analyzed as organic carbon and nitrogen source like rice husk, wheat bran, rice bran, soybean meal, yeast extract, corn steep liquor, casein and beef extract were screened. Moreover, a series of salt solutions were used too, the best composition for protease production they found to be the substrate prepared with soybean meal, wheat bran, $(NH_4)_2SO_4$ and a certain size of inoculum. They found that protease activity increased for two-fold with screened composition than in random one, all the components used during their study were low price and easy found which means that cost production will be decreased **(Shabbiri *et al*., 2012)**.

Otherwise, to increase the production and to produce the specific proteases, recombinant transformation was used as technique. As example the work of **(Gama Salgado *et al.*, 2013)**, where *Pichia pastoris* was transformed to produce extracellular protease, found in *Mucor circinelloides.* This portease was highly capable for milk coagulation, even though the good result of transformation, solid state was not used as a substrate for recombinant cells. Moreover, authors **(Mukhtar & Haq, 2013)** compared the production of protease during SSF and SmF by using wild and mutant strains of *B. subtilis*, by treating the wild types of *B. subtilis* with ethyl methane sulfonate they prepared mutant types which they selected based on casein agar plates. Around ten agro-industrial byproducts were checked out for their indication in protease production, wheat bran with 2% addition of soybean was found to give the best result for protease production in both wild and mutant strains, furthermore, protease activity was around 1.96 fold higher by mutant strains than the wild strain.

Inoculum preparation conditions were shown to have a significant indication in protease and amylase production during solid state fermentation. During inoculum propagation, they used different carbon and nitrogen ratio when maltose and peptone were used as a source of them. A high ratio of carbon and nitrogen during inoculum preparation of *Aspergillus awamori* IOC-3914 bring a notable improvement in both amylase and protease production during solid state fermentation in babassu cake with 70% initiated controlled water content **(de Castro *et al.*, 2011)**.

Interestingly, concomitant production of enzymes (when two or more enzymes are produced in same conditions from the same microorganism in the same time) are successfully proceed by SSF. Protease and amylase are known as concomitant enzymes and was study point of the article **(Saxena & Singh, 2014)**, in their study they tried improvement of the production of this concomitant enzyme from *Bacillus megaterium* B69 by using different agro-industrial waste as a substrate statistical optimized by using designed screening method Placket-Burman. The best production of the concomitant enzyme was by using the mustard oilseed cake, they enriched activity of both of the enzyme for around two fold by using response surface methodology (RSM) to screen the amount of the variables. Table 2 summarizes optimal condition for maximal production of protease by different microorganisms.

Table 1 Summary and optimum conditions of various studies focusing on Protease production using SSF

Microorganisms	substrate	Opt temp(ºC)	Opt pH	Opt moisture (%)	Opt inoc. size (%)	Opt Reactor type	Time of ferment (h)	Activity rez. ($U.g^{-1}$)	Reference
Bacillus cereus	Cow dung Animal tissue 5 (g/l) Beef extract 1.5 Yeast extract 1.5 Sodium chloride 5 Skim milk 10	37	8	120	6	250ml Erlen 10g	72	4813	**(Vijayaraghavan *et al.*, 2014)**
***Bacillus megateriu m* B69**	Mustard oil cake Basal media	37		45		250ml Erlen 10g	84	1242	**(Saxena & Singh, 2014)**
Bacillus subtilis	Wheat bran Soy bean meal	37		50	10	250ml Erlen	42	85.03	**(Mukhtar & Haq, 2013)**
***Brevibacterium linens* DSM 20158**	soybean male	37	7.2	90	3.5		120	2051	**(Shabbiri *et al.*, 2012)**

Legend: inoc.-inoculum, Erlen- Erlenmeyer

AMYLASE PRODUCTION BY SOLID STATE FERMENTATION

Amylase enzyme hydrolyses polysaccharides, in particular starch into smaller (mono or oligo) sugars. Amylase is an important enzyme used in food, fuel, environmental, and pharmaceutical industries

Similarly to protease, the use of SSF in amylase production has drawn increasing attention. In 2000 a group of authors **(Mulimani & Patil Ramalingam, 2000)** published an article about α-amylase production under SSF using the fungus *Gibberella fujikuroi* using wheat bran , soy meal, rice bran were used as substrate. To moisten substrate, a mixture of different salt solution was used. At the end of fermentation, 0.2 M acetate buffer was used to wash the fermentation product. By using the same condition for each solid substrate during fermentation and assaying for amylase activity, they reported that wheat bran as substrate yields the highest amylase activity.

Filamentous fungi are known for its high production of a large portfolio of enzymes, and it is also accepted as a very suitable host for solid state fermentation. *Aspergillus flavours* were used to produce α-amylase during solid state process in which *Amaranthus paniculatas* (velvet flower) grains was used as basic solid matter. The medium optimization experiments aimed for maximum amylase production and these have been designed following a Plackett-Burman fractional factorial design in order to select four significant ingredients among fifteen available ones. They concluded from the results that most significant factor in medium optimization are corn steep liquor, $NH_4H_2PO_4$, NaCl, and $CaCl_2$.

The screening study was followed by detailed Response Surface Methodology approach, and the optimization resulted in an eight fold increase in activity when compared to control case **(Viswanathan & Surlikar, 2001)**. Bogar *et al* reported the use of 11strains of *Aspergillus oryzae* to produce amylase via SSF, using spent brewing grain or corn fiber as solid matter in tray mode **(Bogar *et al.*, 2002)**. Aiming for medium optimization, they first screened the significant medium components starting from 11 components, and they found that most significant medium components are starch, corn steep liquor, ammonium nitrate, maltose and soybean meal. The strain that resulted with highest amylase activity was *Aspergillus oryzae* NRRL1808 in spent brewing grain at 67% moisture and 25ºC reaching up to 4519 $U.g^{-1}$ of dry matter. All in all, *Aspergillus spp.* have been well studied for amylase production: *A. oryzae* NRRL 6270 using spent brewing grains in a lab scale reactor, reaching 6870 $U.g^{-1}$ after optimizing temperature, moisture content, inoculum size and incubation time **(Francis *et al.*, 2002)**. The use of rice bran, supplemented with soybean flour and cassava starch and *A. oryzae* culture, focusing on shelf-life of the resulting enzyme for which both supplements (soyban flour and cassava starch) increased the shelf-life of the enzyme. Using molecular biology tools, Biesebeke *et al.*, deleted the endo-protease encoding gene pclA and pg/pi-tp gene encoding phosphatidylinstol Transfer proteins, resulting 50% increase in amylase production **(Biesebeke *et al.*, 2005)**. Another fungus used for amylase production was *Neurospora crass* CFR 308 and as solid substrate coffee pulp, coffee cherry husk, spent coffee and their combination was used. The highest activity per dry matter was gained when coffee pulp was used, with 60% moisture, after five days of fermentation **(Murthy *et al.*, 2009)**.

In addition to fungi, bacteria is also used for the production of amylase, in using SSF. Bacteria, in turn needs generally higher moisture content in fermentation media, though there are occasional reports, typically with extremophiles stating otherwise an example is reported by **(R. Kumar *et al.*, 2010)**, where *Bacillus* was used for keratinase production. An advantage of bacteria is the faster growth rate, shortening the fermentation time and making overall process economically more attractive **(Baysal *et al.*, 2003)**. Half of the fermentation time with fungi Similar comparison was reported between *Bacillus* and *Aspergillus* stating that bacteria reaches the highest activity after 48h while fungi reaches its maximum at 96h **(Soni *et al.*, 2003)**.

Thermo stable amylase are important for food industry, in particular in drink making. *B. subtilis* PS7 isolated from the hot springs are used to produce a high amount of thermo stable amylase, using wheat bran as solid substrate. They reported that optimum condition were, to use tap water instead of distilled or mineralized water, 60% of water content at 37ºC **(Sodhi *et al.*, 2004)**. Successfully alpha amylase production by using wheat bran at 37ºC for 48h by *Bacillus subtilis* MTCC121 in solid state fermentation was reported by authors **(Raul *et al.*, 2014)**. The downstream process for purifying the raw enzyme requested 30-70% $(NH_4)_2SO_2$ even though the purifying process need to be improved. The effect of using different substrates on amylase production has also been reported e.g. **(Shukla & Kar, 2006)**.

Recently, considerable attention has been given to medium optimization using agricultural byproducts for SSF, in amylase production, typically at lab scale, using 5-40g of solid matter. Most frequently studied parameters are temperature, time of incubation, particle size, pH, moisture content, inoculum size and time of incubation **(Dojnov *et al.*, 2015; Mustafa *et al.*, 2016; Saha *et al.*, 2014; Sahnoun *et al.*, 2015)**, while the problem of aeration and metabolic overheating is scarcely studied. Table 3 summarizes optimal conditions reported in various studies for maximal production of amylase.

Table 2 Summary and optimum conditions of various studies focusing on Amylase production using SSF

Microorganisms	substrate	Opt temp (ºC)	Opt pH	Opt moisture (%)	Opt inoc. size	Opt Reactor type	Time of ferment (h)	Activity ($U.gds^{-1}$)	Reference
***Aspergillus oryzae* NRRL1808**	Spent brewing grain	25	6	67	$2x10^7$ spores	500ml Erlen 5g	72	4519	**(Bogar *et al.*, 2002)**
Aspergillus niger	Triticale grains	28		30	19%	250ml Erlen 16g	120	158	**(Dojnov *et al.*, 2015)**
Penicillium chrysogenum	Wheat bran	30		75	20%	250ml Erlen	144	750	**(Ertan *et al.*, 2006)**
Penicillium chrysogenum	Wheat bran Sunflower oil meal Sugar beet oil cake (1:3:1)	30		75	20%	250ml Erlen	144	845	**(Ertan *et al.*, 2006)**
***Aspergillus oryzae*NRRL 6270**	Spent brewing grains	30		70	$1x10^7$ spores/ml	250ml Erlen 5g	96	6870	**(Francis *et al.*, 2002)**
Gibberella fujikuroi	Wheat barn	32			50	250ml Erlen 20g	120	42	**(Mulimani &Patil Ramalingam, 2000)**
***Neurospora Crassa* CFR308**	Coffee pulp	28	4.6	60	$1x10^7$ spores/ gds	250ml Erlen 10g	120	3908	**(Murthy *et al.*, 2009)**

Legend: gds-gram dry substrate, Erlen- Erlenmeyer , inoc.-inoculum

PECTINASE PRODUCTION BY USING SOLID STATE FERMENTATION

Pectinases represents a collection of enzymes, able to break down pectin which is a polysaccharide composed of galacturonic acid. Pectin is found in cell walls of plants, is therefore of central importance to food industry, in particular for fruit and vegetable processing. Examples include, clarification, decreasing the viscosity of juices, facilitating flavor extraction process, filtration *etc.* **(Solis-Pereyra *et al.*, 1993)**. Based on their mechanisms, pectinases are categorized into several subgroups, and include polygalacturonases, pectate lyases, and pectin methylesterase. Polygalacturonase, pectate lyase, and pectin methylesterase hydrolyze glycosidic bonds in the carbon chain; pectin esterase segregates off methoxyl groups **(Acuna-Arguelles *et al.*, 1995; L. Castilho *et al.*, 2000)**.

Pectinase are produced using microorganisms via both SmF and SSF, the latter being more preferred **(Solis-Pereyra *et al.*, 1993)**. Solis-Pereyra reported that pectinase production is around 11 times higher in SSF than in submerged fermentation, using *A. niger* as host. SSF is performed using sugarcane bagasse as substrate (supplemented with glucose at the beginning) and small amount of pectin for initiating the pectinase production, in glass columns as packed bed reactors. The submerged fermentation, chemically defined medium is used and performed in batch mode in flask. In the SSF, high sugar concentration increased pectinase production, while the opposite occurred in SmF **(Solis-Pereyra *et al.*, 1993)**. Focusing back on the effect of initial glucose level in SSF with *A. niger* strains, **(Solis-Pereyra *et al.*, 1996)** reported that medium amount of glucose yields higher pectinase activity when compared to the high initial glucose levels, the reason is attributed to extreme pH decrease on the medium.

Patil *et al* compared production of pectinase between two *A. niger* strains (DMF27 and DMF45) in SmF where sunflower seeds are used as addition to defined medium while to SSF deseeded sunflower was used as substrate. The paper reported that *A. niger* DMF45 has higher productivity of pectinase in SSF, while *A. niger* DMF27 shows better productivity in submerged fermentation. **(Patil & Dayanand, 2006b)**. Same authors later optimized pectinase production in SSF and SmF by using the same strains, by changing pH, moisture content, temperature, inoculum size and particle size. SSF case improved more than SmF and the produced enzyme by using SSF reached higher activity and was more stable **(Patil & Dayanand, 2006a)**. Similar outcomes are also reported by **(Martin *et al.*, 2010)** for *Thermomucor indicae-seudaticae* N31 and by **(S. Kumar *et al.*, 2011)** for *A. niger* as a culture and by **(Acunaarguelles *et al.*, 1994)** again for A. niger, but specifically studying the effect of water activity on pectinase production. Several other notable works on fungi (including *Penicillium spp.*, *Thermoascus* and *Moniliella*) in SSF, studying the effect of various medium components, washing solutions, process conditions are reported **(Castilho *et al.*, 2000; Gomes *et al.*, 2002; Gomes *et al.*, 2002; Gomes *et al.*, 2004)**

Alternative to fungi, bacteria is also used for pectinase production via SSF to decrease fermentation time leading to economically more attractive process. In this context, Raj Kashyap reported in **(Raj Kashyap *et al.*, 2003)** the use of *Bacillus sp*. DT7 to produce pectinase via SSF in different substrates optimized with a different salt solutions. Highest production rate is achieved with wheat bran with 75% moisture content, after 36h of fermentation. The salt solution included $CaCl_2$, $MgSO_4$ and a multivitamin B solution which increases the productivity around 50%. Similar reports are published by **(Sharma & Satyanarayana, 2012)**, where they used *Bacillus pumilis* and wheat barn and other agro-industrial residues as substrate in SSF. They suggested that manipulating the substrate level, moisture content and pH in SSF, the production rate would increase around 14 fold compared to SmF. Lastly, using recombinant DNA technology and importing the activator aepA from *Erwinia carotovora* to *Escherichia coli* HB101, resulted in an increase in pectinase, cellulase and protease production. The production was also significantly affected from the choice of substrate **(Mei *et al.*, 2013).** Table 4 summarizes optimal conditions for maximal production of pectinase via bacteria and fungi.

Table 3 Summary and optimum conditions of various studies focusing on Pectinase production using SSF

Microorganisms	substrate	Opt temp (ºC)	Opt pH	Opt moisture (%)	Opt inoc. size	Opt Reactor type	Time of ferment (h)	Activity rez.	Reference
Bacillus pumilus	Sesame oilseed cake, Wheat bran Citrus pectin (1:1:0.01)	40	9	70	25%	250ml Erlen 5g material	120	348($U.g^{-1}$) Dry bacterial bran	**(Sharma & Satyanarayana, 2012)**
Aspergillus niger	Lemon peel pomace	30	5	70	$2x10^7$ spore /g	Column tray bioreactor	96	2181 ($U.l^{-1}$)	**(Ruiz *et al.*, 2012)**
***Aspergillus niger* NCIM 548**	Wheat bran corn bran kinnow peel (2:1:2)	30	5	65		Erlen	144	179.83 ($U.g^{-1}$)	**(Kumar *et al.*, 2011)**
Thermomucor indicae seudaticae	wheat bran and orange bagasse (1 : 1)	45		70	10ml in 5g	250ml Erlen 5g material		120 ($U.ml^{-1}$)	**(Martin *et al.*, 2010)**
Aspergillus niger	deseeded sunflower head green gram husk sucrose 6%	34	5	65	$1x10^7$ spore/g	250ml Erlen	90-120	30($U.g^{-1}$)	**(Patil & Dayanand, 2006b)**
***Bacillus* sp. DT7**	Wheat bran polygalacturonic acid 1%w/V	37		75	50%	250ml Erlen 5g	36	8050 ($U.gds^{-1}$)	**(Raj Kashyap *et al.*, 2003)**
Thermoascus aurantiacus	Polygalactouranase Wheat bran Pectin lyase 10%sugarcane bagasse 90%organe bagasse	50	5 10.5	67	5mg dry micelial mass per g dry substrate	250ml Erlen 5g material	96	Pg 43 ($U.g^{-1}$) Pl 40180 ($U.g^{-1}$)	**(Gomes *et al.*, 2002)**
***Penecillium viridicatum* RFC3**	Wheat bran banana and mango peel for PG Orange peel + banana and mango peel for PL	30	Approximately 5	40			96-120	Pg 55 ($U.g^{-1}$) Pl 3540 ($U.g^{-1}$)	**(Castilho *et al.*, 2000)**
***Aspergillus niger* CH4**	sugar cane bagasse pith pectin sucrose urea, ammonium sulphate,	35	4.5	70		Column packed bed			**(Acunaarguelles *et al.*, 1994)**
***Aspergillus niger* F3**	Dried grinded and sifted citrus peel ammonium nitrate Sodium sulfate Magnesium sulfate Zinc sulfate	30	5	60	10%	horizontal drum bioreactor 2kg substrate	72	Around 4($U.mlh^{-1}$)	**(Rodriguez-Fernandez, *et al.*, 2011)**
Aspergillus awamori	Grape pomace	30	-	65		Petri dish			**(Botella *et al.*, 2007)**
Aspergillus niger	Peashrub Supplemented with Wheat bran Ammonium sulfate Rice dextrose	30 for 30h 23for 42h	6.5	60	4%	plate		36 ($U.g^{-1}$)	**(Debing *et al*, 2006)**
***Bacillus* sp. DT7**	Wheat bran polygalactouronic acid	37		75	40%	250ml Erlen 5g	36	8050 ($U.gds^{-1}$)	**(Kashyap *et al.*, 2003)**

Legend: Pg-Polygalacturonase, Pl- Pectin lyase, gds- gram dry substrate, inoc.-inoculum, Erlen-Erlenmeyer

SCALING-UP IN SOLID STATE FERMENTATION

Considerable attention has spent on either process optimization or selection of substrates in lab scale for SSF, while studies on scale-up are comparatively untouched. We consider scale-up to be a significant problem in SSF, since optimum production process in larger scale drastically change when compared with the lab-scale conditions. The most momentous parameters in large scale SSF are the temperature gradient through the solid content, evaporation and aeration control, and local pH changes.

Koji is a traditional scaled-up SSF fermentation process, which is used to produce a Japanese food, which consist of soybean, wheat grains or rice fermented with fungi *Aspergillus oryzae* **(Zhu &Tramper, 2013)**. During Koji fermentation there are several enzymes produced, most known are protease, amylase and lipase, which increased the nutritional value of fermented foods and also improved the flavor via the production of aroma compounds **(Chancharoonpong *et al*., 2012; Feng *et al*., 2014).** Tray reactors with different dimension and designs (e.g. sieve-like) are used during Koji fermentation to support different parameters to increase the fermentation rate. Typically, the tray reactors filled with a (thick) layer of substrate are placed in a temperature and moisture controlled room, called Koji room. A solid state fermentation with tray reactor was analyzed by **(Ghildyal *et al*., 1992)** for gaseous concentration gradient and changed the thickness of the substrate layer. Even though the design of the trays would support the air distribution, the tray with thicker substrate layer (larger than 80mm), the distribution of gas decreased and similarly the activity of the produced enzyme also decreased. When the thickness of the layer is less than 18mm, the moisture decreases considerably faster **(Jao *et al*., 2011)**.

In spite of the fact that though Koji fermentation is a large scale fermentation compared to other types for SSF reactor, Ghildyal *et al.* assessed the temperature gradients in substrate layers, resulting from the substrate depth **(Ghildyal *et al*., 1993)**. In their study they included four range of substrate loads between 17.8-126.1kg/m^2 which consists of 40-270mm initial bed heights. Their results showed an increase in temperature gradient with the increase of the bed heights and the enzymatic production was decreased accordingly. They concluded that in scaling-up process for SSF reactors, bed depth is an important parameter to be optimized, considering temperatures gradients **(Ghildyal *et al*., 1993**). A successful story of reproducible lab-scale result to scaled-up 30kg Koji for protease production was

published by **(Malathi & Chakraborty, 1990).** During their research they did a gradual scaling-up starting form Erlenmeyer flask with 10g substrate to tray fermentation with 500g and finally to the Koji room with 1-30kg wheat bran.

Meanwhile, there have been several studies for different types of scaled-up reactors for SSF process. In 1997, a 50L fed-batch reactor was prepared as a pilot plant for production of giberellic acid. The amount of the wheat bran, in the beginning, was 6 kg plus for each kg of wheat bran 250 g of soluble starch have been added. The humidity, temperature and aeration flow temperature were monitored with probes inserted into the reactor. The reactor was fed with 500g of corn starch and 500ml of distilled water, every 24 hours for 72 hour. The aim of the work was to minimize the temperature gradient (both vertically and horizontally) and control the dry matter percentage of the solid medium inside the reactor by using aeration, agitation and the jacket from the outside of the reactor. They report that during initial stages (up to 75-100 h), maintaining the outlet temperature is easy, while the inlet air temperature should be decreased considerably in later stages of the fermentation. The amount of giberellic acid per dry matter produced was around 3g per kg dry matter, and it is worth mentioning aseptic auto-sampler is used to take samples every time during fermentation process **(Bandelier *et al.*, 1997)**.

Oostra *et al*. reported a cooling system in SSF to produce *Coniothyrium minitans* spores which are used as biopesticide. For bioreactor selection and scale-up, they used lab experiments and mathematical simulation. Based on their results, conductive cooling through the wall in the packed-bed reactor (non-mixed) is unproductive but it gives a good effect in decreasing the temperature in scarped drum reactor (mixed). Additionally, they reported forced aeration for temperature control inside the reactor, which is found to be effective but with a side effect as substrate shrinkage resulting from evaporation. Saturated air was found to be not effective. They come into the conclusion that mixed reactor with conductivity cooling seems the best option for better production in large scale reactors **(Oostra *et al.*, 2000)**. In contrast with previous work, by using Koji room 200kg authors **(Liu *et al.*, 2003)** suggested that increasing the humidity during the aeration step decreases the temperature (accumulated from metabolic activity) and decreases the losses in water content. Based on their outcome authors **(Abraham *et al.*, 2013)** reported higher enzyme production from scaled up process than unscaled one, were they used soy fiber to produce alkaline protease in 500ml Erlenmeyer with 100g solid material for lab scale and in a 4.5L bioreactor with 1.25kg of solid matter. They suggested that there is no negative effect on enzyme activity from scaling up the process. This interestingly contradicts to the report in **(Siumara & Da Silv, 2013)** where they concluded that the production is 45 times lower in tray reactor than in lab scale, attributed to the metabolic heat accumulation in tray reactors. They concluded that tray reactor was not the appropriate for polygalacturonases production when cashew apple bagasse is used as substrate.

Alternative to tray reactors in SSF, fixed bed forced aerated reactor was proposed in **(Castro *et al.*, 2015)**. They used 2.3L capacity bioreactor aerated with humidified air. From their work, they suggested that babassu cake was well adopted for production of hydrolases enzymes in fixed bed reactor. Moreover, inappropriate aeration in large scale reactors can cause additional problems in gas emission during fermentation, as reported in **(Maulini-Duran *et al.*, 2015)**. The most problematic contamination gas emitted during the fermentation process were CH_4 and ammonia, typically related to temperature accumulation.

CONCLUSIONS

SSF is typically considered to be an easy and cheap method for various metabolite and enzyme production with concentrated products and high stability; and as such, it attracted increasing attention in food industry. A considerable number of research is devoted in enzyme production takes into consideration several abundant agro-industry byproducts, especially suitable for SSF. Moisture content, temperature, and variety of the substrate from the literature are well studied.

The challenges of SSF becomes more and more pronounced in later stages of fermentation, even more so in scale-up, when the actual production (of the enzyme or metabolite) occurs. Aeration and agitation are manipulated variables that are used in scaling-up the production, to avoid unwanted effects e.g. particle shrinkage, excess evaporation and metabolic heat accumulation.

At last, increased number of studies in genetically modified strains leads even more increased production capacity. Considering the literature, the following items needs further focus: aeration with saturated air, agitation, and cooling by conductivity in combination with the use of (possibly modified) species, well adopted to solid state fermentation.

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