Chapter 2

LITERATURE REVIEW

2.1 Overview

As fungal infections are becoming more prevalent in the medical field as well as food or agricultural sector too (post harvest loss), novel and more efficient antifungal agents are needed at once. Within the scope of developing new production strategies for antifungal compounds, the management of fungal infections and target essential fungal cell wall components are highly preferable. This review summarized the current knowledge of the various sources and also addressed some new important findings of related proteins, classification, structure, mode of action/expression and highlights the potential application of antifungal protein in the combat against food spoilage contaminations and deterioration.

However, few antimicrobial proteins have been reported, namely; AFP from *Aspergillus giganteus* strain lfGB0203 **[78]**; ANAFP from *Aspergillus niger*, ANAFP1 from *Aspergillus niger* strain KCTC 2025; ANAFP2 from *Aspergillus niger* strain CBS 513.88 **[79];** PAF from *Penicillium chrysogenum* strain Q176 **[80]**; NAF from *Penicillium nalgiovense* **[81];** ACLA from *Aspergillus clavatus* **[82]**; AFP_{NN5353} from *A. giganteus* strain A3274 **[83];** PgAFP from *P. chrysogenum* strain RP42C **[84];** strain bubble protein (BP) from *Penicillium brevicompactum* Dierckx **[85];** AcAFP and AcAMP from *Aspergillus clavatus* **[86];** NFAP from *Neosartorya fisheri* **[87],** anticandidal protein from *Aspergillus giganteus* MTCC 8408 **[88];** F2 from *Bacillus licheniformis* **[89];** baciamin from *Bacillus amyloliquefaciens* **[90];** BL21 from *Escherichia Coli* **[91]**; bacisubin from *Bacillus subtilis* B-916 **[92];** LA1-SCS *from Lactobacillus acidophilus* **[93]***;* acrocin (*Enterobacter aerogenes*), pesticin (*Yersinia pestis*),

marcescin (*Serratia marcescens*), piocin (*P. aeruginosa*); lactococcin A (*Lactobacillus lactis*), thermophilin A (*Streptococcus thermophilicus*), and bovicin (*Streptococcus bovis*) **[94]**.

Around 300 fungal species are reported to be origins of major diseases, whereby, the filamentous fungus *Aspergillus fumigatus* and the dimorphic yeast *Cryptococcus neoformans* and *Candida albicans* are the three predominant causative agents of human diseases **[95, 96].** In addition, indoor moulds have been recognized as important risk factors for allergic diseases and for the damage of building materials **[97, 98].** Another important aspect is that many filamentous fungi such as *Fusarium* species and *Magnaporthe grisea* are destructive pathogens of plants and are thus responsible for enormous crop losses worldwide **[99, 100].**

Fungal infections and contaminations have, therefore, led to an increasing demand for antifungal drugs. Still, antifungal treatment is nowadays limited to only a small number of antifungal drugs such as azoles, echinocandins and polyenes **[95, 101, 102].** The application of these antifungals is, however, often restricted because of several reasons. Antifungal compounds have often low efficacy rates, as their activity is rather fungistatic than fungicidal. They show frequently severe side effects and can even be toxic to man and, moreover, frequently interact unfavorably with other medications **[95, 101].**

In addition, a steady increase in fungal pathogens that do no longer respond to antifungal treatment is observed presumably because of resistance mechanisms evolved in consequence of long-term antifungal treatment **[103-106].** Ideally, they should meet several criteria to overcome the limitations described above, such as (1) specific mode of antifungal action; i.e., structures or functions unique to fungi have to be targeted to prevent any side effects on man and environment, (2) high efficacy and (3) inexpensive and sustainable way of production.

The development of new antifungal drugs applied in the agricultural and/or medical field is therefore mandatory. Interestingly, filamentous fungi themselves can be considered as valuable sources for the production of antifungal compounds. One prominent example is the commercial use of mycoparasitic *Trichoderma* strains that antagonise plant pathogens.

Here, the fungus itself is applied as biocontrol agent in the combat against soilborne fungal plant pathogens. The mechanisms behind biocontrol are complex and involve the synthesis and secretion of synergistically acting agents such as cell-wall degrading enzymes (chitinases, glucanases and proteases) and antibiotics **[107]**.

Hence, filamentous fungi do have significant capacities to secrete compounds with antifungal activities **[108].** In general, it is thought that these compounds are part of their defense system to protect themselves against other fungi that might act as nutrient competitors in the same environment. Among the antifungal compounds produced by filamentous fungi, small-sized antifungal peptides with suppressive effects on fungal growth have attracted considerable interest. One prominent peptide bearing great potential for future antifungal strategies is the antifungal protein AFP secreted by *Aspergillus giganteus*.

2.2 Classification and sources

Antifungal proteins have been categorized according to their enzymatic properties (glucanases, chitinases), their structure (e.g. cysteine rich) or their similarity to a known 'type' of protein. Antifungal proteins from plants, mammals, insects/amphibians and bacteria/fungi have been organized based on serological and sequence analysis namely; cysteine-rich and small proteins (5–27 kDa), glucanases, chitinases, chitin-binding proteins, thaumatin-like proteins, membrane-acting antifungal proteins **[109].**

2.2.1 Mammalian peptides

Defensins: α-Defensins ("classic defensins") and β-defensins **(Table 2.1)**, which are present in many organisms, are predominantly β-sheet structures stabilized by three disulfide bonds that distinguish them from other antimicrobial peptides that form amphipathic helices **[110].** Rabbit, guinea pig, rat, and human neutrophils contained defensins within azurophilic granules **[111-113]**. Rabbit granulocytes contained six α-defensins structurally homologous to human defensins **[114].** Three such peptides, NP-1, NP-2, and NP-3a, were highly effective against *Candida albicans* **[113].** Although NP-5 lacked candidacidal properties alone, at submicromolar concentrations it potentiates the anti-Candida effects of other rabbit defensins **[114].**

This effect of NP-5, however, was not observed with NP-3b or NP-4. NP-1 had MICs ranging from 3.75 to 15 mg/ml for encapsulated strains of *Cryptococcus neoformans*, while the MICs for acapsular strains were much lower (0.93 mg/ml) (3). NP-1 and other rabbit defensins were also lethal for *Coccidiodes immitis*, as well as hyphae and germinating conidia, but not dormant conidia, of *Rhizopus oryzae* and *Aspergillus fumigatus* **[115, 116].**

As measured by the yellow tetrazolium salt assay, NP-1, NP-2, and NP-3 killed all *A. fumigatus* hyphae at 25, 25, and 100 mg/ml, respectively **[115].** At 100 mg/ml, NP-4 killed only 11% of the hyphae, while NP-5 had no effect. Resting conidia of *A. fumigatus* were resistant to 100 mg of these peptides per ml. Purified chitin and its fragments chitobiose and chitotrose bound to NP-1 and prevented the death of *A. fumigatus*, suggesting that the lethality of NP-1 was through binding to cell wall chitin **[115].**

Protegrins and gallinacins: They are cationic, cysteine-rich molecules with two intermolecular, parallel, disulfide bridges which stabilize an amphipathic β-sheet structure comprising two antiparallel strands **[117-119].**

The protegrins, which are related to the β-defensins, are produced by porcine leukocytes.Zone inhibition studies showed that protegrins 1, 2, and 3 inhibited *C. albicans* growth at 60, 8, and 3 mg/ml, respectively **[119].** Chicken leukocytes produce the gallinacin peptide family **(69).** Gallinacins have three intramolecular cystine disulfide bonds, are relatively cationic, and are rich in lysine and arginine. Gallinacin-1 and -1a inhibited *C. albicans* in a radial diffusion assay **[120].** However, gallinacin-2 showed no activity at up to 400 mg/ml in this assay.

Tritrpticin and lactoferricin: Precursors of many antimicrobial peptides of porcine, bovine, and rabbit origin share highly conserved regions with antifungal properties **[121-123].** Tritrpticin corresponds to 13 amino acids of the C-terminal portion of cathelin, a putative proteinase inhibitor from porcine blood leukocytes. In vitro, it was weakly inhibitory for *Aspergillus flavus* and *C. albicans* **[124].** Lactoferricin was active against *C. albicans*; however, its antimicrobial properties were diminished by Ca^{2+}, Mg^{2+}, and Fe^{2+}. The optimum pH for this peptide was 6.0, and it bound to outer bacterial membranes, causing disruption of normal permeability functions of the cytoplasmic membrane and ultrastructural damage **[125, 126].**

BPI protein domain III analogs: Several potent antifungal peptides with activity against *Candida* spp., *C. neoformans*, and *A. fumigatus* were derived from BPI protein functional domain III **[127, 128].** These constructs produced significant, dose-dependent reductions in the numbers of *C. albicans* CFU in the kidney and significant protection from mortality in murine candidiasis models **[129].** Three small synthetic peptides (XMP.284, XMP.366 and XMP.391) based on BPI protein domain III were found to be fungicidal for several *Candida* species, while sub-inhibitory concentrations of these peptides enhanced the anti-*Candida* activities of fluconazole **[130].** XMP.391 was effective against murine disseminated aspergillosis and enhanced the effectiveness of amphotericin B **[131].**

Table 2.1 Mammalian antifungal peptides

Peptide	Source	No. of amino acids	Mode of action	Typical target organism	In vitro MIC (μg/ml)
Defensins					
NP-1	Rabbit granulocytes	33	Lysis	*C. neoformans*	3.75–15.0[a]
NP-2	Rabbit granulocytes	33	Lysis	*A. fumigatus*	25.0
NP-3A	Rabbit granulocytes	34	Lysis	*A. fumigatus*	100.0
NP-3B	Rabbit granulocytes	33	Lysis	*A. fumigatus*	100.0
NP-4	Rabbit granulocytes	33	Lysis	*A. fumigatus*	>100.0
NP-5	Rabbit granulocytes	33	Lysis	*A. fumigatus*	Inactive alone
HNP-1	Human neutrophils	30	Lysis	*C. albicans*	50.0
HNP-2	Human neutrophils	29	Lysis	*C. albicans*	50.0
HNP-3	Human neutrophils	30	Lysis	*C. neoformans*	50.0 (LD_{50}[b])
Gallinacin-1	Chicken	39	Lysis	*C. albicans*	25.0
Lactoferricin-B	Human, bovine	18	Lysis	*C. albicans*	0.8
Protegrins 1 to 3	Human, porcine	16–18	Lysis	*C. albicans*	3.0–60.0
Tracheal antimicrobial peptide	Human, bovine	38	Lysis	*C. albicans*	6.0–12.0
Tritrptcin	Human, porcine	13	Lysis	*A. flavus*	250.0

[a] MICs based on assays with multiple isolates.
[b] LD_{50}, lethal dose for 50% of the population.

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2.2.2 Insect derived peptides

Cecropins: Cecropins, which form a-helices in solution, are linear peptides **(Table 2.2)** in the hemolymph of the giant silk moth (*Hyalopora cecropia*) **[132, 133].** They are positively charged and form time-variant and voltage-dependent ion channels in planar lipid membranes **[134].** At between 25 and 100 mg/ml it is fungicidal for pathogenic *Aspergillus* species **[135, 136].** *Fusarium moniliforme* and *Fusarium oxysporum* were especially sensitive to cecropin A, with total killing attained at 12.4 mg/ml. An insect defensin with significant homology with plant antifungal peptides isolated from seeds of members of the family *Brassicaceae* **[137]**. It was similar in structure to the radish antifungal peptide, Rs-AFP1, and was particularly effective against *F. oxysporum* isolates **[138].**

Antifungal peptide, holotricin 3, and thanatin: Antifungal protein, a histidine-rich peptide that causes cellular leakage, was purified from the third instar larval hemolymph of *Sacrophaga peregrina*, and in vitro, it was lethal for *C. albicans* **[139].**

Holotricin 3, a glycine- and histidine-rich peptide purified from the larval hemolymph of *Holotrichia diomphalia*, inhibited *C. albicans* growth **[140].** Thanatin, produced by *Podisus maculiventris*, is nonhemolytic and is active against *F. oxysporum* and *A. fumigatus* **[141].**

Table 2.2 Insect and amphibian antimicrobial peptides

Peptide	Source	No. of amino acids	Mode of action	Typical target organism	In vitro MIC (μg/ml)
Antifungal peptide	*S. peregrina*	67	Lysis	*C. albicans*	25.0
Cecropins					
A	*H. cecropia*	37	Lysis	*F. oxysporum*	12.0
B	*H. cecropia*	35	Lysis	*A. fumigatus*	9.5
Dermaseptins					
b	*P. sauvagii*	27	Lysis	*C. neoformans*	60.0
s	*P. sauvagii*	34	Lysis	*C. neoformans*	5.0
Drosomycin	*D. melanogaster*	44	Lysis	*F. oxysporum*	5.9–12.3[a]
Magainin 2	*X. laevis*	23	Lysis	*C. albicans*	80
Thanatin	*P. maculiventris*	21	Unknown	*A. fumigatus*	24–48[a]

[a] MICs based on assays with multiple isolates.

2.2.3 Amphibian derived peptides

Magainins: The African clawed frog (*Xenopus laevis*) produces the magainins, which are a-helical ionophores that dissipate ion gradients in cell membranes, causing lysis **[142].** Their helical, amphiphilic structure was responsible for affinity to membranes **[143].** An increase in the magainin concentration caused the artificial lipid bilayer thickness to decrease, suggesting adsorption within the head-group region of the lipid bilayer **[144]**. Magainin 2 was nonhemolytic and inhibited *C. albicans* growth **[145]**.

This nonhemolytic property may result from a peptide-cholesterol interaction in mammalian membranes that inhibits the formation of peptide structures capable of lysis **[146].**

Dermaseptin: The South American arboreal frog (*Phyllomedusa sauvagii*) produces the dermaseptin family of nonhemolytic antifungal peptides **[147, 148].** Dermaseptins are linear cationic, lysine-rich peptides and are believed to lyse microorganisms by interacting with lipid bilayers, leading to alterations in membrane functions responsible for osmotic balance **[149-151]**. Zone inhibition assays demonstrated that 10 mg/ml suppresses the growth of *A. fumigatus* **[152].** Dermaseptins s1 to s5 were potent antifungal agents that inhibited a wide range of fungi **[150].** Dermaseptin b inhibited the in vitro growth of yeasts and some filamentous fungi; however, the dermaseptin s group was more effective**.**

2.2.4 Bacterial and fungal peptides

Iturins. Various strains of *Bacillus subtilis* produce the iturin peptide family **(Table 2.3).** They are small cyclic peptidolipids characterized by a lipid-soluble b-amino acid linked to a peptide containing D and L amino acids **[153].**

Iturins affected membrane surface tension, which caused pore formation and which resulted in the leakage of K1 and other vital ions, paralleling cell death **[154-156].** In a disc assay, iturin A inhibited *A. flavus* and *F. moniliforme* growth **[157].** Initial clinical trials involving humans and animals showed that iturin A was effective against dermatomycoses and had a wide spectrum of antifungal properties and low allergenic effects **[158-159].**

Syringomycins and related peptides: Members of the *Pseudomonas syringae* pv. *syringae* group produce small cyclic lipodepsipeptides known as syringomycins **[160],** the major form being syringomycin E (SE).

SE increased transmembrane K1, H1, and Ca21 fluxes and the membrane potential in plasma membranes of plants and yeasts **[161-165]**.

These compounds were fungicidal for *Candida*, *Cryptococcus*, and *Aspergillus* isolates **[166]**. A 12% (wt/vol) ointment of SE was effective in controlling vaginal candidiasis in a murine model **[167]**. *P. syringae* also produced the pseudomycins, another family of peptides with broad-spectrum antifungal activity **[168].**

Nikkomycins: Nikkomycins, which are produced by *Streptomyces tendae*, enter target cells via dipeptide permeases and inhibit chitin biosynthesis in *C. albicans* both in vitro and in vivo **[169-174].** Nikkomycins provided antifungal protection to infected kidneys, while other organs were unprotected. Nikkomycin Z at high dosages prolonged the survival of mice with disseminated candidiasis **[175, 176].** Nikkomycins X and Z were active against pathogenic dimorphic fungi but showed only modest to poor activity against yeast and filamentous fungi **[177, 178].** However, they were highly efficacious in murine models of coccidioidomycosis and blastomycosis, with moderate efficacy against histoplasmosis. Given orally, the nikkomycins prevented the deaths of mice infected with a 100% lethal challenge of *C. immitis*, with nikkomycin Z being more active than nikkomycin X.

Polyoxins: Polyoxins, which are produced by *Streptomyces cacaoi*, were active against isolated chitin synthases but had variable activity against intact organisms **[179-182]**. Polyoxin D was fungistatic for *C. albicans* at concentrations of 500 to 2,000 mg/ml, depending on the strain, and inhibited *C. neoformans* growth. Notably, polyoxin D reduced the ability of *C. albicans* to bind to buccal epithelial cells by as much as 58% compared to the binding ability of controls **[183].**

***Bacillus licheniformis* peptides:** CB-1 is a chitin-binding peptide containing fatty acids bound to amino acids and has an IC50 for *F. oxysporum* of 50 mg/ml (130). A *B. licheniformis* isolate, M-4, produces fungicin M-4 **[184].**

It is a hydrophilic, narrow-spectrum antifungal peptide that is resistant to proteolytic enzymes and lipase and that inhibited the growth of *Microsporum canis*, *Mucor* species, and *Sporothrix schenckii*. However, fungicin M-4 was ineffective against *C. albicans*, *C. neoformans*, *A. niger*, and *Trichophyton mentagrophytes. B. licheniformis* also produces A12-C, a fungal cell growth and hyphal proliferation inhibitor. A12-C inhibited *S. schenckii*, *T. mentagrophytes*, and *M. canis* growth, as observed in zone-ofinhibition studies **[185].**

Schizotrin A: A cyanobacterium, *Schizotrix* (TAU strain IL- 89-2), produces schizotrin A, a cyclic undecapeptide **[186].** Zone-of-inhibition assays demonstrated that it has activity against *C. albicans* and *C. tropicalis*. It also inhibited the radial growth of *F. oxysporum* at 0.05 mg/ml.

Cepacidines: Cepacidines A1 and A2 are glycopeptides that have similar structures and that are produced by *Burkholderia cepacia* **[187, 188].** Together, they displayed potent antifungal properties superior to those of amphotericin B**.** In vitro, the MICs of cepacidine A ranged from 0.049 to 0.391 mg/ml for *Candida* species, *C. neoformans*, *A. niger*, *T. mentagrophytes*, *Trichophyton rubrum*, *M. canis*, and *F. oxysporum***.** Its activity was diminished significantly against *C. albicans* and *C. neoformans* in the presence of 50% human serum, which may limit its clinical potential **[188]**.

1907-II and 1907-VIII: *P. lilacinus* produces two antifungal peptides, 1907-II and 1907-VIII, consisting of several amino acids, a methylamine, and a fatty acid **[189]**. In vitro, both peptides have a MIC of 6.25 mg/ml for *C. albicans*, while *C. neoformans* was very susceptible (MICs, 0.78 and 1.56 mg/ml for 1907-II and 1907-VIII, respectively).

Table 2.3 Bacterial and fungal antifungal peptides

Peptide	Source	Structure	Mode of action	Typical target organism	In vitro MIC (μg/ml)
1901-II	*P. lilacinus*	Amino-lipopeptide	Unknown	*C. tropicalis*	12.5
1907-VIII	*P. lilacinus*	Amino-peptide	Unknown	*C. tropicalis*	50.0
A12-C	*B. licheniformis*	Peptide	Hyphal proliferation	*M. canis*	Unknown
Aculeacins	*Aspergillus aculeatus*	Lipopeptide	Glucan synthesis	*C. albicans*	0.2–6.3[a]
Aureobasidin A	*A. pullulans*	Cyclic depsipeptide	Actin assembly	*C. neoformans*	0.63
Bacillomycin F	*Bacillus subtilis*	Lipopeptide	Lysis	*Aspergillus niger*	40.0
CB-1	*B. licheniformis*	Lipopeptide	Chitin binding	*F. oxysporum*	50.0 (IC_{50}[b])
Cepacidine A_1	*B. cepacia*	Cyclic glycopeptide	Unknown	*A. niger*	0.098
Cepacidine A_2	*B. cepacia*	Cyclic glycopeptide	Unknown	*A. niger*	0.096
Echinocandin B	*A. nidulans*	Lipopeptide	Glucan synthesis	*C. albicans*	0.625
Fungicin M-4	*B. licheniformis*	Cyclic peptide	Unknown	*Mucor sp.*	8.0
FR900403	*Kernia* sp.	Lipopeptide	Chitin synthesis	*C. albicans*	0.4
Helioferin A	*M. rosea*	Lipopeptide	Unknown	*C. albicans*	5.0
Helioferin B	*M. rosea*	Lipopeptide	Unknown	*C. albicans*	5.0
Iturin A	*B. subtilis*	Lipopeptide	Lysis	*S. cerevisiae*	22.0
Leucinostatin A	*P. lilacinum*	Amino-lipopeptide	Unknown	*C. neoformans*	0.5
Leucinostatin H	*P. marquandii*	Amino-lipopeptide	Unknown	*C. albicans*	10.0
Leucinostatin K	*P. marquandii*	Amino-lipopeptide	Unknown	*C. albicans*	25.0
Mulundocandin	*A. syndowi*	Lipopeptide	Glycan synthesis	*C. albicans*, *A. niger*	0.97 31.25
Nikkomycin X	*Streptomyces tendae*	Peptide-nucleoside	Chitin synthesis	*C. immitis*	0.125
Nikkomycin Z	*S. tendae*	Peptide-nucleoside	Chitin synthesis	*C. immitis*	0.77
Pneumocandin A_0	*Z. arboricola*	Lipopeptide	Glucan synthesis	*C. albicans* isolates	0.12–2.0[a]
Polyoxin D	*S. cacaoi*	Trinucleoside peptide	Chitin synthesis	*C. immitis*	0.125
Pseudomycin A	*P. syringae*	Lipodepsinonapeptide	Lysis	*C. neoformans*	1.56
Schizotrin A	*Schizotrix* sp.	Cyclic undecapeptide	Unknown	*C. albicans*	0.02
Syringomycin E	*P. syringae*	Lipodepsipeptide	Lysis	*C. neoformans*	0.8–12.5[a]
Syringostatin A	*P. syringae*	Lipodepsipeptide	Lysis (?)	*A. fumigatus*	5.0–40.0[a]
Syringotoxin B	*P. syringae*	Lipodepsinonapeptide	Lysis (?)	*C. albicans*	3.2–50.0[a]
Trichopolyn A	*T. polysporum*	Amino-lipopeptide	Unknown	*C. neoformans*	0.78
Trichopolyn B	*T. polysporum*	Amino-lipopeptide	Unknown	*C. neoformans*	0.78
WF11899 A	*Coleophoma empetri*	Lipopeptide	Glucan synthesis	*C. albicans*	0.16
WF11899 B	*C. empetri*	Lipopeptide	Glucan synthesis	*C. albicans*	0.008 (IC_{50})
WF11899 C	*C. empetri*	Lipopeptide	Glucan synthesis	*C. albicans*	0.008 (IC_{50})

[a] MICs based on assays with multiple isolates.
[b] IC_{50}, inhibitory concentration for 50% of the population.

Leucinostatin-trichopolyn group: The leucinostatin-trichopolyn group is structurally related to 1907-II and 1907-VIII. Leucinostatins A and B are produced by submerged cultures of *Penicillium lilacinum* **[190, 191].** Leucinostatin A and 1907-VIII have the same molecular weight (1,217), while leucinostatin B and 1907-II have a molecular weight of 1,203 **[192, 193].** Leucinostatin A and B acted as uncouplers on rat mitochondria **[194]**.

Leucinostatins D, H, and K were isolated from *Paecilomyces marquandii* (Massee) Hughes and had a wide spectrum of antimicrobial properties against *Candida* species, *C. neoformans*, and other clinically important fungi **[195, 196]**.

Unfortunately, it is rather cytotoxic, with the following 50% inhibitory doses: 850 ng/ml for HeLa cells, 0.95 ng/ml for KB cells, and 1.00 ng/ml for P388/S cells. Trichopolyns A and B are produced by *Trichoderma polysporum* **[197, 198].** The MICs of trichopolyns A and B for *C. albicans, C. neoformans, A. niger, A. fumigatus,* and *T. mentagrophytes* were 0.78 to 6.25 mg/ml.

Helioferins: *Mycogone rosea* produces helioferins A and B, which are members of the leucinostatin-trichopolyn group that also may not have clinical utility **[199]**. They inhibited *C. albicans* (MIC, 5.0 mg/ml) but were toxic to chicken embryos at levels greater than 0.5 mg/kg and caused hemolysis at concentrations greater than 100 mg/ml. They also displayed cytotoxic activities, with IC50s for the L-1210 leukemia cell line and the L0929 mouse fibroblast cell line of 0.01 to 0.4 mg/ml.

AFP: The mould *Aspergillus giganteus* MDH 18894 was obtained from the soil of a farm in Michigan during an antitumor screening program. This imperfect ascomycete mainly produced two extracellular proteins **[78, 200]** One of them, a-sarcin, is a cytotoxic ribonuclease and The other one described as a very basic small-sized protein, which was said to display an inhibitory activity against the growth of fungi is a small basic protein which has been shown to inhibit the growth of several filamentous fungi, mainly from the genera *Fusarium* and *Aspergillus* **[1, 2, 72].** The protein consists of 51 amino acids and is folded into a small compact structure with four stabilizing disulfide bridges **[200-203].**

AFP causes membrane permeabilization in AFP-sensitive fungi by an as yet poorly understood mechanism, and its minimal inhibitory concentrations (MICs) range from 0.1 μg/ml for *Fusarium moniliforme* IfGB 39/1402 up to 200 μg/ml for *Aspergillus nidulans* G191. However, the antifungal activity of AFP is strongly diminished in the presence of cations.

AFPNN5353: An antifungal protein AFPNN5353, a defensin-like protein was produced by *Aspergillus giganteus* **[83].** It belongs to a group of secretory proteins with low molecular mass, cationic character and a high content of cysteine residues. AFPNN5353 inhibits the germination and growth of filamentous ascomycetes, including important human and plant pathogens and the model organisms *Aspergillus nidulans* and *Aspergillus niger*.

Furthermore, they provide evidence about calcium (Ca^{2+}) signaling which plays an important role in the mechanistic function of this antifungal protein. AFPNN5353 treated transgenic A. niger strain increased about 2-fold the cytosolic free Ca^{2+} of a transgenic A. niger strain expressing codon optimized aequorin. Supplementation of the growth medium with $CaCl_2$ counteracted AFPNN5353 toxicity, ameliorated the perturbation of the $[Ca^{2+}]c$ resting level and prevented protein uptake into Aspergillus sp. cells. The minimal inhibitory concentration (MIC) (the concentration that completely inhibited conidial germination in liquid growth assays) was 0.2 μg/ml for *A. nidulans*, 0.5 μg/ml for *N. crassa* and 1 μg/ml for *A. niger*. Two strains were unaffected at the protein concentrations tested: *M. circenelloides* and *M. genevensis* were insensitive against AFPNN5353 when concentrations up to 500 μg/ml were used.

Baciamin: In 2008, Wong JH, et.al, **[90, 216]** examined and reported that an antifungal protein from Bacillus amyloliquefaciens is a broad-spectrum antifungal protein, baciamin. It induces membrane permeabilization in fungi but not in rabbit erythrocytes. Its antifungal activity is relatively thermostable, pH and trypsin-stable.

It demonstrates antiproliferative activity towards various tumour cells, nitric oxide-inducing activity towards macrophages, and inhibitory activity towards HIV-1 RT.

The antifungal protein designated as baciamin was isolated and exhibited a molecular mass around 50 kDa. Baciamin could induce membrane permeabilization of tested fungi.

Its antifungal activity was retained after incubation with trypsin and EDTA. Various ions tested did not affect its antifungal activity. Baciamin reduced the activity of HIV-1 reverse transcriptase (RT). It also inhibited proliferation of hepatoma, breast cancer and colon cancer cell lines. Baciamin augmented nitric oxide production by mouse macrophages.

Escherichia coli BL21: A cytosolic protein was first purified from Escherichia coli BL21 and reported in 2007, by V. Yadav, R. Mandhan **[91]** that demonstrated potent antifungal activity against pathogenic strains of Aspergillus fumigatus, Aspergillus flavus, Aspergillus niger and Candida albicans.

The MIC of purified protein from E. coli BL21 (PPEBL21) against Aspergillus species and C. albicans was 1.95–3.98 and 15.62 mg per ml, respectively. In vitro toxicity tests demonstrated no cytotoxicity of PPEBL21 to human erythrocytes up to the tested concentrations of 1250 mg per ml. Amphotericin B was lethal to 100% of human erythrocytes at a concentration of 37.5 mg per ml. The N-terminal amino acid sequence of PPEBL21 was found to be DLAEVASR, which showed 75% sequence similarity with alcohol dehydrogenase of yeast. Mass fingerprinting by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry also substantiated these observations. The results suggested that E. coli BL21 might be an important bioresource of lead molecules for developing new peptide-based therapies for treating fungal infections.

Bacisubin: An antifungal protein, with a molecular mass of 41.9 kDa, and designated as bacisubin, was isolated from a culture of *Bacillus subtilis strain* B-916 in 2007 by Yongfeng Liu, et.al., **[92]**. The isolation procedure consisted of ion exchange chromatography on DEAE-Sepharose Fast Flow, and fast protein liquid chromatography on Phenyl Sepharose 6 Fast Flow and hydroxyapatite columns. The protein was adsorbed on all three chromatographic media.

Bacisubin exhibited inhibitory activity on mycelial growth in *Magnaporthe grisease, Sclerotinia sclerotiorum, Rhizoctonia solani, Alternaria oleracea, A. brassicae* and *Botrytis cinerea*. The IC_{50} values of its antifungal activity toward the last four fungal species were 4.01 mM, 0.087 mM, 0.055 mM and 2.74 mM, respectively. Bacisubin demonstrated neither protease activity, nor protease inhibitory activity. However, it manifested ribonuclease and hemagglutinating activities.

PAF: Active Internalization of the *Penicillium chrysogenum* Antifungal Protein PAF in Sensitive Aspergilli was observed **[204].** The *Penicillium chrysogenum* antifungal protein PAF inhibits the growth of various filamentous fungi. PAF was found to localize to the cytoplasm of sensitive aspergilli by indirect immunofluorescence staining. The internalization process required active metabolism and ATP and was prevented by latrunculin B, suggesting an endocytotic mechanism **[84].**

2.2.5 Plant derived peptides

Plant defensins: Plant defensins (Table 5), which are not elated to either the mammalian or the insect defensins, have eight disulfide-linked cysteines comprising a triple-stranded antiparallel b-sheet structure with only one a helix **[205].**

Their mechanisms of action have not yet been elucidated, although the possibility of permeabilization through direct protein- lipid interactions has been eliminated **[206]** They reduced hyphal elongation without marked morphological distortions **[205].** Hs-AFP1 and Rs-AFP2 were isolated from *Heuchera sanginea* and *Raphanus sativus* seeds, respectively **[207].**

They possess poor lethality for the clinical fungi studied to date. Hs-AFP1 and Rs-AFP2 at a concentration of 125 mg/ml reduced the viability of germinated conidia of *A. flavus* by only 20 and 35%, respectively **[109].**

In contrast, Hs-AFP1 at 125 mg/ml reduced the viabilities of nongerminated and germinating conidia of *F. moniliforme* by 42 and 85%, respectively, while Rs-AFP2 reduced the viabilities of these conidial types by 25 and 95%, respectively. Hs-AFP1 and Rs-AFP2 bound at different rates to mannan, chitin, ergosterol, galactocerebrosides, and sphingomyelin **[135, 136].**

Lipid transfer proteins: Some plants produce lipid transfer proteins, a family of homologous peptides having eight disulfide-linked cysteines. Onion seeds (*Allium cepa* L.) produce the lipid transfer peptide ACE-AMP1, which inhibited *F. oxysporum* **[208].**

Zeamatin: *Zea mays* seeds produce the peptide zeamatin, which belongs to a third class of plant antifungal compounds **[209].** Peptides in the zeamatin family are also present in *Avena sativa*, *Sorghum bicolor*, and *Triticum aestivum* seeds **[210]**. Zeamatin caused the release of cytoplasmic material from *C. albicans* and *Neurospora crassa*, resulting in hyphal rupture. It appears to permeabilize the fungal plasma membrane and inhibited *C. albicans*. Zeamatin activity was reduced by increasing concentrations of NaCl. A flax seed antifungal peptide similar to zeamatin, in synergy with nikkomycin Z, inhibits *C. albicans* **[5]**.

Cyclopeptides: Members of the family *Rhamnaceae* and other plant families produce the basic cyclopeptides in which a 10- or 12-membered peptide-type bridge spans the 1,3 or 1,4 positions of a benzene ring **[211]**. The antifungal properties of many family members have not yet been determined.

Frangufoline, amphibine H, rugosanines A and B, and nummularines B, K, R, and S showed significant activity against *A. niger* but not *C. albicans* in zonal inhibition studies **[212]**.

Phylloplanins: reported that Phylloplanins, secreted surface proteins (a novel AFP On plant leaf source) are an innate immune defense component employed by animals to inhibit invading microbes. Surface proteins have not been documented in plants, even though the aerial leaf surface, or phylloplane, is a major site of pathogen ingress. They have discovered novel proteins, termed phylloplanins **[213]** , which accumulate on leaf surfaces of *Nicotiana tabacum*, and we have isolated the gene *Phylloplanin* that is unique in gene databases. Natural and *E. coli* expressed phylloplanins inhibit spore germination and limit leaf infection by the oomycete pathogen *Peronospora tabacina*. Further study of leaf surface proteins is justified to understand further their roles in plant defense, and to investigate their potential in agricultural biotechnology.

Pr-2: A novel antifungal protein (Pr-2) was identified in 2009, by **Seong-Cheol Park, et. al., [214]** from pumpkin rinds using water-soluble extraction, ultrafiltration, cation exchange chromatography, and reverse-phase high-performance liquid chromatography. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry indicated that the protein had a molecular mass of 14865.57 Da. Automated Edman degradation showed that the N-terminal sequence of Pr-2 was QGIGVGDNDGKRGKR-The Pr-2 protein strongly inhibited in vitro growth of Botrytis cinerea, Colletotrichum coccodes, Fusarium solani, Fusarium oxysporum, and Trichoderma harzianum at 10-20 μM. The results of confocal laser scanning microscopy and SYTOX Green uptake demonstrated that its effective region was the membrane of the fungal cell surface. In addition, this protein was found to be noncytotoxic and heat-stable. Taken together, the results of this study indicate that Pr-2 is a good candidate for use as a natural antifungal agent.

C-FKBP: Purification and Characterization of an Antifungal Protein, C-FKBP, from Chinese Cabbage had also been reported by scientist. In 2007, **Seong-Cheol Park, et.al, [215]** observed and reported that an antifungal protein isolated from Chinese cabbage (Brassica campestris L. ssp. pekinensis) by buffer-soluble extraction and two chromatographic procedures, exihibited a potent antifungal activity against pathogenic fungal strains, including Candida albicans, Botrytis cinerea, Rhizoctonia solani, and Trichoderma viride, whereas it exhibited no activity against *E. coli* and *Staphylococcus aureus*.

The amino acid sequence of C-FKBP exhibits striking degrees of identity with the corresponding mouse (61%), human (60%), and yeast (56%) proteins.

Genomic Southern blot analyses using the full-length C-FKBP cDNA probe revealed a multigene family in the Chinese cabbage genome. These results suggest that recombinant C-FKBP is an excellent candidate as a lead compound for the development of antifungal agents.

Thaumatin-like proteins: From the emperor banana A 20-kDa protein with substantial N-terminal sequence homology to thaumatin-like proteins was isolated and reported by **Vincent S.M. Ho, et.al,. in 2007 [216]**. The isolation procedure entailed $(NH_4)_2SO_4$ precipitation, ion exchange chromatography on DEAE-cellulose, and affinity chromatography on Affi-gel blue gel. The thaumatin-like protein inhibited mycelial growth in Fusarium oxysporum and Mycosphaerella arachidicola. However, it did not affect the mitogenic response of murine splenocytes or [methyl-3H] thymidine incorporation by tumor cells. The activity of HIV-1 reverse transcriptase was slightly inhibited.

Castamollin: castamollin **[217],** a novel antifungal protein purified from Chinese chestnuts possessed a novel N-terminal sequence demonstrating Castamollin exhibited a molecular mass of 37 kDa in gel filtration and SDS–PAGE.

It inhibited the activity of human immunodeficiency virus-1 reverse transcriptase with an IC50 of 7 lM and translation in a cell-free rabbit reticulocyte lysate system with an IC_{50} of 2.7 lM. Castamollin displayed antifungal activity against Botrytis cinerea, Mycosphaerella arachidicola, Physalospora piricola, and Coprinus comatus but was devoid of lectin activity.

Ganodermin: In 2003, Hexiang Wanga, T.B. Ng **[218]** observed that Ganodermin, A 15-kDa antifungal protein from fruiting bodies of the medicinal mushroom Ganoderma lucidum, had been found to exhibit the inhibitory effect on the mycelial growth of Botrytis cinerea, Fusarium oxysporum and Physalospora piricola with an IC50 value of 15.2 mM, 12.4 mM and 18.1 mM, respectively. It was devoid of hemagglutinating, deoxyribonuclease, ribonuclease and protease inhibitory activities.

Pearl Millet: A New Class of Antifungal Protein, Cysteine Protease Inhibitor from Pearl Millet had been reported in 1998, by Bimba N. Joshi**, [219]** exhibiting antifungal activity, purified to homogeneity by ammonium sulphate precipitation and chromatographic procedures involving CM-sephadex and SP-sepharose cation exchange columns. The molecular characterization has revealed its molecular mass as 24 kD and isoelectric point 9.8. The amino acid composition data shows presence of high content of serine and glycine (34 residues / mole) and absence of tryptophan. The inhibitor exhibits potent antifungal activity against *Trichoderma reesei,* a dead wood fungus with minimum inhibitory dose to inhibit mycelial growth or spore germination is as low as 1mg / ml (250 ng/ disc).

In addition to *Trichoderma reesei*, the antifungal activity is observed against some important phytopathogenic fungi, namely, *Claviceps, Helminthosporium, Curvularia, Alternaria* and *Fusarium species.* A cysteine protease inhibitor as an antifungal protein is reported for the first time from a plant system.

Engelmannia pinnatifida: An extract from leaves of *Engelmannia pinnatifida* exhibited potent and broad-spectrum antifungal activity. Isolation and characterization of a 30 kDa protein with antifungal activity from leaves of *Engelmannia pinnatifida* had been done in 1996, by Huynh, et.al, **[220]** In this method a 30 kDa protein from *E. pinnatifida* leaves was purified to homogeneity by ammonium sulphate precipitation, gel filtration, Mono-Q and C" and reverse-phase column chromatographies. The purified protein showed potent antifungal activity against various plant pathogens with as little as 50 ng. The N-terminal amino acid sequence of the purified protein was determined as XXTKFDFFTLALQXPAXF, where X indicates an unidentified residue. This sequence showed 35±50% sequence identity with purified style glycoproteins associated with self-incompatibility from wild tomato, tobacco and petunia, a phosphate-starvation induced ribonuclease from cultured tomato cells and the SIR 63.4 kDa protein from yeast.

RsAFP1 and RsAFP2: Radish seeds had previously been shown to contain two homologous, 5-kD cysteine-rich proteins designated *Raphanus sativus*-antifungal protein 1 (RsAFPl) and RsAFP2, both of which exhibit potent antifungal activity in vitro. Research on Small Cysteine-Rich Antifungal Proteins from Radish had been explored in 1995, by Terras,et.al., **[221].** The amount of released proteins is sufficient to create a microenvironment around the seed in which fungal growth is suppressed.

Both the cDNAs and the intron-containing genomic regions encoding the Rs-AFP preproteins were cloned. Transcripts (0.55 kb) hybridizing with an RsAFP1 cDNA-derlved probe were present in near-mature and mature seeds. Such transcripts as well as the corresponding proteins were barely detectable in healthy uninfected leaves but accumulated systemically at high levels after localized fungal infection.

The induced leaf proteins (designated RsdFP3 and RsdFP4) were purified and shown to be homologous to seed RsAFPs and to exert similar antifungal activity in vitro. A chimeric RsAFP2 gene under the control of the constitutive cauliflower mosaic virus 35s promoter conferred enhanced resistance to the foliar pathogen Alternaria *longipes* in transgenic tobacco. The term "plant defensins" is proposed to denote these defense-related proteins.

PR-1: "Pathogenesis-Related PR-1 Proteins Are Antifungal in nature" statement was first appeared in 1995 , by Thierry Niderman **[222]** when isolation and characterization of three 14-Kilodalton Proteins of Tomato and of a Basic PR-1 of Tobacco with inhibitory activity against *Phyfophfhora infestans,* revealed that three distinct basic 14-kD proteins, P14a, P14b, and P14c, isolated from tomato (Lycopersicon esculentum Mill. cv Baby) leaves infected with *Phytophthora infestans*, exhibited antifungal activity against *P.* infestam both in vitro (inhibition of zoospore germination) and in vivo with a tomato leaf disc assay (decrease in infected leaf surface) (1995). Serological cross-reactions and amino acid sequence comparisons showed that the three proteins are members of the PR-1 group of pathogenesis-related (PR) proteins. P14a and P14b showed high similarity to a previously characterized P14, whereas P14c was found to be very similar to a putative basic-type PR-1 from tobacco predicted from isolated DNA clones. This protein, named PR-1 g, was purified from virus-infected tobacco (Nicotiana *tabacum* Samsun NN) leaves and characterized by amino acid micro sequencing, along with the well-known acidic tobacco PR-la, PR-lb, and PR-lc. lhe various tomato and tobacco PR-1 proteins were compared for their biological activity and found to display differential fungicidal activity against *P.* infestam in both the in vitro and in vivo assays, the most efficient being the newly characterized tomato P14c and tobacco PR-1 g.

2.3 Fermentation strategy

The successful production strategy of a fungal metabolite requires a detailed knowledge of the Filamentous fungi are eukaryotic microorganisms that influence our everyday lives in areas as diverse as medicine, agriculture, and basic science. Filamentous fungi have been widely employed in the food industry, biopharmaceutical sector and become a principal source of enzymes and bioactive metabolites, and therefore have been widely investigated **[223, 224]**. The problem encountered is that the production levels by fungi are much lower usually only reaching a few tens of milligrams per liter of culture medium **[225]**.

However, in industry, fermentation usually implies an emphasis on submerged liquid cultivation systems. Growth and production are affected by a wide range of parameters, including cultivation medium, inoculum, pH, temperature, aeration agitation, shear stress, etc. Compared to many unicellular microbes, filamentous fungi fermentation processes present special challenges in optimization and scale-up because of the varying fungal morphological forms **[226].**

The genus Aspergillus in particular has been used with success as a host for the production of various bioactive proteins of both fungal and non-fungal origins, e.g., glucoamylase, bovine chymosin, human lactoferrin, hen egg-white lysozyme, human interleukin-6, and thaumatin. Other host strains include *Trichoderma reesei*, *Chrysosporium lucknowense*, *Mortierella alpinis*, etc. **[225, 227-230]** has been cited in several research works.

Several factors that negatively affect the production levels of proteins have been reported, showing that the production can be limited at any level, i.e., transcription, translation, secretion, and extracellular degradation **[231]**.

However, few studies of protein secretion have been made with filamentous fungi; the molecular understanding of the protein secretion in fungi is still lacking **[232]**. Some recent studies showed that gene expression and protein secretion in solid-state fermentation might be quite different from that in submerged fermentation **[233]**. For example, in submerged cultures, some enzyme activities are found mainly in the cell wall of mycelia. However, in solid state cultures, these enzyme activities are observed in the medium, while little activity is observed in the cell wall **[234]**. This suggests that the secretion of proteins is strongly affected by culture conditions. It is known that the secretory pathway performs the functions of protein folding, glycosylation, processing, etc. The cell wall acts as a barrier to all secreted proteins to some extent and many fungal bioactive metabolites are partially cell wall-associated **[231, 235]**.

Protein secretion in filamentous fungi is believed to occur mainly at the tips of growing hyphae, since the growing hyphal tips are more porous, making it easier for the exoenzymes to pass through the cell wall **[232]**. Therefore, factors that increase the number of active tips, such as the properly controlled fungal morphology, may improve the protein yield **[236, 237]**.

Apergillus species can secrete a diversity of extracellular proteases, and it has been shown that proteases are responsible for the degradation of many bioactive proteins **[230]**. Proteolytic degradation by fungal proteases is recognized as one of the major problems interfering with efficient heterologous protein production. To improve the non-fungal protein production, several bioprocess strategies have been developed and shown to play a significant role **[226, 238, 239]**.

2.3.1 Carbon and nitrogen sources

The carbon and nitrogen source are two important factors affecting cell growth and product formation of microorganisms. It was reported that the production of fructo-furanosidase by A. japonicus could vary by 100 times on different carbon sources, i.e., corn starch, maltose, fructose, glucose, galactose, sorbital, and sucrose, the latter of which was found to be the best for h-fructofuranosidase production **[240, 241]**.

Carbon and nitrogen sources may have either repressing or inducing effects on enzyme productions. Glucoamylase, amylase, and a-glucosidase are all dupregulated (induced) by starch and ddown-regulatedT (repressed) by glucose **[231]**, starch is better than maltose, while maltose is better than glucose as the substrate (inducer).

Most bioactive fungal metabolites are repressed under the conditions of high glucose and ammonium levels in the medium. For example, the extracellular proteases of A. nidulans are subjected to carbon, nitrogen and sulfur metabolite reprocessing. The depletion of low molecular weight sources of nitrogen, carbon, phosphorus, and sulfur elevates protease activity **[230]**. Since proteases were found to be responsible for the loss of many proteins, the regulation of proteases by carbon and nitrogen sources would benefit the protein production. Some researchers applied statistical or factorial designs for the culture media optimization **[242]**.

Papagianni investigated the effect of microelements on fungal cell morphology and reported as influencing factors for enhanced fungal metabolite production even at constant carbon and nitrogen sources **[240]**.

Therefore to improve production and promote economical design at the industrial level, it has become essential to optimize all the culture conditions (physical parameter) and composition (chemical parameter) for improved metabolite production **[241, 242].**

Carbon is required as the skeletal element of all organic molecules, and molecules serving as carbon sources normally also contribute both oxygen and hydrogen. Gao et al. **[243]** studied the effect of different C/N ratio on the growth and sporulation of several biocontrol agents. Mg^{2+} ion is widely perceived as an essential element for fungal growth and its biochemical functions **[244]** involved in a major way through transducing and the control of cell division in many filamentous ascomycetes through its control of microtubule assembly **[245]**.

2.3.2 Essential microelements sources

It has also been reported that passage of the Mg^{2+} and Ca^{2+} ions through the plasma membrane shows many of the characteristics of an active transport system with K^{+} ion which extruded to preserve electrical neutrality as Ca^{2+} and Na^{+} could act as a growth inhibitor through competition with Mg^{2+}. The C/N ratio is said to significantly affect the number of conidia produced and conidial characteristics as well as on growth, sporulation and biocontrol efficacy **[246-248].**

2.3.3 Cell morphology

The understanding of morphology is lagging behind in biopharmaceuticals producing filamentous organisms whereas the relationship between morphology and productivity as a challenge **[249]**. To select a suitable production strategy, the direct and indirect roles of morphological parameters in controlling bioprocess variables are observed. Some progress has been made in quantitation of morphology.

Useful correlations have been reported in the several literatures and of course, some of the models, with proper modification, can be used to explain the morphological phenomena.

Therefore, fungal morphology is regarded as a qualitative tool for bioprocess optimization of fungal metabolite production **[242]**.

It is generally believed that protein secretion in filamentous fungi mainly occurs at the tips of growing hyphae **[229, 232]**, and those factors that increase the number of active tips may improve yield **[236, 237]**.

The dependence of protein production on fungal morphology has been investigated by several researchers in shake flasks cultures which carries a gene for the glucoamylase-green fluorescence protein-fusion protein **[239]** production by employing the recombinant A. niger strain AB4.1. Different inoculum levels were used to obtain different sizes of pellet or free mycelia. The extracellular protease activity of the cultures varied with the pellet size and decreased dramatically when the morphology was changed from free mycelia to pellets, resulting in a dramatic 3.4-fold increase in the GFP yield. The results indicate that a morphology control strategy can be effective in inhibiting protease activity in filamentous fungal fermentation, thereby enhancing protein production.

2.3.4 Inoculum effect

Many parameters influence pellet formation, including inoculum level, initial pH, agitation, medium composition, and use of polymer additives or surface-active agents **[250]**. Among them, the inoculum level is generally recognized as one of the most important factors. When the medium is inoculated with spores, filamentous growth is observed at high initial spore levels, while pellets of increasing size are formed as the inoculum level is reduced **[251, 252]**.

2.3.5 pH effect

The initial medium pH also plays an important role in fungal morphology **[253]**. Higher pH values (5–6) produce pellets, while low pH values (2–3) lead to filamentous mycelium. The surface properties of the spores are influenced by pH and are responsible for this effect **[254]**.

2.3.6 Agitation effect

Fungal morphology to a large extent is affected by agitation in a rotary shaker/bioreactor. Strong agitation will form free filaments. When pellets are formed, the pellet size, structure and survival are also affected by agitation. In general, more agitation yields smaller and more compact pellets **[255, 256]**. Polymer additives can also influence the fungal morphology. Some polymer additives (sodium alginate, dextran, polyacylic acid, and carbopol) cause a more dispersed filamentous growth in comparison with the pronounced pellet growth under normal conditions **[257, 258]**. However, some researchers found an increase in pellet size with the addition of Carboxy-methylcellulose (CMC) **[250]**.

2.3.7 Mathematical modeling

Several mathematical models have been presented in the literature to elucidate the interdependence of morphological properties attributed to filamentous growth in fermented broths and kinetics phenomena related to better bioprocess strategy to optimize operating conditions for process improvement. For example, the Tubular reactor analogy model **[259]**, population model **[251, 252]**, fractal model **[260]**, etc. have been developed for micro-morphology; and cube-root growth kinetics **[261]**, pellet size distribution model **[262]**, fractal morphology **[263]**, etc. have been developed to explain macro-morphology.

Although many models have been proposed to describe fungal morphology and few simultaneously deal with the influence of morphology on product formation. In fact, the relationship between fungal morphology and product formation is difficult to investigate, as there are many interrelated factors that exist in a fermentation system that affect both morphology and protein production.

2.3.8 Design of experiment (DOE)

In conventional approach, an optimization process usually involves one factor at a time (OFAT), **[264]** this procedure is more time consuming and cumbersome which usually involves more experimental data sets and do not provide information about the mutual interactions of the parameters. In contrast, statistically planned Taguchi method reduces the number of experiments through developing a specific DOE, which also minimize the error in determining the values for most decisive parameters **[265, 266].** Although, the response surface methodology (RSM) have received much attention in previous scientific researches **[267].**

Taguchi method is appeared as powerful optimization techniques as it possesses some advantages, such as extraction of much quantitative information by only a few experimental trials and provides a systematic and efficient plan for performing experiments under the consideration of the interactive effects among the control factors and requires half of the time than RSM, **[268, 269]** hence, has gained much popularity in engineering as well as in the field of biotechnology applications **[270-272].** Taguchi method is based on orthogonal array experiments, provides quantitative elucidation and gives much reduced variance with optimal settings of control parameters **[272, 273]** and appreciated as one of the powerful optimization techniques that requires half of the time than RSM **[269].**