Approaches Towards Improvement of Microbial Strains Used in Solid State Fermentation

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The low water activity, the complex nature of the support and/or substrate, the concentration of substrates used and products formed, and the heterogeneity within the system, influence the growth and physiology of the microbial strains used in solid state fermentation (SSF). As a result, the quality and quantity of the products formed from SSF processes significantly differ from those of submerged fermentation (SmF). Some strains that performed well in SmF processes do not perform efficiently in SSF process. Therefore, it is necessary to maximize the potential of the organism through genetic manipulation to suit the SSF process. Some of the desirable characteristics to be considered for the improvement of the microbial strains for SSF are: tolerance to low water activity and elevated temperature, deregulation of stringent metabolic controls, insensitivity to concentrations of the substrates and products. The genes responsible for counteracting the effect of increased osmotic condition, temperature, carbon and nitrogen catabolite repression are to be identified in the SSF organisms. They may be suitable manipulated in the producers or/and may be transferred to the producer organism.

Solid state fermentation (SSF) process differs from Submerged fermentation (SmF) in several ways. The luxurious growth of microorganisms and formation of products occur on the solid support or substrate in SSF process while a low yield of biomass concentration and product formation occur at the liquid phase in SmF process. The low water content is a critical condition in SSF process and this condition favors the growth of several filamentous fungi. The bacteria generally require high water activity for growth whereas the yeast and fungi can grow in a low water activity conditions. Thus, the filamentous fungi are best suited for SSF because of their inherent growth characteristics.

The SSF process have not been explored extensively because of the difficulty in controlling parameters such as pH, temperature, aeration and microbial growth as compared to the submerged culture fermentation. For example, the aeration condition is important in SSF to maintain the cultures in aerobic state, to ventilate CO_2 produced and to remove the heat produced in fermentation process (1). Aeration is also important for the onset of sporulation of the fungi and inoculum production (2).

Most of the studies on the SSF process so far are centered on the process optimization and scale up of the process. Several investigators have demonstrated that the fermentation performance and yield of product concentration is higher in SSF than in SmF. However, some investigations revealed that certain strains performing well in SmF failed to show better performance in SSF. Generally the strains used in SmF process are conveniently used in SSF process. Therefore, it should be realized that strains specialized for SSF process have to be developed. Improvement of product yields in SSF process could be achieved if standardized and improved inoculum adapted to SSF conditions is used. There are limited studies on the strain development for SSF process due to heterogeneity and complex nature of the substrates used in fermentation. However, certain strains are isolated for SSF process through random selection procedure.

Some of the example for the improved strains for SSF process reported are: a) strains developed for *tempeh* fermentation (5,6), an *Aspergillus* strain developed for animal feed production from wheat, sorghum and cassava fermentation (7). Generally mutation and selection method was used for the selection of strains of *Trichoderma reesei* for cellulase production (8), a strain of *Aspergillus niger* for pectinase production (9) and strain of *A. oryzae* for protease production (10). More relevant strain with improved characteristics for SSF can

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be developed if the genetics of the organism used is known and the nature of the substrate and fermentation conditions are defined. Approaches for the selection of strains for SSF process are presented in this paper.

MICROORGANISMS USED IN SSF PROCESS

The microorganisms used in SSF process for production of variety of products are listed by Nigam and Singh (4). Mostly filamentous fungi and certain yeast strains are well adapted for the SSF process because of their inherent ability of preferential growth at reduced water activity levels. However certain bacterial strain, *Bacillus subtilis* has also been used in SSF process (4). Further, the inoculum level used is higher in SSF process resulting higher cell mass production in SSF process than in SmF (11). Therefore, the metabolites and macromolecules which are growth and cell mass associated could be efficiently produced in SSF process.

The strains used in SSF studies were the same used in SmF process and no special attention have been made except in few instances as mentioned above. If suitable strains are not developed and used in SSF process, the product yield may not be at its maximum despite its yield may be relatively higher in SSF than in SmF. Therefore, there is a need for development of suitable strains well adapted for SSF process. Some of the mutants of filamentous fungi desirable for SSF process are listed in Table 1.

Table 1. Mutants desirable for SSF process

- Osmotolerant
- Insensitive to catabolite repression
- Tolerant to elevated temperature
- Temperature sensitive asporogenous
- Tolerant to low water activity
- Insensitive to water activity depressors
- Efficient sporulation at low temperature
- Insensitive to product concentrations
- Insensitive to feed back repression or inhibition

To perform effective screening of suitable strains for SSF, it is essential to understand the physico-chemical characteristics of the substrates and support used, fermentation conditions, physiology and biochemical characteristics of the strains used and also the biochemical pathways and genetic regulation of the production of the metabolite of interest (12). Once these details are available, then it is plausible to design suitable strategy for improvement of the organisms for desirable characteristics for SSF process and also develop a suitable screening method for scoring potential strains.

The enzymes such as pectinase, protease, amylase, cellulase and lipase etc. are produced at the high level in the SSF process (13,14,15,16). The level of these enzymes considerably decreased after the onset of sporulation. The reduction of these enzyme level could be due to the synthesis or release of non specific proteases during sporulation or from the cell lysis and their effect on the target enzymes. Therefore, the level and stability of these enzymes can be improved if the sporulation is prevented during the SSF process. In such case, the fermentation may be stopped before the sporulation begins or sporulation may be controlled by selective conditions. Alternatively, mutants that could be isolated and used in SSF process are asporogeonous at higher temperature (temperature sensitive mutants) and that are high productive strains. If such mutants are employed, the inoculum of spores could be developed at lower temperature and the fermentation may be carried out at elevated temperature. Such strains will result in the production of stable enzyme preparations from SSF process. Moreover, the use of asporogenous strains in SSF would minimize the problem of the allergic effect of spores during handling fermented mash containing spores and their health hazards. Further, the stability of the final product is also improved if the asporogenous mutant is used in the SSF process.

GENETIC SYSTEMS IN THE SSF ORGANISMS

The organisms used in SSF are those of yeast and fungi with exception of certain bacterial strains (4) which show tolerance of growth at low water activity level. The genetics of yeast *S. cerevisiae* is well studied and of the other yeasts are being developed. Among the filamentous fungi only the genetic system of *A. nidulans*, though it is not industrially important strain, is studied in detail. But the genetics of other fungal system have to be developed. The parasexual cycle first demonstrated in *A. nidulans* by Pontecorvo et al (17) become a valuable tool in genetic studies of other filamentous fungi particularly fungi imperfecti. Several *Aspergillus sp* are known to exhibit parasexual cycle.

VARIABLES IN SSF PROCESS

The physico-chemical variables of SSF process that are to be considered during genetic improvement of the strain are a) water activity b) nature of substrates and

their concentrations c) nature of products and their effect on the producer d) osmotic pressure e) temperature raise f) viscosity g) oxygen requirement of the organism.

WATER ACTIVITY

The SSF process is always performed with low water activity and therefore the water activity plays an important role in regulating the fermentation process. The metabolism of the microorganisms as well as the production of the primary or secondary metabolites can be strongly influenced by the water activity condition (18). Therefore, it is important to know the optimal water activity for individual physiological phenomena such as microbial growth, sporulation and production of primary and secondary metabolites. By controlling the water activity condition one can regulate the fermentation and the production of desirable products.

The fungi are known to endogeneously accumulate compatible solutes which function as osmoregulators (19,20,21). These solutes are water activity reducing polyols such as erythrol, glycerol and trehalose. These compounds counter act the water activity decrease caused by external water activity depressors. They are also known to impart protective function to enzymes and increase the half life of the enzymes produced in SSF process (22). Alternatively, the proline, is an amino acid usually accumulated when the cells are exposed to water stress condition (23). However, the signals for the synthesis of the polyols and other osmoregulators are not well understood. In bacteria, a mutation that affected the synthesis of trehalose resulted in increasing the sensitivity of the strain to osmotic condition (24). Therefore, increasing the synthesis of these osmoprotectors in fungi through modern genetic manipulation methods is a reliable method for the improvement of the strain for SSF process. Studies on this aspect on filamentous fungi may significantly contribute to the development of strains for SSF process. Several studies on SSF were carried out using water activity depressing compounds to reduce the water activity in the fermentation medium. Therefore, suitable water activity depressing compounds are added in the selection media to isolate strains for SSF process (25).

SUBSTRATE CONCENTRATION

The concentration of substrate used in SSF process is considerably higher as it is not diluted with excess amount of water. It may result in affecting growth and production of metabolites through some of the control mechanisms. Such mechanism are substrate inhibition (carbon or nitrogen catabolite repression), product inhibition (feedback repression or feedback inhibition) and osmotic pressure. These effect could reduce the product yield in SSF if the organism used are sensitive to these effects. Attempts are being made to improve the organisms for SSF considering these physiological effect on the organisms.

DEREGULATED MUTANTS FOR CATABOLITE REPRESSION

Several enzymes that are capable of breaking down complex polymeric substrates are widely produced by filamentous fungi in SSF process. Biosynthesis of these enzymes are often controlled by catabolite repression (26,27). A widely used approach to develop strains that are deregulated of this control is through the selection of mutants that are resistant to glucose analogues such as sorbose, glucosamine or deoxy glucose. For example the glucose analogue, 2-deoxy glucose inhibits the growth of the fungi through a) interfering with the sugar uptake b) inhibiting the hexokinase activity or c) inhibition of one of the glycolytic enzymes such as phosphoglucose isomerase or glucose 6 phosphate dehydrogenase (28). In addition to these, the analogues may also mimic the regulatory role of glucose in the enzyme biosynthesis. Therefore, the resistance towards the glucose analogues can arise in the strains due to: a) the altered permeability of membrane towards the analogue b) altered phosphorylation of sugars or c) enhanced phosphatase activity that cleave the phosphorylated analogues. At the same time some of the mutant resistant to these analogue may also exhibit relaxed control mechanism for the biosynthesis of glucose repressible enzymes. Some of the 2- deoxy glucose resistant mutants isolated that over produce catabolite repressible enzymes are listed in Table 2.

Mutants of filamentous fungi altered in carbon metabolism were isolated (33). Genetic analysis of such mutants of *A. nidulans* revealed that there are genes (*creA*, *creB* and *creC*) responsible for carbon catabolite repression (34,35). The *creA* mutants overproduce certain enzyme that are carbon catabolite repressible in wild type strain. The *creB* and *creC* mutants show increased level of sensitivity to fluoroacetate, fluoroacetamide and allyl alcohol in the presence of glucose (36). The CreA is a regulatory protein negatively acting and has DNA binding domain. It is able

to bind at several promoters of *A. nidulans*, *T. reesei* and *N. crassa* (37).

Table 2. Deoxy glucose resistant mutants isolated for overproduction of enzymes

Strain	Enzyme overproduced	Reference
T. reseei	Cellulase	8
S. cerevisiae	Invertase	29
A. niger	Pectinase	9
	(citric acid)	30
A. terreus	Glucoamylase	31
Rhizopus sp	Glucoamylase	32
A. oryzae	Protease	10

In *T. reesei*, a deletion or mutation of upstream CreA binding site completely abolished the glucose repression of *cbh1* gene specifically without affecting the regulation of other catabolite repressed genes (38).

Similarly the *areA* gene is involved in the control of pathways of catabolism of nitrogen sources and mutation in this locus can lead to the inability of the strain to grow on wide range of nitrogen sources such as acetamide, proline and glutamine (39) (Table 3). Therefore the *areA* gene product, AreA is transcriptional regulator in *A. nidulans*. Similar such gene product, Nir2 was also identified in *N. crassa*. Both the proteins have the ability to bind DNA at specific upstream region of the gene (40).

Table. 3. Growth characteristics of catabolite repression insensitive mutants

	Mutant strains			
Growth on	1	2	3	4
Acetamide	+	+	+	+
Glucose+acetamide	-	+	+	+
Proline	+	+	-	+
Glucose+proline	-	+	-	-
Glutamate	+	+	+	+
Glucose+Glutamate	-	-	-	-

1- *areA*; 2- *areA*, *creA*; 3- *areA*, *creB*; 4- *areA*, *creC*

The regulatory protein AreA interact with its C-terminal with other transcription factor such as NirA to regulate the gene expression. The presence of overlapping region or adjacent sites for AreA, CreA in *amdS* and *prn*B promoters of *A. nidulans* control the transcription of genes (35). Therefore when strains insensitive to catabolite repression are developed or catabolite repressible genes are cloned in filamentous fungi, these upstream regulatory region may be eliminated. Such strains will be useful in SSF process with high initial substrate concentrations.

POST TRANSLATIONAL MODIFICATION

There are several reports indicating that the enzymes produced in SSF are relatively stable than that are produced in SmF. This tempt to suggest that if any post translational modification of the enzyme contribute to the stability of enzyme produced in SSF process. The filamentous fungi being eukaryotic organism, are known to produce glycosylated proteins. Glycosylation can influence solubility, resistance to proteolytic attack, *in vivo* activity and the half life of the proteins (41,42). For a given protein, the glycosylation pattern may vary depending on the cell type and culture condition. O-glycosylation and N-linked glycosylation are the two ways of glycosylation of protein. O-glycosylation is mediated in secretory pathway and occur during early translocation of the protein into the Endoplasmic reticulum (ER). The N-linked glycosylation appears to occur at the Golgi complex. However not much information is available on the glycosylation machinery of the filamentous fungi.

MANIPULATION OF FUNGAL GENES

Genetic engineering provides new opportunities for the improvement of strains for overproduction of desirable metabolites. Plasmids and cloning vehicles have been constructed for cloning of chromosomal DNA fragment of certain fungi and transfer to other fungal strains and express efficiently. Efficient methods for cloning of genes by have been developed for *A. nidulans* and *N. crassa* (43,44) Selectable genetic markers such as *argB*, *prn*, *BenR*, *pabaA* and *nialD* are effectively used in gene cloning studies of fungi. These successful methodology developed for *A. nidulans* and *N. crassa* can be followed for other industrially important filamentous fungi (45). Further novel methods for detection of genes that are expressed under certain conditions (by differential display) amplification (by PCR) and cloning of them in desirable organism is for SSF process. Therefore it is possible now to produce new metabolites by introducing new genes encoding enzymes in the producing organisms. The area of genetic improvement of the

organism for SSF process is benign and of recent interest.

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Development of Scale-up Criteria for Bioreactors of Solid State Fermentation

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In this work, biological and engineering aspects concerning scale-up of bioreactor of solid state fermentation are discussed. Fundamentals of mathematical modeling are presented as an alternative for scaling-up of bioreactors. Scientific criteria based on mass and heat balances, similarity principles and dimensionless numbers are proposed.

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