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Recent Advances and Technologies in Chitinase Production Under Solid-State Fermentation

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Our target is to evaluate recent literature on chitinase production from different sources via solid-state fermentation and to analyze several strategies to improve chitinase production via solid-state fermentation. Plant pathogen biocontrol, sequential transformation of chitin into bioactive molecules such as chito-oligosaccharides and N-acetylglucosamine, protoplast synthesis from filamentous fungi, and single-cell protein production are some of the applications for chitinase. Despite their enormous biological importance, chitinases have received little commercial importance due to the smaller percentage of microbes with high efficiencies, the enzymes' decreased activity and consistency, and the cost of production. Solid-state fermentation (SSF) is less expensive, requires fewer vessels, uses less water, requires fewer wastewater treatments, produces a greater product yield, has a lower risk of bacterial contamination, and requires less energy expenditure. Despite its higher productivity and lower cost, the SSF technique is now mostly limited to lab scales. Furthermore, the crude SSF products can be used as an enzyme source for biotransformation. There are many findings on different microorganisms that produce chitinase by SSF. So it is very critical to isolate new organisms for such production. So we assessed the traditional approach to medium optimization, which focuses on changing one factor at a time while leaving the others constant, and statistical optimization techniques such as response surface methodology (RSM), artificial neural networks (ANNs), and genetic algorithms (GA).

Keywords: Chitin; Chitinase; Optimization; Substrate; Response Surface Methodology; Solid state fermentation.

Chitinase

Chitin is a structural element found in mollusks, crustaceans, algae and fungi.[1] Chitin is the 2nd most prevalent biopolymer in nature, next to cellulose [2]. Chitinase (E.C. 3.2.1.14, Poly 1, 4-N-acetyl D-glucosaminide glucanohydrolase) is a glycosidase enzyme that specifically degrades chitin. The cleaving site for chitinase is the bond between CI and C4 of two consecutive N-acetyl glucosamine monomers[2]. Chitinases occur in microorganisms, plants and animals. Chitinolytic microorganisms are abundant in nature and are ideal producers of chitinase because of their low cost of production and the accessibility of raw materials for their cultivation. Bacteria like *Bacillus* sp. BG-11, *Bacillus laterosporous* MML2270 and *Paenibacillus illinoisensis* [3-5], while *Myrothecium verrucaria* [6] and *Trichoderma*

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sp. [7] were found as main sources for chitinase in fungi. Many prospective applications of chitinase include biocontrol entities for fungal pathogens [5, 6], separation of fungal protoplasts [8, 9], mosquito control by degrading insect cuticle, which contains chitin as an essential component [10], production of SCP [11], and synthesis of oligosaccharides and N-acetyl glucosamine [12]. Because of their extensive applications in biocontrol, waste management, medicine, and biotechnology, chitinase enzymes are classified as enzymes of rising interest. Although chitinase was isolated and characterized from different sources, it is still necessary to search for new sources of chitinase with higher economic values and improved properties to broaden their utility. The culture conditions have a significant impact on the growth of microorganisms, the by-products of their metabolism, and the cost of production. The classic one-variable-at-a-time strategy was initially employed to optimise the process parameters due to its simplicity and ease. The loss of the interactive effects between different parameters, the time and number of trials needed, and other disadvantages of this method could increase production costs. In fermentation technology, statistical models have been employed to overcome these limitations.

Isolation of chitinolytic microorganisms

Chitinolytic organisms are usually isolated from coastal soil enriched with crab shells [13], agricultural fields [14], degraded stalk of mushroom [15], intestine of the *Patagonian sea lion* [16], kimchi juice [17], lily plant [18], marine environment [19] and gut of red palm weevil [20]. Substrate Phydrolysis, pathogen inhibition, biochemical assays of enzymes, and particular PCR techniques to show the existence of the concerned genes can be used to isolate chitinolytic microbes.

Chitinase production by solid state fermentation (SSF)

Due to high production costs where culture media can account for up to 40% of overall production costs, commercial chitinase is still used on a limited scale. In the past few years, there has been a great deal of interest in enhancing chitinase production by employing fermentation techniques. Microbial chitinase was produced using submerged fermentation (SmF) [21-24] and solid substrate fermentation (SSF). Because it uses readily available agro-industrial residues such as wheat bran, rice husk, and sugar cane bagasse, SSF is the best option for cost-effective enzyme synthesis. The availability and cost of substrate are the most important criteria in choosing a substrate for enzyme synthesis in SSF. The solid substrate provides crucial nutrients to encourage microbial growth while also providing support for the microorganisms [25]. SSF is less expensive and more closely resembles the natural environment of most microorganisms (mainly fungi and mold). It also takes less energy for sterilisation (due to decreased water activity). It is less vulnerable to bacterial contamination and substrate inhibition, allowing for higher end product concentration levels and a multitude of environmental benefits, such as creating less effluent[26-28]. Although challenges with substrate sterilization, temperature and pH control, culture purity maintenance, and process time are encountered, SSF benefits from the relative ease of process operations[29]. Under SSF, the microorganisms that generate chitinase are listed in Table 1.

Optimization of process parameters

Traditional approach

Effect of medium component on chitinase production

Extracellular chitinase production is determined by a multitude of physical parameters such as pH, aeration, temperature, and components of media such as carbon sources, nitrogen sources, and other micronutrients. Chitinous substances such as prawn waste, commercial chitin, or colloidal chitin were revealed as the principal carbon, nitrogen, and energy sources in the chitinase production process. As per Felse and Panda, shrimp shell waste contains 21.4% chitin, 40% calcium carbonate, 27.9% protein, 20% moisture and 6% ash[38]. It also contains a lot of feeding enhancers (peptides, beatine, and polynucleotide compounds) that may help to improve nutritional benefit. However, Yesim (2000) made the argument that shellfish waste materials may be insufficient in many essential components, such as amino acids, which may have a substantial impact on microbial growth[39]. *Enterobacter* sp. NRG4 [40], *Fusarium oxysporum* [41], used chitin and wheat bran as substrate to produce chitinase in solid-state fermentation, while *K. gibsonii* Mb126 [31] and *B. thuringiensis* R 176 [34] used shrimp shell waste. Chitin (1–1.5%) and ball

Table 1. Microbial chitinase production under SSF

Microorganisms	Substrateand supplements	Incubation Time	Moisture level	Temperature	pH	Maximal activities	References
Trichoderma harzianum TUBF 781	wheat bran yeast extract (2%,), colloidal chitin (1%)	96 h	65.7%	30°C		3.18 U/gds	30
Kurthia gibsonii Mb126	Prawn shell powder (o.6mm size)	60h	75%	40°C	8	426U/gds	3 31
Oerskovia xanthineolytica NCIM 2839	wheat bran & 10% colloidal chitin	96h	60%	45°C	-	148 Ug-1	32
Streptomyces champavatii AZ-1	Wheat bran, sugar cane bagasse, colloidal chitin(1%)	168h	-	37°C	-	12.5 U/gds	33
Bacillus thuringiensis R 176	shrimp shell, rice straw & chitin 0.5 % ammonium sulfate	14 days		37 °C	7	3.86 U/gds	34
Trichoderma koningiopsis UFSMQ40	Wheat bran, colloidal chitin (15%), 100% of corn steep liquor	72h	55%	30 °C		10.76Ugds"1	35
Beauveria felina RD 101	wheat bran, 0.1% chitin	6 days	100%	28°C	5	6.34 U g^{-1}	36
Chromobacterium violaceum	Prawn shell waste, 1.5 % starch,1 % urea	60h	60%	34°C	7	531.66U/gds	37

milled chitin [33,34] are activators of the chitinase enzyme, which is produced by microorganisms. Yeast extract has been reported as the ideal organic N_2 source for *T. harzianum* TUBF 781 [30], while *B. thuringiensis* R 176 used ammonium sulphate [34] and *T. koningiopsis* UFSMQ40 used corn steep liquor in the chitinase production process[35].

Incubation period

The time period of incubation has a significant influence on chitinase production, as it increases to a certain maximum level after a time period and then decreases by further incubation. Nutrient depletion in the fermentation medium could be the biggest factor in the drop in production. It could also be the result of the medium's creating inhibitory products, which lead to the down regulation of the enzymatic secretory framework or the enzyme's own collapse. *Penicillium aculeatum*, produced maximum chitinase at 72 h [42]. *T. harzianum* TUBF 781 [30], *O. xanthineolytica* NCIM 2839 [32] and *T. koningiopsis* UFSMQ40 [35] produced highest chitinase after 96 h of fermentation, and *S. champavatii* AZ-1 required 168 hours of incubation to produce maximal chitinase[33]. Chitinase production reached maximum on the 6th day in *B. felina* RD 101 [36], whereas it peaked on the 14th day in *B. thuringiensis* R 176 [34].

Inoculum size

The period and amount of chitinase production are also influenced by the nature of the inoculum that has been used. The concentration of the inoculum determines the production of total biomass on the solid medium. A higher spore count in the inoculum would result in faster growth and biomass conversion. Moreover, due to nutrient competition, the organism's metabolic activity decreases after a certain point. There is a compromise between proliferating biomass and nutrient availability that supports production of enzymes with the optimal inoculum size. The maximal chitinase production (1.26 U/gds) was noted when an inoculum size of 2 ml (4×10^7 spores) was used [30]. The optimum inoculum size for chitinase production by *K. gibsonii* Mb126 was 3×10^8 CFU/mL [31].

pH and Temperature

The pH and temperature of incubation are important criteria in chitinase production. In the case of SSF, the commonly used substrates being agro-residues with very good buffering capacity, medium pH adjustment is not required [25]. The optimal pH was 8 for *K. gibsonii* Mb126 [31], 7.0 for *B. thuringiensis* R 176 [34] and *C. violaceum* [37], and pH 5 for *B. felina* RD 101[36]. Chitinase production generally highest at temperatures ranging from 25 to 40 °C (Table 1). An exception to this was *O. xanthineolytica* NCIM 2839, which had a 45 °C optimal temperature for chitinase synthesis[32].

Fig. 1. Optimization of Chitinase production under solid state fermentation

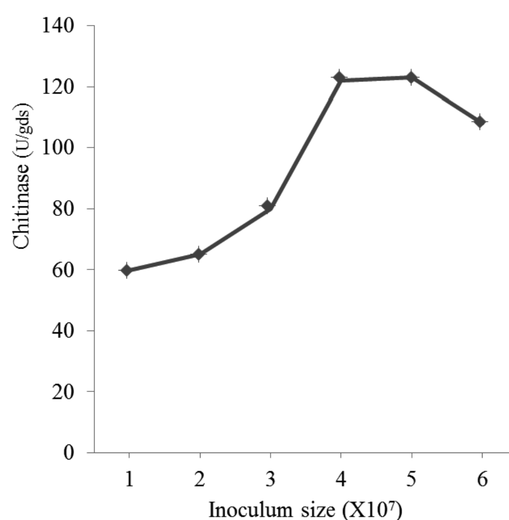

Fig. 2. Effect of inoculums size on chitinase production by *K. gibsonii* Mb126 [31]

Table 2. Microorganisms used to synthesize chitinase under SSF using statistical method.

Experimental design	organism	Substrate	Optimized conditions	Predicted response for chitinase synthesis	Reference
PBD &CCRD	*T. koningiopsis* UFSMQ40	wheat bran 15%,colloidal chitin powder,flakes.100% of corn steep liquor	Moisture-55%2discs of inoculum Temperature -30 °C Incubation time- 72 h	10.76 U gds”1	35
CCD	*Enterobacter* sp. NRG4	Wheat bran-to-flake chitin-	Moisture level-80% Inoculum size-2.6 mL Incubation time-168 h.	1475 U · g”1	40
PBD &CCRD	*Serratia marcescens*	Rice bran(2.5 g/l)	pH -5 Temperature- 35° C, Inoculum size- 1.8%	61.12 U/ml	47
PBD and BBD	*A. terreus*	fish-scales	Medium contains- $FeSO_4$. $7H_2O$, glucose, $MnSO_4$. $2H_2O$ Incubation time- 90 h	4.309 u/min,	48
PBD	*Citrobacter* strain	Wheat bran & powdered fish scales	Moisture content -61% , Temperature -34 °C	94.3 U/gds	50
CCD	*Trichoderma virens*	Shrimp waste	Moisture level- 54% Incubation time-6 days Temperature-27.83°C	0.48738 U/g of	52
CCRD	*Metarhizium anisopliae*	Sugarcane bagasse.	Temperature-33°C g Moisture content- 60%. Chitin mass- 0.75	-	53
CCRD	*M. anisopliae* strains IBCB 360	Silkworm chrysalis	Age of inoculum-8 to 12-days old cultures Moisture content -45% to 62% Temperature -26°C	7.14 U/g of substrate	54
PBD	*Metarhizium anisopliae* IBCB 348	Sugarcane bagasse	Moisture content-40 % Inoculum-10%(v/w)Temperature - 28 °C Incubation time- 8 days	12.07 ± 0.50 U.g”1	55
PBD, RSM and DANNs	*Alternaria* sp	Wheat bran and sugarcane bagasse	Temperature - 25°C Incubation time- 16 days	28.931U/g	56

Particle size

An optimal particle size must be established to balance the adsorbent's surface area of contact and inter-particle distance for effective growth, mass transfer, and gaseous exchange. Suresh and Chandrasekaran optimised the particle size to 425-600 nm [43], and Paul et al., 2018 [31] used prawn shell powder with a 0.6 mm size for the best chitinase output.

Moisture content

Moisture optimization would be employed to control SSF's chitinase production as well as regulate and adjust the microorganism's metabolic processes. Increased moisture results in lower porosity, changes in the particle structure of the substrate, and a reduction in oxygen transport.

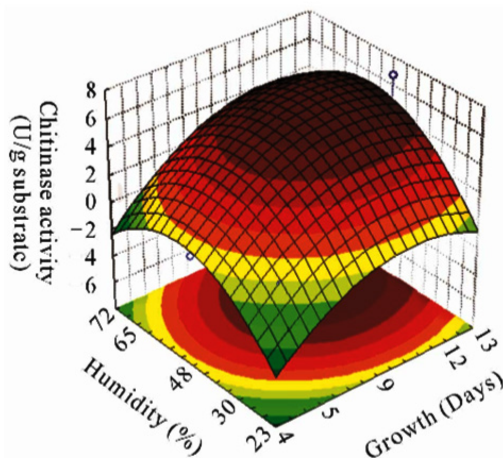

Fig. 3. Response surface plot of the factorial design for the influence of the independent variable time of growth and humidity on chitinase production by *M. anisopliae* IBCB 167 under SSF using silkworm chrysalis as substrate [54]

Lowering the moisture level results in increased water tension, reduced swelling, and decreased nutrient solubility of the solid substrate [44]. The majority of chitinase production necessitates a moisture level of 60-80 percent. Thus, moisture content of 60% was found to be optimal for chitinase synthesis by the organism *O. xanthineolytica* NCIM 2839 in SSF [32], 75% for *K. gibsonii* Mb126 [31], and 80% for *Enterobacter* sp. NRG4 [40].

Statistical optimization

Response Surface Methodology

The traditional approach to medium optimization means altering one factor at a time while retaining others constant. However, because it does not consider the cumulative effect of all factors, it is time-consuming and costly, and it frequently fails to ensure the sampling of optimal conditions[45]. Yet another solution to tackle this issue is to use a successive experimental conceptual framework. Box and Wilson first established response surface methodology (RSM) in 1951, and it has since been successfully used in the domains of biological sciences. The RSM method creates a computational formula that precisely captures the whole process by evaluating the influence of numerous variables or factors, either separately or in combination, on it. Full factorial designs, for example, provide more comprehensive information, but they necessitate a huge number of tests (Lk, where k is the factor number and L is the level number for each variable), making them unsuitable for studying a lot of variables. As a two-level fractional factorial screening design, the Plackett-Burman design (PBD) is especially useful for determining the significant effects of k variables in only k + 1 tests by a linear model. However, the interplay of parameters is not taken into account

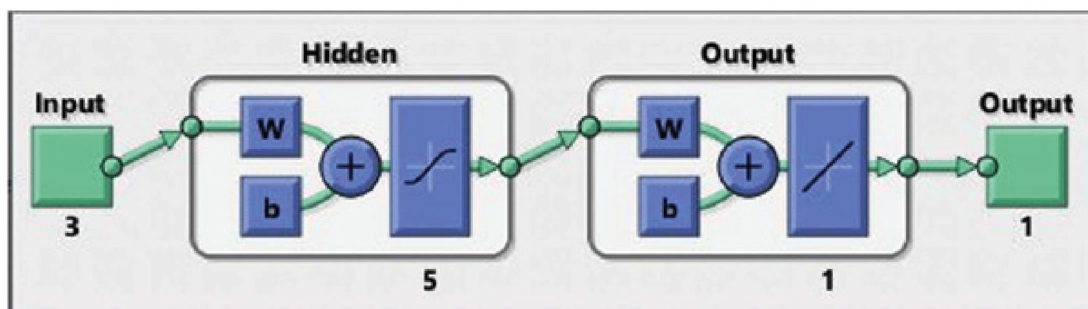

Fig. 4. Artificial Neural Network with input, output and hidden layers applied for optimization of chitinase production[65]

in this strategy. PBD variables could be optimised using statistical and mathematical optimization methods such as Response Surface Methodology (RSM)[46, 47]. This empirical method allows us to assess the relationship among variables and predict the response through an efficient design of experiments. In order to offer the most information on the effects of the experimental variables and the overall experimental error in the fewest runs possible, a central composite design (CCD) is highly useful. A small number of investigations are utilised to determine the relevance of essential elements using the well-known and regularly used statistical technique known as CCD. This design has certain star or axial points that are distributed radially and have an identical spacing in order to fit the quadratic polynomials.

Optimisation of bioprocesses using statistical approaches was also used to maximise chitinase production. Ghanem *et al*. used PBD and Box Behnken Design (BBD) to increase chitinase production of *Aspergillus terreus* by 1.81 fold [48]. By using CCD [49], *Basidiobolu*s *ranarum* produced 7.71 times more chitinase. A comparable study indicated a 1.1-fold increment in enzymatic activity for chitinase synthesis from the shell of *Parapeneopsis hardwickii* (spear shrimp) via solid-state fermentation[50]. The interplay of factors in the microbial degradation of shrimp bio-waste and simultaneous chitinase and GlcNAc synthesis by *Vibrio* sp. CFR173 M were explored by statistical methods. Statistical optimization increased chitinase yield by twofold and GlcNAc output by nine-fold[51]. Table 2 lists the microorganisms used to produce chitinase under SSF using statistical methods.

Artificial neural networks (ANNs)

ANNs can indeed be utilised for parameter optimization operations in various academic fields. ANN has numerous advantages, including the capacity to use noisy data and fine-tune incomplete and highly non-linear behaviours[57-59]. Aside from the numerous advantages of ANN, the emergence of deep learning will result in more reliable information[60]. Artificial neural networks (ANNs) have been successfully used in system design, modelling, prediction, optimization, and control because of their ability to acquire, filter noisy impulses, and extrapolate knowledge during the training process. The application of ANN increases the yield of several enzymes including galactosidase [61], L-asparaginase [62], protease [63], and-amylase [64]. Ismail *et al*., 2019 compared deep artificial neural networks (DANN) and RSM to find the optimised parameters for exochitinase synthesis under SSF[56]. They found that using deep artificial neural networks, measurable enzyme production increased by roughly 8.5 folds, from 3.4 to 28.931U/gds, with a coefficient of determination (R2) value of 0.996 compared to 0.76 utilising RSM. DANNs have been shown to be more reliable than response surface methodologies in predicting the activity of enzymes.

The ANN can be combined with a genetic algorithm (GA) to optimise the media for enhanced fermentation. The genetic algorithm (GA), which mimics the process of mutation, is based on the "survival of the fittest" theory. In order to forecast the conditions under which the targeted variable (response) would reach its maximum benefit, it is employed to tackle numerous optimization challenges.

Suryawanshi *et al*. compared three different statistical designs for chitinase synthesis by *Thermomyces lanuginosus* MTCC 9331; RSM, ANN, and genetic algorithm (GA) [65]. The expected values of ANN produced more chitinase activity, 102.24 U/L, than the RSM predicted values, which produced 88.38 U/L. The expected production of chitinase was closer to the observational reality at these concentrations. As per the validation tests, the maximum level of chitinase by ANN prediction equates to experimental analysis. In terms of optimization results, a comparative analysis of three distinct statistical designs indicated that ANN outshines the GA and RSM studies.

CONCLUSION

In-depth study is being carried out in order to tap into hitherto untapped SSF sources. In SSF, bioreactor modelling and careful manipulation of physical-chemical parameters can result in a higher chitinase production. The majority of the research comprise on strain separation, operating parameter optimization, and simple reactor design. The main issue is that a simple, efficient, and automated SSF fermenter is still required. Despite enormous biotechnological potential, chitinase have received little industrial attention due to the limited number

of microorganisms with high efficiency, poor activity and stability and high manufacturing costs of chitinase. Because of the widespread use of chitinase in a variety of industries, additional research is needed.

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Conflict of Interest

There is no conflict of interest.

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