ISSN No:-2456-2165

Industrial Applications of CRISPR/Cas9 Genome Editing Technology in Major Filamentous Fungal Families

Manpreet Kaur, Vishwajeet Singh Bumrah
Department of Botany
Shri Guru Teg Bahadur Khalsa College,
University of Delhi New Delhi, India -110007

Abstract:- Manipulating fungi to enhance the yield of industrially important products is a fairly common practice and methods to do so are increasingly improving to maximize the yield of the product more than the presently used methods. Fungi form competent hosts for such manipulations and procedures because of their smaller genome sizes that can be studied and exploited for various research and industrial endeavors.The CRISPR/Cas9 system, a recently developed genome editing tool, has been utilised to change and edit the genes in the species of many fungal families, including Mucoraceae, Saccharomycetaceae, and Trichocomaceae. This technique is commonly known to improve the regulation of lipid metabolism, production of fermentation products, heterologous proteins and genome evolutionary editing in Saccharomyces cerevisiae, Aspergillus sp., Rhizopus sp., Mucor sp. and Mortierella alpina. This review encompasses the present day progress of the renowned genome editing technology CRISPR/Cas9 and its usage in scientific study and various industrial and biotechnological fronts.

Keywords:- *gene editing techniques; CRISPR/Cas9; Aspergillus oryzae; Aspergillus niger; Saccharomyces cerevisiae; Ogataea thermomethanolica; Rhizopus arrhizus; Mucor circinelloides; Mortierella alpina; heterologous protein production; lipid metabolism; fermentation products; evolutionary engineering; transformation.*

I. INTRODUCTION

Fungi are a diverse group of organisms that have continued to provide many scientific breakthroughs that find application in the industrial, biotechnological and pharmaceutical world.

Mucoraceae constitutes a wide genus of fungus, including the most generally occurring species like *Mucor* and *Rhizopus*. Order Mucorales members are frequently called as peg forms.They are distinguished by their regular development and their lengthy, fibrous hyphae that are septa-free. Asexual sporangiospores with an upright hyphae structure are found inside sac-like sporangiophores. As they are haploid in nature, members of this order often reproduce asexually via sporangiospores, which is why they are known as heterothallic [1]. Nevertheless, they may sometimes multiply sexually through zygospores, when reductional divison takes place. For zygospores to reproduce, one (+)-strain and one (-)-strain are needed. This order of fungus has several species that have been connected to human diseases, but many of them are also connected to industries and agriculture. Species like *Mucor* are dimorphic fungi that could also take on isotropic and polarized versions and develop as yeast or hyphae depending on the surroundings [2]. The most predominant growth form of Mucorales is filamentation, yet some of the conditions favor yeast growth as well [3]. Species belonging to Mucoromycota like *Mortierella alpina* can be utilized for applications of lipid metabolism and in the production of industrially important fermentation products while other species of *Mucor* and *Rhizopus* also contribute to the production of several fermented products and organic acids. Filamentous fungi of the family Trichocomaceae have a vital role in commercial processes and food manufacturing. *Aspergillus*, among the more common members of this family, releases a lot of conidia and disperses them all over the surroundings. The more than 300 species that make up this group vary from one another in terms of their structural, physiological, and genealogical traits [4]. Many organisms in this group function as pathogens and decomposers in their native environments, filling an important ecological niche. It is well known that *Aspergillus* species are significant natural decomposers of organic materials in terrestrial environments [5]. Due to its extraordinary capacity to create copious amounts of hydrolytic enzymes and many other organic products, it also plays a crucial role in the conventional fermentation and food industries. This is why it is referred to as a microbial cell factory. For instance, *Aspergillus niger* and *Aspergillus oryzae* strains generate a variety of useful products, including citric acid, sake, miso, and more [7-9]. Due to the long-term use of A. oryzae and A. niger in the food industry, they are listed as generally recognized as safe (GRAS) [6]. *Saccharomyces cerevisiae* and *Ogataea thermomethanolica* are two members of the Saccharomycetaceae family that have gained more attention due to their great tolerance to industrial environments, potential for molecular modification, and inherent resistance to phage invasion, among other benefits [10-13]. There are two forms of yeast cells:- haploid and diploid. Growth rates of yeast vary enormously between strains and between environments. A Lot of successful efforts, including the construction of heterogeneous pathways and their optimization, along with the modification of chassis cells to obtain superior chemical producers [14-17]. As a result, *S. cerevisiae* has emerged as one of the most promising models of chassis cells for both industrial and biological study. Genetic differences that alter the phenotype, including as insertions, substitutions, and deletions, are inevitably introduced by such processes.Yeast species presently are

greatly manipulated through genetic engineering for various applications in heterologous protein production, lipid metabolism, and evolutionary genome engineering.

The CRISPR/Cas9 system was first discovered in bacteria and archaea as an immunological defense system[18]. It has been used effectively to modify the genes in both prokaryotes and eukaryotes [19,20,21]. It is an economical technique used in a variety of species, including plants and animals [25]. Six primary kinds and two categories might be used to categorize the CRISPR/Cas system [23,24]. Of these, the *Streptococcus pyogenes* type II CRISPR/Cas9 system is most frequently exploited in organisms that have the CRISPR-associated protein 9 (Cas9), which is essential for nearly all phases of *S. pyogenes* response [22]. Along with Cas9, single guide RNA (sgRNA) is also an important component of the CRISPR/Cas System. This method for genetic modification can be conducted by a range of approaches in filamentous fungi . Until now, two kinds of CRISPR/Cas9 systems were being employed for gene editing purposes.. The former system contains the elements for in vivo expression of the Cas9 and gRNA where multiple plasmids are formed and transformed via expression vector which have the Cas9 gene and sequences that encodes some portions of the guide RNA (gRNA), including crRNA, tracrRNA, etc. The creation of the plasmid expressing cas9 and a gRNA takes a lot of time, and this method has limitations in terms of its applicability because this genome editing system permits plasmids to persevere and multiply even after editing and produces mutants that may contain some bacterial antibiotic resistance genes that can be used for plasmid selection. Also the long time presence of plasmid and foreign DNA could cause the undesirable degradation and reorganization of the DNA and can form off-target effects [26, 33]. However, the ribonucleoprotein (RNP) complex is formed by the Cas9 enzyme, in the second system , along with the trancribed gRNA (in vitro) which, in order to design the desired gene, is converted into a cell. This approach has many advantages such as, by the use of it the off-target impacts can be prevented, RNP can be degraded readily, the additional cloning processes is eliminated neglecting the off-target effect because of the temporary exposure of transformed cells to Cas9, and finally the capacity of Cas9 to become functional right away after transformation [32].

II. APPLICATIONS OF CRISPR/CAS9 GENE EDITING

A. Fermentation / Industrial Processes

Important strains employed in fermentation and subsequent industrial food processing phases include Aspergillus oryzae, Aspergillus niger, Rhizopus arrhizus, and Mucor circinelloides.

A. oryzae has recently undergone fast development for the flexible genome-editing method known as CRISPR/Cas9 system, for enhanced utilization and performance in industrial processes [34]. The genome editing system CRISPR/Cas9 was effectively created in A. oryzae for the first time by Katayama et al. [35]. The team created plasmids that produce codon-optimized Cas9 and include an SV40 nuclear localization sequence at both the N- and C-termini of the Cas9 gene. Near about 10-20% mutation rate was seen in transformed strains,where most of the mutations are either insertion or deletion of 1bp. The increase in target effectiveness of CRISPR/Cas9 in an industrial strain of A. oryzae was therefore shown to be caused by the mutation of lig D (DNA ligase gene), which is engaged in NHEJ [37], by evaluating the deletion impact of sclerotial formation associated gene an ecdR [36]. Additional study was conducted on the industrial varieties of A. oryzae for its improved uses in studies and manufacturing by employing more of these instruments [40].

Due to its involvement in the manufacture of several industrial enzymes and organic acids, A. niger is one of the constituents of an advanced industrial cell factory[40]. Galactaric acid was produced effectively by interrupting the genes responsible for their catabolism using a combination of the CRISPR/Cas9 technique and in vitro-created sgRNA [41]. By using this method, 2-keto-L-gulonate is effectively built up due to the loss of the gluD gene, which encodes for a D-glucuronic acid catabolizing enzyme that requires NADPH [42]. In a similar vein, Kuivanen et al. investigated the loss of the strain's capacity to catabolize D-glucuronate in A. niger due to breakdown or deletion of the gluF gene [43]. Resultantly, it is said that this approach is successful in investigating the unexplored metabolic pathways as well as more knowledge about functional genes Also, it has been proven suitable in metabolic engineering through multiplexed genome editing in A.niger causing the increase in galactarate production. [44]. A. niger's mycelial development is associated with the production of several proteins, including their secretions and organic acids. The relationship between the expression of proteins and filamentous outgrowth was examined using CRISPR-based genome editing techniques. For the objective of reawakening the biosynthesis of natural products in this organism, a conditional expression system was added upstream of the Tet-on system of the linked genes ageB, secG, and geaB [45]. With the use of this CRISPR/Cas9 and Tet-on system pairing, we may be able to boost both the creation of organic acids and proteins. So, by interrupting the pyrG gene, several studies are carried out in A. niger for the investigation of the synthesis and research of enzyme preparations [46,47,48] and metabolites such as pectinases, trehalases, citric acid, and succinic acid, among others [49,50].

Although Rhizopus arrhizus is one organisms, generally recognised as safe for the manufacture of alcoholic beverages like ragi or tempeh—it has mostly been utilised in industrial fermentation. Based on the synthesis of the principal organic acid when the strains are cultured on D-glucose, they are divided into two categories [82]. Whereas the first group creates lactic acid, the other produces fumaric and L(+)-malic acids. These substances are generated in high quantities on a range of carbon-containing substrates. For instance, compared to their respective theoretical yields, the output of L(+)-lactic acid and ethanol rose by 80%, while fumaric acid production increased by 65%.

Chemical mutagenesis is employed to create auxotrophic mutants in R.arrhizus that use N-methyl-N-nitro-N-nitrosoguanidine to augment L(+)-lactic acid synthesis with diethyl sulphate [80,81]. By combining all the necessary elements in a single vector, Baldin et colleagues used a plasmid-based CRISPR/CAS method in R.arrhizus to interrupt

the target gene [32]. Using a small interference RNA strategy in genes ldhA and ldhB increases the production of ethanol manifolds.[129]

In addition, M.circinelloides has received a great deal of experimental use for the industrial synthesis of enzymes, pigments [56], and other compounds. Because of its capacity to create important proteins, enzymes, organic acids, polyphenols, and bacterial lipids, it is sometimes referred to as a well-established "microbial factory" [59,60]. Conventional gene deletion constructs have also been developed for Mucor in order to knock out the specific gene [32]. For instance, the significance of the crgA gene in the production of carotenoid-induced light pigments has been demonstrated by analysis of its null mutant using gene replacement techniques. The detrimental effect on carotenogenesis was lessened by the crgA gene disruption. [62,63]. In M.circinelloides, Nagy et al. recently developed a unique CRISPR-Cas9 system that demonstrated its use as a site-specific mutagene since it did not need the use of plasmids or RNP synthesis [61]. They employed this strategy to disrupt the carB and hmgR2 genes, which causes viable mutants to arise. The Cas9 enzyme creates an RNP complex here to construct the gene of interest that is translated into gRNA (in vitro), which is then converted into a cell.

B. Transformation

Rhizopus arrhizus is a flexible organism that serves a number of functions. Three separate transformation mechanisms have been established in Rhizopus arrhizus, including the Agrobacterium tumefaciens-mediated transformation (ATMT) [64], the CaCl2/PEG method protoplast generation and transformation with exogenous DNA [64], and the particle bombardment DNA delivery method [65]. The most current metabolic engineering technologies for integrating heterologous genes with the goal of creating novel products or a pathways include RNAi, spontaneous mutagenesis, gene knockout procedures, etc. Numerous auxotrophic selection markers permit the insertion of several genes, which may be employed to find out what happens to inserted DNA as it seldom gets incorporated into the genome, according to hundreds of research that have been published. A plasmid-based strategy utilized by Baldin et al. in Rhizopus to disrupt certain genes. For this, the team merged each of the critical elements into a solitary vector [52]. Meanwhile, the other research team, Fuller and colleagues, employed this CRISPR/Cas9-based gene editing approach to explore gene-related functionalities by focusing on the loss of function in PKS [32] since PKS is a critical enzyme that creates toxins within filamentous fungus [53]. Genetically modified fungi are produced when this gene is disrupted, which lessens the negative impact on the hosts [54].As one molecular method for gene silencing, RNAi is utilized to manipulate Rhizopus's genetic makeup. The existence of 2 Argonaute copies, 1 Dicer, and 5 RdRP-encoding genes in RNAi is shown by the genome sequencing of R. arrhizus variant 99-880. Ibrahim et al. tested the effectiveness of RNAi by using it to silence the ftr1 gene, that encodes a high-affinity iron permease. Nevertheless, a plasmid design was created using sense and antisense forms of this gene that are split by a spacer element. This construct, when translated, creates hairpin loops (hpRNA), which constitute the initial step in the RNA interference (RNAi) silencing process. The iron intake by transformants was reduced by roughly 50%[55] as a result of this being silenced.

Van Heeswijck and Roncero were the first scientists to employ calcium chloride and a technique based on polyethylene glycol to transform the plasmid in Mucor circinelloides in 1984 [68,69]. Using approaches like Agrobacterium tumefaciens-mediated transformation [70], biolistic-mediated transformation [71], and limited enzyme-mediated transformation (REMI) [72] has enhanced the transformation process. For Mucor, although, the protoplast-mediated approach was the most effective [69]. Important advances in the control of fungi's genes have been made in M. circinelloides as a result of genetic tools and knocking off specific genes [66]. In M. circinelloides, Nagy et al. recently created a new CRISPR-Cas9 system without the need for plasmids or in-vitro RNP production, and they demonstrated its effectiveness as a site-specific mutagenesis method [61]. And as was already noted in the beginning, this in-vitro technique offers a number of significant benefits over the other in-vivo approaches.

This CRISPR/Cas9 technique is used in the industry for a variety of purposes, including gene editing of fungus species known as "microbial cell factories," such as Aspergillus fumigatus and other Aspergillus species. The A.fumigatus pksP gene was first employed as a case analysis for the explanation of the reason of the melanin production that this gene triggers as well as for the initial validation of the documented CRISPR/Cas9 system effectiveness in genome editing [76]. Based on this, the very effective, reliable, and exact genome editing method known as microhomology-mediated end joining (MMEJ) was developed, which uses an incredibly small homologous arm that is around 35 bp long [77]. This method allowed for the editing and integration of a pksP, a cnaA, the catalytic component of the calcineurin gene, and an exogenous GFP at several target locations. Umeyama and associates used this method to substitute the gene cyp51A in the azole-resistant clinical A.fumigatus [78]. They concurrently introduced Cas9/gRNA ribonucleoprotein structures into the cells while also delivering the donor template. The testing of azole susceptibility in transformants is then conducted after Ser138 has been replaced with glycine in order to demonstrate the increased susceptibility [78]. Furthermore, by reusing the genome editing-based plasmids AMA1, an enhanced CRISPR/Cas9 method in A. oryzae was created, enabling the efficient multiple gene INDEL (insertion and deletion). Moreover, these plasmids carry the "ptrA" indicator for drug resistance [35]. Using circular DNA as a source improves the efficacy of HDR-mediated genome editing. A.oryzae has effectively built both an in vitro assembly and an instantaneous genome editing technology. The cas9-gRNA RNP complex constituted the foundation for this method [36].

Mortierella alpina has also undergone effective transformation during the recent past. In order to create a resistant strain, Mackenzie originally described the transformation strategy in 2000. He used the homologous histone (hisH4.1) promoter to transform the hygromycin resistance gene [67]. The rate of transformation and mitotic

durability were enhanced in close to 60 to 80% of the transformants using the microprojectile bombardment and ATMT procedures developed by Ando et al. [73,74]. DNA from an external source that has been merged with the chromosome by homologous recombination (HR) and non-homologous end-joining (NHEJ) is processed by the DNA dual-strand break repair. Targeting and disrupting genes for the introduction of non-native genes is frequently done using the HR method. The effectiveness of the mechanism has also been increased by the removal of crucial proteins involved in NHEJ [75].

C. Lipid Metabolism and Production

All esters of intermediate to long-chain fatty acids make up the naturally existing class of organic molecules known as lipids, which are soluble in non-polar organic solvents but indissoluble in water. All life forms, including bacteria, fungi, algae, plants, and others, have fatty acid production as one of their common metabolic processes. These fatty acids serve as the foundations for membranes or are used to store energy as poly hydroxyalkanoates or triacylglycerols in lipid droplets. To improve this production of fatty acids through lipid metabolism, CRISPR/Cas9 techniques are being employed extensively.

Commercial-scale production of a variety of physiologically active polyunsaturated fatty acids (PUFAs), including eicosapentaenoic acid and arachidonic acid (ARA), may be possible thanks to M.alpina, a filamentous fungus[83]. Due of the high-value PUFAs contained in M.alpina's triacylglycerol profiles, this species has been employed in the industrial manufacture of high-value PUFAs since 1987[84]. Considering the amount of arachidonic acid (>50% of its entire fatty acid composition) in M. alpina lipids, these compounds are frequently used as dietary supplements.Both n-3 and n-6 PUFA can be synthesized by M.alpina, and fermentation and genetic modification can be used to regulate output. Modern techniques like CRISPR/Cas9 are utilized to dramatically enhance the quantity of lipid output, and studies on M.alpina lipid metabolism are increasingly being undertaken to utilize the organism to produce specific fatty acids [85]. A common approach for enhancing M.alpina's lipid output continues to be the coupling of mutation and genetic alteration with fermentation optimization [86, 87]. In 2000, Mackenzie published the first description of an M.alpina modification in which the hygromycin resistance gene was inserted into the protoplast of M.alpina CBS 224.37. This produced a resistant strain using the traditional polyethylene glycol technique [67].

It has been revealed that M.alpina has a fair amount of genes involved in the synthesis of fatty acids and reducing power. In an increased expression investigation, Δ12 desaturase was abundantly expressed in M.alpina JT-180, and the synthesis of ARA significantly increased (66.7% of overall fatty acids versus the wild-type strain 1S-4) [88]. Being known that enhancing the accessibility of the reducing power NADPH would promote lipid synthesis in M.alpina, Hao et al. abundantly expressed the malic enzymes malE1 and malE2, glucose-6-phosphate dehydrogenase (G6PD2), and 6-phosphate gluconate dehydrogenase in M.alpina. Although malE2 overexpression increased the level of ARA by 1.5 times, overexpression of G6PD2 caused the synthesis of total fatty acids to rise by 1.7 times [89]. In a different investigation, the -linolenic acid elongase gene was thrivingly inserted into a desired variety of the fungus. This gene produces the fatty acid elongase EL2, which promotes the conversion of -linolenic acid into dihomo-linolenic acid. In contrast to the parent strain, this led to a two-fold rise in ARA formation [90].To enhance the fungus's use of glycerol, Hao et al. genetically altered it. They observed that overexpressing glycerol kinase (GK) and glycerol-3-phosphate dehydrogenase (G3PD) boosted overall fatty acid content upwards to 35%, although G3PD had no discernible effect on lipid build-up. In M.alpina grown in pure glycerol, co-overexpression of GK and malic enzyme (ME1) was reported to enhance fatty acid formation by 81%. MaLeuB, the gene that controls IPMDH production, was overexpressed homologously by Tangs et al. As a consequence of a fourfold elevation in the MaLeuB transcriptional level, they revealed that the overall amount of fatty acids of the recombinant strain was 20.2% greater compared to the control strain [83] [32,91,92,93].

Saccharomyces cerevisiae lipids are valuable as sustainable substitutes for crude oil in the synthesis of chemicals and fuels, and yeasts that have had their metabolism altered to increase output may create significant amounts of sustainable compounds from basic, easily accessible starting materials. In yeast, acetyl-CoA is converted to fatty acids through a multi-enzyme system with six active enzymes that is found in the cytoplasm. Condensation, reduction of the carbonyl group, dehydration, and reduction of the double bond are all steps in this operation. In S.cerevisiae, targeted gene or pathway modifications typically result in higher lipid content; however, this improvement is modest, and for more significant improvement in lipid production, it is necessary to combine strategies that push the central carbon flux towards fatty acid biosynthesis by improving the biosynthesis of precursors like acetyl-CoA, malonyl-CoA, and the fatty acyl-CoA pool or pull the free fatty acid towards stored lipid. [94,95]. By interrupting neutral lipid recycling, which involves -oxidation and FA accumulation FAA2, PXA1, POX1, FAA1, FAA4, FAT1, and co-expression of DGA1 and TGL3, the Da Silva group was able to produce 2.2 g/L external free fatty acids in S. cerevisiae [96]. The Nielsen group obtained 10.4 g/L extracellular FFAs in 2016 [97] by advancing the acetyl-CoA route, malonyl-CoA pathway, and reverse pathway and interrupting FA production. In addition, the Nielsen group has achieved an important development by changing the metabolism of yeast to convert from alcoholic fermentation to lipogenesis, developing a synthetic oil yeast that could produce up to 33.4 g/L FFA. The Nielsen group published the maximum TAG concentration of 254 mg TAG/g DCW in S.cerevisiae and reached 27.4% of the greatest yield possible [95] based on the gene pairing for internal deposition. The engineering strains were created in these advancements utilizing the most recent CRISPR genome editing techniques, and the lipid content and titer were further improved using these approaches [98].

D. Heterologous Protein Production

Heterologous protein expression has formed a crucial backbone of industrial and pharmaceutical biotechnology across a wide array of production platforms for years. Since heterologous proteins are usually stored intracellularly, endoplasmic reticulum (ER) strain and ineffective secretion follow, and heterologous protein synthesis is frequently lower than predicted in yeast despite attempts to develop protein secretory pathways. It has been commercially exploited with different types of host systems, expression systems, and strategies to maximise or increase the yield of the desired product above a threshold or above its natural production capacity. Significant animal and plant-derived proteins were synthesised utilising Aspergillus oryzae in heterologous protein synthesis, and various strains with high productivity were utilised. Many heterologous proteins have been produced by continually eliminating 10 protease genes [107-108] relying on transformation marker recycling [109-110] and editing genes with CRISPR systems linked to protein secretion transport [104-106]. A.oryzae has been used in the production of heterologous proteins because of its capacity to secrete a sizable amount of proteins into the environment and the guarantee of its safety provided by its broad use in the food industry [111]. Although A.oryzae is a hygienic host that produces very little accompanying metabolites, it is nonetheless used in biosynthesis research because, when foreign biosynthetic enzyme genes are added to it, it may synthesize a sizable amount of heterologous herbal products. Until recently, only wild strains of A.oryzae were capable of this type of genetic modification (RIB40). The creation of sake, soy sauce, and miso, for example, may all be accomplished using different commercial strains of A.oryzae, each of which has distinctive features and qualities. The application of genome editing to produce heterologous proteins utilizing A.oryzae has risen significantly. The quadruple auxotrophic strain NSAR1 of the fungus A. oryzae is widely utilized as a host when natural products are synthesized heterologously [114,115]. The amount of times that the quadruple auxotrophic strain NSAR1 can be genetically modified is currently limited, but metabolic engineering employing effective multiple-gene modification technology has the ability to indefinitely raise the quantity of natural product synthesis. The formation of the secondary metabolite kojic acid was suppressed by altering the expression of the genes kojiA and kojiR involved in kojic acid biosynthesis as a result of endogenous metabolic change in A.oryzae [112]. Fan J, Zhang Z., et al. employed the CRISPR/Cas9 system to eliminate Aogld3, encoding a potential glycerol dehydrogenase.The Aooch1 gene, which is crucial for the formation of high-mannose N-glycan, was deleted using the CRISPR/Cas9 system, and Huynh, H.H. et al. reported that this impacted the structure of the N-glycan in the human antibody generated by A.oryzae. The ability to modulate antibody function by emulating the mammalian N-glycan structure will be facilitated by further genome editing of the gene for the expression of several heterologous glycotransferases, which will bolster the potential of A.oryzae as a system for the generation of heterologous proteins [113].

An effective host that can be utilized for the commercial manufacture of proteins is the thermotolerant methylotrophic yeast O.thermomethanolica TBRC 656. Clustered interspaced short palindromic repeats (CRISPR)-CRISPR-associated protein 9 (Cas9) techniques were employed to produce its mutants lacking the proteins' secretory expression. As a result, the model protein xylanase was secreted at significantly lower levels, which resulted in the loss of function of the oxidative stress (sod1Δ), vacuolar and protein sorting (vps1Δ and ypt7Δ), and sod1 genes in the mutants. But in the autophagy-related atg12A mutant, xylanase secretion was unaffected. SOD1, VPS1, and YPT7 genes were activated using a technique for sequence-specific activation of target gene expression (CRISPRa) in O.thermomethanolica. The gene-activated mutants produced increased non-glycosylated xylanase and glycosylated phytase, suggesting the effectiveness of the CRISPR/Cas9 system as a tool for deciphering O.thermomethanolica protein secretion genes and their potential use in enhancing heterologous protein secretion in this yeast. [116,117]

E. Genome Evolutionary Engineering

The ability to efficiently generate characteristics that are customized to an organism's requirements as well as the identification of pertinent gene targets that control physiological processes, such as stress resistance, metabolic pathway optimization, and organismal adaptation, have significantly benefited from the advancement of genome editing and evolutionary technologies in recent years. Particularly in species like S. cerevisiae, directed genome evolution has proven to be an adaptable technique that enables scientists to extract desired characteristics and investigate the evolution of these characteristics.Alterations in gene transcriptional levels are seen through repression, activation, and deletion. In order to access evolved strains and conduct genetic searches to comprehend genotype-phenotype correlations, these aberrations are indispensable. Techniques that accurately identify specific genes are needed for this. In this situation, the DNA endonuclease Cas9 is guided by the clustered regularly interspaced short palindromic repeats (CRISPR) system to cleave targets by utilizing all guide RNAs (sgRNAs) with similarity to certain loci [118, 119], and this method offers a flexible framework for evolutionary engineering. Integrating up- and down-regulation with sgRNA design has been the focal point of recent developments in CRISPR/Cas phenotypic evaluation. CRISPR interference (CRIPSRi) occurs when RNA polymerase's ability to associate with and/or extend itself is sterically inhibited by catalytically inactive Cas9 (dCas9), which lacks cleavage activity [119]. The TEF1 promoter-driven green fluorescent protein (GFP) fluorescence level in S. cerevisiae served as a demonstration of the concept for this strategy. sgRNA targeting the TEF1 promoter in combination with the expression of dCas9 led to an 18-fold reduction in fluorescence intensity [120]. Furthermore, coupling dCas9 with the transcriptional repressor Mxi1 increases fluorescence suppression by a factor of 53. Contrary to various transcriptional repressor expressions in CRISPRi, stringent merger with the activation domain is necessary for CRISPR aviation (CRISPRa) to enhance transcriptional levels. [121]. To enhance transcriptional activity, the VP64 activator was linked to dCas9 and directed to both natural and synthetic promoters. In S. cerevisiae, a

more effective hybrid VP64-p65-Rta tripartite activator (VPR) was fused to dCas9, yielding activation ranging from 5 to 300 times greater than the VP64-based activator [122]. Similarly, the target gene's impact on a particular phenotype was examined using full open reading frame (ORF) deletion. By employing fully catalytic Cas9, synthetic chips made of gRNA, and arbitrarily knocking off recombinant donors, the CHaNGE technique was developed. To avoid loss while culturing, the gRNA, and donor were.

Both integrated into the plasmid. The optical density (OD) at 600 nm in 10 mM furfural was elevated 8.1 times by two rounds of synthetic DNA transformation [123]. The aforementioned techniques have each been used effectively for both up- and down-regulation. On the other hand, complex phenotypes frequently comprise multiplex genes that are both up-and down-regulated, necessitating the use of techniques that can concurrently achieve overexpression, repression, and deletion. The targeted genes are expressed in a graded manner using the STEPS technique, which is composed of two fusion proteins, dCas9Mxi1 and dCas9-VPR. Xylose breakdown, 3-dehydroshikimate synthesis, and glycerol fermentation have all shown efficacy using STEPS, which has been utilized in determining the limiting steps of these reactions [124]. The MAGIC technique combines three functions to regulate the degrees of gene expression across the S. cerevisiae genome, which is a less complex approach [125]. Three distinct sgRNA libraries are used to direct three separate structures of proteins, dLbCas12a-VP, dSpCas9-RD1152, and SaCas9, with activation, repression, and deletion functionalities, to genomic loci [126].

III. CONCLUSION

This review highlights a number of key applications and methodologies for manipulating the genomes of fungal species in order to conduct meaningful investigations on metabolite synthesis and gene regulation. Even though strides have been made in overcoming the difficulties of genetic modification in these species, we are still a long way from completely comprehending them. This is because some of these fungi are difficult to genetically manipulate because there aren't as numerous genetic tools available to us. Improved research is required to better understand the potential of these fungi and to alter advantageous channels to increase the production of diverse beneficial compounds. CRISPR-based methodologies have been identified as significantly more advantageous than other strategies for a variety of reasons, including the fact that they enable a relatively simple design process and more cost-effective and quicker execution when compared to other methods. making them extremely preferable engineering tools for industrially significant microorganisms that have little or no genetic features for transformation. For example, using strategies like genome reduction to get rid of unwanted byproducts and the use of the CRISPR/CAS9 system to make sets of deletion mutant strains over the fungal gene interaction machinery, respectively, could make the advancement of fungal cell factories for the production of high-value-added compounds and metabolic products more cost-effective. These genetic approaches have been successful in extracting valuable bioactive substances from crucial fungi for industry.

Table 1: Stratergies of Crispr/Cas9 genome editing tool employed to manipulate various genes/enzymes to enhance their functions, to be utilized in various applications

Applications	Species	Strategy Used	Genes/ Enzymes	Result	Ref.
Fermentation	*Aspergillus oryzae*	Deletion or Insertion	ecdR	Enhanced the production of products by 10-20%.	[36]
	Aspergillus niger	Gene Disruption and Deletion	*pyrG*, glu F, gluD	Enhance protein and organic acid production.	[43, 44]
	Rhizopus arrhizus	Smallinterfering RNA	ldhA and ldhB	Improvement in ethanol output by 15.4%.	[127]
	Mucor circinelloides	Mutation and deletion of crgA gene.	crgA	Increased the production of Carotenoids.	[62]
Transformation	*Rhizopus arrhizus*	RNA interference, random mutation, and gene knockout techniques.	Polyketide synthase (PKS)	Together with a 50% drop in iron intake, the detrimental effects of toxins on the hosts are reduced.	[53, 54,55]
	Mucor circinelloides	Gene Disruption	carB and hmgR2	Generation of stable Mutants	[62]
	Aspergillus fumigatus and other species	Gene replacement with RNP	Ser138	Increased azole susceptibility in transformants	[78]
	Mortierella alpina	Gene Disruption	Hygromycin resistant gene	Improvement in transformation frequency.	[67, 73,74]
Lipid Metabolism	*Mortierella alpina*	i.Co-overexpression ii.Overexpression iii.Homologous overexpression iv.Overexpression	i.Glycerol Kinase(GK) and malic enzyme (ME1) ii.GK and glycerol-3p hosphate dehydrogen ase (G3PD) iii.MaLeuB iv.Δ12 desaturase	i.Increased fatty acid accumulation by 81%. ii. This raises the amount of total fatty acids by about 35%. iii.Fatty acid content 20.2% higher in recombinant strain. iv.66.7% higher fatty acid than wild strain	[83,88,91 ,92,93]
	Saccharomyces cerevisiae	i.Deletion or gene knockout ii.Deletion or gene knockout iii.Co-expression	i) oxidation FAA2, PXA1, POX1, ii.FAA1, FAA4, FAT1	i.Elevation of over 55% in intracellular fatty acids. ii. Rise in free fatty acids outside of cells. iii. Increased1.3 g/L extracellular FFAs	[98]
Heterologous Protein Production	*Aspergillus oryzae*	Deletion	i.kojA and kojR genes ii.*Aooch1* gene	i.Production of kojic acid was decreased ii.N-glycan structure altered	[112,113]
	Ogataea thermomethanolia	Sequence-specific activation of target gene expression (CRISPRa)	Genes like SOD1, VPS1 and YPT7.	Enhancement in the production of both non-glycosylated xylanase and glycosylated phytase.	[116,117]

ACKNOWLEDGMENT

We would like to express our gratitude to Professor Surinder Kaur Walia and Dr Gurpreet Kaur of Shri Guru Teg Bahadur Khalsa College, University of Delhi, for guiding us and inspiring us to write this article. Also, we are very thankful to both our teachers for making us understand the importance of correct information in publications and scientific writing.

REFERENCES

[1.] Morin-Sardin, S.; Nodet, P.; Coton, E.; Jany, J.L. Mucor: A Janus-faced fungal genus with human health impact and industrial applications. Fungal Biol. Rev. 2017, 31, 12–32. [CrossRef]
[2.] Lübbehüsen, T.L.; Nielsen, J.; McIntyre, M. Characterization of the Mucor circinelloides life cycle by on-line image analysis. J. Appl. Microbiol. 2003, 95, 1152–1160. [CrossRef]
[3.] Lee, S.C.; Li, A.; Calo, S.; Heitman, J. Calcineurin Plays Key Roles in the Dimorphic Transition and Virulence of the Human Pathogenic Zygomycete Mucor circinelloides. PLoS Pathog. 2013, 9, e1003625. [CrossRef]
[4.] Samson, R.A.; Visagie, C.M.; Houbraken, J.; Hong, S.B.; Hubka, V.; Klaassen, C.H.; Perrone, G.;Seifert, K.A.; Susca, A.; Tanney, J.B.; et al. Phylogeny, identification and nomenclature of the genus Aspergillus. Stud. Mycol. 2014, 78, 141–173. [CrossRef]
[5.] Challacombe, J.F.; Hesse, C.N.; Bramer, L.M.; McCue, L.A.; Lipton, M.; Purvine, S.; Nicora, C.; Gallegos-Graves, V.; Porras-Alfaro, A.; Kuske, C.R. Genomes and secretomes of Ascomycota fungi reveal diverse functions in plant biomass decomposition and pathogenesis. BMC Genom. 2019, 20, 976. [CrossRef] [PubMed]
[6.] Kobayashi, T.; Abe, K.; Asai, K.; Gomi, K.; Juvvadi, P.R.; Kato, M.; Kitamoto, K.; Takeuchi, M.; Machida, M. Genomics of Aspergillus oryzae. Biosci. Biotechnol. Biochem. 2007, 71, 646–670.[CrossRef]
[7.] Kitamoto, K. Cell biology of the Koji mold Aspergillus oryzae. Biosci. Biotech. Biochem. 2015, 79, 863–869. [CrossRef] [PubMed]
[8.] Nishimura, I.; Shinohara, Y.; Oguma, T.; Koyama, Y. Survival strategy of the salt-tolerant lactic acid bacterium, Tetragenococcus halophilus, to counteract koji mold, Aspergillus oryzae, in soy sauce brewing. Biosci. Biotech. Biochem. 2018, 82, 1437–1443. [CrossRef] [PubMed]
[9.] Papagianni, M. Advances in citric acid fermentation by Aspergillus niger: Biochemical aspects, membrane transport and modeling. Biotechnol. Adv. 2007, 25, 244–263. [CrossRef] [PubMed]
[10.] Avalos, J.L.; Fink, G.R.; Stephanopoulos, G. Compartmentalization of metabolic pathways in yeast mitochondria improves the production of branched-chain alcohols. Nat. Biotechnol. 2013, 31, 335–341. [CrossRef] [PubMed]
[11.] Shen, B.; Zhou, P.; Jiao, X.; Yao, Z.; Ye, L.; Yu, H. Fermentative production of Vitamin E tocotrienols in Saccharomyces cerevisiae under cold-shock-triggered temperature control. Nat. Commun. 2020, 11, 5155. [CrossRef] [PubMed]
[12.] Meadows, A.L.; Hawkins, K.M.; Tsegaye, Y.; Antipov, E.; Kim, Y.; Raetz, L.; Dahl, R.H.; Tai, A.; Mahatdejkul-Meadows, T.; Xu, L.; et al. Rewriting yeast central carbon metabolism for industrial isoprenoid production. Nature 2016, 537, 694–697. [CrossRef] [PubMed]
[13.] Paddon, C.J.; Westfall, P.J.; Pitera, D.J.; Benjamin, K.; Fisher, K.; McPhee, D.; Leavell, M.D.; Tai, A.; Main, A.; Eng, D.; et al. High-level semi-synthetic production of the potent antimalarial artemisinin. Nature 2013, 496, 528–532. [CrossRef] [PubMed]
[14.] Xie, W.; Lv, X.; Ye, L.; Zhou, P.; Yu, H. Construction of lycopene-overproducing Saccharomyces cerevisiae by combining directed evolution and metabolic engineering. Metab. Eng. 2015, 30, 69–78. [CrossRef]
[15.] Zhu, M.; Wang, C.; Sun, W.; Zhou, A.; Wang, Y.; Zhang, G.; Zhou, X.; Huo, Y.; Li, C. Boosting 11-oxo-β-amyrin and glycyrrhetinic acid synthesis in Saccharomyces cerevisiae via pairing novel oxidation and reduction system from legume plants. Metab. Eng. 2018, 45, 43–50. [CrossRef]
[16.] Yu, T.; Zhou, Y.J.; Huang, M.; Liu, Q.; Pereira, R.; David, F.; Nielsen, J. Reprogramming Yeast Metabolism from Alcoholic Fermentation to Lipogenesis. Cell 2018, 174, 1549–1558. [CrossRef]
[17.] Dai, Z.; Huang, M.; Chen, Y.; Siewers, V.; Nielsen, J. Global rewiring of cellular metabolism renders Saccharomyces cerevisiae Crabtree negative. Nat. Commun. 2018, 9, 3059. [CrossRef]
[18.] Hsu, P.D.; Scott, D.A.; Weinstein, J.A.; Ran, F.A.; Konermann, S.; Agarwala, V.; Li, Y.; Fine, E.J.; Wu, X.; Shalem, O.; et al. DNA targeting specificity of RNA-guided Cas9 nucleases. Nat. Biotechnol. 2013, 31, 827–832. [CrossRef]
[19.] DiCarlo, J.E.; Norville, J.E.; Mali, P.; Rios, X.; Aach, J.; Church, G.M. Genome engineering in Saccharomyces cerevisiae using CRISPR-Cas systems. Nucleic Acids Res. 2013, 41, 4336–4343. [CrossRef]
[20.] Copeland, M.F.; Politz, M.C.; Pfleger, B.F. Application of TALEs, CRISPR/Cas and sRNAs as trans-acting regulators in prokaryotes. Curr. Opin. Biotech. 2014, 29, 46–54. [CrossRef]
[21.] Stojkovic, M.; Han, D.; Jeong, M.; Stojkovic, P.; Stankovic, K.M. Human induced pluripotent stem cells and CRISPR/Cas-mediated targeted genome editing: Platforms to tackle sensorineural hearing loss. Stem Cells 2021, 39, 673–696. [CrossRef]
[22.] Marraffini, L.A. The CRISPR-Cas System of Streptococcus Pyogenes: Function and Applications; University of Oklahoma Health Sciences Center: Oklahoma City, OK, USA, 2016.
[23.] Shmakov, S.; Smargon, A.; Scott, D.; Cox, D.; Pyzocha, N.; Yan, W.; Abudayyeh, O.O.; Gootenberg, J.S.; Makarova, K.S.; Wolf, Y.I.; et al. Diversity and evolution of class 2 CRISPR-Cas systems. Nat. Rev. Microbiol. 2017, 15, 169–182. [CrossRef] [PubMed]
[24.] Makarova, K.S.; Wolf, Y.I.; Alkhnbashi, O.S.; Costa, F.; Shah, S.A.; Saunders, S.J.; Barrangou, R.; Brouns, S.J.; Charpentier, E.; Haft, D.H.; et al. An updated evolutionary classification of CRISPR-Cas systems. Nat. Rev. Microbiol. 2015, 13, 722–736. [CrossRef] [PubMed]
[25.] Doudna, J.F.; Charpentier, E. The new frontier of genome engineering with CRISPR-Cas9. Science 2014, 346, 1258096. [CrossRef]
[26.] Ibrahim, A.S.; Skory, C.D. Genetic Manipulation of Zygomycetes. In Medical Mycology: Cellular and

Molecular Techniques; Wiley: New York, NY, USA, 2007; ISBN 9780470019238.
[27.] Zalatan, J.G.; Lee, M.E.; Almeida, R.; Gilbert, L.A.; Whitehead, E.H.; La Russa, M.; Tsai, J.C.; Weissman, J.S.; Dueber, J.E.; Qi, L.S. et al. Engineering complex synthetic transcriptional programs with CRISPR RNA scaffolds. Cell 2015, 160, 339–350. [CrossRef]
[28.] Nielsen, J.C.; Nielsen, J. Development of fungal cell factories for the production of secondary metabolites: Linking genomics and metabolism. Synth. Syst. Biotechnol. 2017, 2, 5–12[CrossRef]
[29.] Calderone, R.; Sun, N.; Gay-Andrieu, F.; Groutas, W.; Weerawarna, P.; Prasad, S.; Alex, D.; Li, D. Antifungal drug discovery: The process and outcomes. Future Microbiol. 2014, 9, 791–805. [CrossRef]
[30.] Hara, S.; Jin, F.J.; Takahashi, T.; Koyama, Y. A further study on chromosome minimization by protoplast fusion in Aspergillus oryzae. Mol. Genet. Genom. 2012, 287, 177–187. [CrossRef]
[31.] Roemer, T.; Boone, C. Systems-level antimicrobial drug and drug synergy discovery. Nat. Chem. Biol. 2013, 9, 222–231. [CrossRef]
[32.] Mohamed, H.; Naz, T.;Yang, J.; Shah, A.M.; Nazir, Y.; Song, Y.Recent Molecular Tools for the Genetic Manipulation of Highly Industrially Important Mucoromycotina Fungi. J.Fungi 2021, 7, 1061.
[33.] Papp, T.; Csernetics, Á.; Nyilasi, I.; Ábrók, M.; Vágvölgyi, C. Genetic transformation of zygomycetes fungi. In Progress in Mycology;Springer: Singapore, 2010; ISBN 9789048137138
[34.] Maruyama, J.I. Genome Editing Technology and Its Application Potentials in the Industrial Filamentous Fungus Aspergillus oryzae. J. Fungi 2021, 7, 638. [CrossRef]
[35.] Katayama, T.; Tanaka, Y.; Okabe, T.; Nakamura, H.; Fujii, W.; Kitamoto, K.; Maruyama, J. Development of a genome editing technique using the CRISPR/Cas9 system in the industrial filamentous fungus Aspergillus oryzae. Biotechnol. Lett. 2016, 38,637–642. [CrossRef]
[36.] Jin, F.J.; Nishida, M.; Hara, S.; Koyama, Y. Identification and characterization of a putative basic helix-loop-helix transcription factor involved in the early stage of conidiophore development in Aspergillus oryzae. Fungal Genet. Biol. 2011, 48, 1108–1115. [CrossRef]
[37.] Nakamura, H.; Katayama, T.; Okabe, T.; Iwashita, K.; Fujii, W.; Kitamoto, K.; Maruyama, J.I. Highly efficient gene targeting in Aspergillus oryzae industrial strains under ligD mutation introduced by genome editing: Strain-specific differences in the effects of deleting EcdR, the negative regulator of sclerotia formation. J. Gen. Appl. Microbiol. 2017, 63, 172–178. [CrossRef]
[38.] Katayama, T.; Nakamura, H.; Zhang, Y.; Pascal, A.; Fujii, W.; Maruyama, J.I. Forced Recycling of an AMA1-Based Genome-Editing Plasmid Allows for Efficient Multiple Gene Deletion/Integration in the Industrial Filamentous Fungus Aspergillus oryzae. Appl. Environ. Microbiol. 2019, 85, e01896-18. [CrossRef]
[39.] Zou, G.; Xiao, M.; Chai, S.; Zhu, Z.; Wang, Y.; Zhou, Z. Efficient genome editing in filamentous fungi via an improved CRISPR-Cas9 ribonucleoprotein method facilitated by chemical reagents. Microb. Biotechnol. 2021, 14, 2343–2355. [CrossRef]
[40.] Jin, F.-J.; Wang, B.-T.; Wang, Z.-D.; Jin, L.; Han, P. CRISPR/Cas9-Based Genome Editing and Its Application in Aspergillus Species. J.Fungi 2022, 8, 467. [Ref]
[41.] Kuivanen, J.; Wang, Y.M.J.; Richard, P. Engineering Aspergillus niger for galactaric acid production: Elimination of galactaric acid catabolism by using RNA sequencing and CRISPR/Cas9. Microb. Cell Factories 2016, 15, 210. [CrossRef] [PubMed]
[42.] Kuivanen, J.; Arvas, M.; Richard, P. Clustered Genes Encoding 2-Keto-l-Gulonate Reductase and l-Idonate 5-Dehydrogenase in the Novel Fungal d-Glucuronic Acid Pathway. Front. Microbiol. 2017, 8, 225. [CrossRef]
[43.] Kuivanen, J.; Richard, P. NADPH-dependent 5-keto-D-gluconate reductase is a part of the fungal pathway for D-glucuronate catabolism. FEBS Lett. 2018, 592, 71–77. [CrossRef]
[44.] Kuivanen, J.; Korja, V.; Holmstrom, S.; Richard, P. Development of microtiter plate scale CRISPR/Cas9 transformation method for Aspergillus niger based on in vitro assembled ribonucleoprotein complexes. Fungal. Biol. Biotechnol. 2019, 6, 3. [CrossRef]
[45.] Cairns, T.C.; Feurstein, C.; Zheng, X.; Zhang, L.H.; Zheng, P.; Sun, J.; Meyer, V. Functional exploration of co-expression networks identifies a nexus for modulating protein and citric acid titres in Aspergillus niger submerged culture. Fungal Biol. Biotechnol. 2019, 6, 18. [CrossRef]
[46.] Dong, L.; Lin, X.; Yu, D.; Huang, L.; Wang, B.; Pan, L. High-level expression of highly active and thermostable trehalase from Myceliophthora thermophila in Aspergillus niger by using the CRISPR/Cas9 tool and its application in ethanol fermentation.J. Ind. Microbiol. Biotechnol. 2020, 47, 133–144. [CrossRef] [PubMed]
[47.] Alazi, E.; Niu, J.; Otto, S.B.; Arentshorst, M.; Pham, T.T.M.; Tsang, A.; Ram, A.F.J. W361R mutation in GaaR, the regulator of D-galacturonic acid-responsive genes, leads to constitutive production of pectinases in Aspergillus niger. Microbiologyopen 2019,8, e00732. [CrossRef] [PubMed]
[48.] Dong, L.; Yu, D.; Lin, X.; Wang, B.; Pan, L. Improving expression of thermostable trehalase from Myceliophthora sepedonium in Aspergillus niger mediated by the CRISPR/Cas9 tool and its purification, characterization. Protein Expr. Purif. 2020, 165, 105482.[CrossRef] [PubMed]
[49.] Yang, L.; Henriksen, M.M.; Hansen, R.S.; Lubeck, M.; Vang, J.; Andersen, J.E.; Bille, S.; Lubeck, P.S. Metabolic engineering of Aspergillus niger via ribonucleoprotein-based CRISPR-Cas9 system for succinic acid production from renewable biomass.Biotechnol. Biofuels 2020, 13, 206. [CrossRef] [PubMed]
[50.] Zhang, L.; Zheng, X.; Cairns, T.C.; Zhang, Z.; Wang, D.; Zheng, P.; Sun, J. Disruption or reduced expression of the orotidine-5'-decarboxylase gene pyrG increases

citric acid production: A new discovery during recyclable genome editing in Aspergillus niger. Microb. Cell Factories 2020, 19, 76. [CrossRef] [PubMed]

[51.] Roden, M.M.; Zaoutis, T.E.; Buchanan, W.L.; Knudsen, T.A.; Sarkisova, T.A.; Schaufele, R.L.; Sein, M.; Sein, T.; Chiou, C.C.; Chu,J.H.; et al. Epidemiology and outcome of zygomycosis: A review of 929 reported cases. Clin. Infect. Dis. 2005, 41, 634–656.[CrossRef].

[52.] Baldin, C.; Soliman, S.; Jeon, H.; Skory, C.; Edwards, J.; Ibrahim, A. Optimization of the CRISPR/Cas9 System to Manipulate Gene Function in Rhizopus delemar. Open Forum Infect. Dis. 2017, 4, S116. [CrossRef]

[53.] Langfelder, K.; Jahn, B.; Gehringer, H.; Schmidt, A.; Wanner, G.; Brakhage, A.A. Identification of a polyketide synthase gene (pksP) of Aspergillus fumigatus involved in conidial pigment biosynthesis and virulence. Med. Microbiol. Immunol. 1998, 187,79–89. [CrossRef]

[54.] Yang, G.; Rose, M.S.; Turgeon, B.G.; Yoder, O.C. A polyketide synthase is required for fungal virulence and production of the polyketide T-toxin. Plant Cell 1996, 8, 2139–2150. [CrossRef]

[55.] Ibrahim, A.S.; Gebremariam, T.; Lin, L.; Luo, G.; Husseiny, M.I.; Skory, C.D.; Fu, Y.; French, S.W.; Edwards, J.E.; Spellberg, B. The high affinity iron permease is a key virulence factor required for Rhizopus oryzae pathogenesis. Mol. Microbiol. 2010, 77, 587–604.[CrossRef]

[56.] Naz, T.; Nosheen, S.; Li, S.; Nazir, Y.; Mustafa, K.; Liu, Q.; Garre, V.; Song, Y. Comparative analysis of β-carotene production by Mucor circinelloides strains CBS 277.49 and WJ11 under light and dark conditions. Metabolites 2020, 10, 38. [CrossRef]

[57.] Orlowski, M. Mucor dimorphism. Microbiol. Rev. 1991, 55, 234–258. [CrossRef]

[58.] Li, C.H.; Cervantes, M.; Springer, D.J.; Boekhout, T.; Ruiz-Vazquez, R.M.; Torres-Martinez, S.R.; Heitman, J.; Lee, S.C. Sporangiospore size dimorphism is linked to virulence of Mucor circinelloides. PLoS Pathog. 2011, 7, e1002086. [CrossRef]

[59.] Nosheen, S.; Yang, J.; Naz, T.; Nazir, Y.; Ahmad, M.I.; Fazili, A.B.A.; Li, S.; Mustafa, K.; Song, Y. Annotation of AMP-activated protein kinase genes and its comparative transcriptional analysis between high and low lipid producing strains of Mucor circinelloides. Biotechnol. Lett. 2020, 43, 1–10. [CrossRef]

[60.] Hameed, A.; Hussain, S.A.; Ijaz, M.U.; Ullah, S.; Muhammad, Z.; Suleria, H.A.R.; Song, Y. Antioxidant activity of polyphenolic extracts of filamentous fungus Mucor circinelloides (WJ11): Extraction, characterization and storage stability of food emulsions.Food Biosci. 2020, 34, 100525. [CrossRef]

[61.] Nagy, G.; Szebenyi, C.; Csernetics, Á.; Vaz, A.G.; Tóth, E.J.; Vágvölgyi, C.; Papp, T. Development of a plasmid free CRISPR-Cas9 system for the genetic modification of Mucor circinelloides. Sci. Rep. 2017, 7, 16800. [CrossRef]

[62.] Zhang, Y.; Navarro, E.; Cánovas-Márquez, J.T.; Almagro, L.; Chen, H.; Chen, Y.Q.; Zhang, H.; Torres-Martínez, S.; Chen, W.; Garre,V. A new regulatory mechanism controlling carotenogenesis in the fungus Mucor circinelloides as a target to generate β-carotene over-producing strains by genetic engineering. Microb. Cell Fact. 2016, 15, 1–14. [CrossRef]

[63.] Navarro, E.; Lorca-Pascual, J.; Quiles-Rosillo, M.; Nicolás, F.; Garre, V.; Torres-Martínez, S.; Ruiz-Vázquez, R. A negative regulator of light-inducible carotenogenesis in Mucor circinelloides. Mol. Genet. Genom. 2001, 266, 463–470. [CrossRef]

[64.] Michielse, C.B.; Salim, K.; Ragas, P.; Ram, A.F.J.; Kudla, B.; Jarry, B.; Punt, P.J.; Van Den Hondel, C.A.M.J.J. Development of a system for integrative and stable transformation of the zygomycete Rhizopus oryzae by Agrobacterium-mediated DNA transfer.Mol. Genet. Genom. 2004, 271, 499–510. [CrossRef]

[65.] Klein, T.M.; Sanford, J.C.; Wolf, E.D.; Wu, R. High-velocity microprojectiles for delivering nucleic acids into living cells. Nature 1987, 327, 70–73. [CrossRef]

[66.] Garcia, A.; Vellanki, S.; Lee, S.C. Genetic Tools for Investigating Mucorales Fungal Pathogenesis. Curr. Clin. Microbiol. Rep. 2018,5, 173–180. [CrossRef]

[67.] Mackenzie, D.A.; Wongwathanarat, P.; Carter, A.T.; Archer, D.B. Isolation and use of a homologous histone H4 promoter and a ribosomal DNA region in a transformation vector for the oil-producing fungus Mortierella alpina. Appl. Environ. Microbiol. 2000, 66, 4655–4661. [CrossRef]

[68.] Van Heeswijck, R.; Roncero, M.I.G. High frequency transformation of Mucor with recombinant plasmid DNA. Carlsberg Res.Commun. 1984, 49, 691–702. [CrossRef]

[69.] Garre, V.; Barredo, J.L.; Iturriaga, E.A. Transformation of Mucor circinelloides f. lusitanicus Protoplasts. Fungal Biol. 2015, 49–59.[CrossRef]

[70.] Nyilasi, I.; Ács, K.; Papp, T.; Nagy, E.; Vágvölgyi, C. Agrobacterium tumefaciens-mediated transformation of Mucor circinelloides.Folia Microbiol. 2005, 50, 415–420. [CrossRef] [PubMed]

[71.] González-Hernández, G.A.; Herrera-Estrella, L.; Rocha-Ramírez, V.; Roncero, M.I.G.; Gutierrez-Corona, J.F. Biolistic transformation of Mucor circinelloides. Mycol. Res. 1997, 101, 953–956. [CrossRef]

[72.] Papp, T.; Csernetics, Á.; Nagy, G.; Bencsik, O.; Iturriaga, E.A.; Eslava, A.P.; Vágvölgyi, C. Canthaxanthin production with modified Mucor circinelloides strains. Appl. Microbiol. Biotechnol. 2013, 97, 4937–4950. [CrossRef]

[73.] Ando, A.; Sumida, Y.; Negoro, H.; Suroto, D.A.; Ogawa, J.; Sakuradani, E.; Shimizu, S. Establishment of Agrobacterium tumefaciens-mediated transformation of an oleaginous fungus, Mortierella alpina 1S-4, and its application for eicosapentaenoic acid producer breeding. Appl. Environ. Microbiol. 2009, 75, 5529–5535. [CrossRef]

[74.] Takeno, S.; Sakuradani, E.; Murata, S.; Inohara-Ochiai, M.; Kawashima, H.; Ashikari, T.; Shimizu, S. Establishment of an overall transformation system for an oil-producing filamentous fungus, Mortierella alpina

1S-4. Appl. Microbiol. Biotechnol. 2004, 65, 419–425. [CrossRef]
[75.] Zhang, H.; Cui, Q.; Song, X. Research advances on arachidonic acid production by fermentation and genetic modification of Mortierella alpina. World J. Microbiol. Biotechnol. 2021, 37, 1–9. [CrossRef]
[76.] .Fuller, K.K.; Chen, S.; Loros, J.J.; Dunlap, J.C. Development of the CRISPR/Cas9 System for Targeted Gene Disruption in Aspergillus fumigatus. Eukaryot. Cell 2015, 14, 1073–1080. [CrossRef] [PubMed]
[77.] Zhang, C.; Meng, X.; Wei, X.; Lu, L. Highly efficient CRISPR mutagenesis by microhomology-mediated end joining in Aspergillus fumigatus. Fungal Genet. Biol. 2016, 86, 47–57. [CrossRef]
[78.] Umeyama, T.; Hayashi, Y.; Shimosaka, H.; Inukai, T.; Yamagoe, S.; Takatsuka, S.; Hoshino, Y.; Nagi, M.; Nakamura, S.; Kamei, K.; et al. CRISPR/Cas9 Genome Editing to Demonstrate the Contribution of Cyp51A Gly138Ser to Azole Resistance in Aspergillus Fumigatus. Antimicrob. Agents Chemother. 2018, 62. [CrossRef]
[79.] Skory, C.D.; Freer, S.N.; Bothast, R.J. Production of L-lactic acid by Rhizopus oryzae under oxygen limiting conditions. Biotechnol. Lett. 1998, 20, 191–194. [CrossRef]
[80.] Bai, D.M.; Zhao, X.M.; Li, X.G.; Xu, S.M. Strain improvement of Rhizopus oryzae for over-production of L(+)-lactic acid and metabolic flux analysis of mutants. Biochem. Eng. J. 2004, 18, 41–48. [CrossRef]
[81.] Ge, C.M.; Gu, S.B.; Zhou, X.H.; Yao, J.M.; Pan, R.R.; Yu, Z.L. Breeding of L(+)-lactic acid producing strain by low-energy ion implantation. J. Microbiol. Biotechnol. 2004, 14, 363–366.
[82.] Oda, Y.; Yajima, Y.; Kinoshita, M.; Ohnishi, M. Differences of Rhizopus oryzae strains in organic acid synthesis and fatty acid composition. Food Microbiol. 2003, 20, 371–375. [CrossRef]
[83.] Tang, X.; Chang, L.; Gu, S.; Zhang, H.; Chen, Y.Q.; Chen, H.; Zhao, J.; Chen, W. Role of beta isopropylmalate dehydrogenase in lipid biosynthesis of the oleaginous fungus Mortierella alpina. Fungal Genet. Biol. 2021, 152, 103572. [CrossRef]
[84.] Totani, N.; Oba, K. The filamentous fungus Mortierella alpina, high in arachidonic acid. Lipids 1987, 22, 1060–1062. [CrossRef]
[85.] Ge, C.; Chen, H.; Mei, T.; Tang, X.; Chang, L.; Gu, Z.; Zhang, H.; Chen, W.; Chen, Y.Q. Application of a ω-3 desaturase with an arachidonic acid preference to eicosapentaenoic acid production in Mortierella alpina. Front. Bioeng. Biotechnol. 2018, 5, 89. [CrossRef]
[86.] Kawashima, H.; Akimoto, K.; Higashiyama, K.; Fujikawa, S.; Shimizu, S. Industrial production of dihomo-γ-linolenic acid by a Δ5 desaturase-defective mutant of Mortierella alpina 1S-4 fungus. J. Am. Oil Chem. Soc. 2000, 77, 1135. [CrossRef]
[87.] Li, X.; Liu, R.; Li, J.; Chang, M.; Liu, Y.; Jin, Q.; Wang, X. Enhanced arachidonic acid production from Mortierella alpina combining atmospheric and room temperature plasma (ARTP) and diethyl sulfate treatments. Bioresour. Technol. 2015, 177, 134–140. [CrossRef]
[88.] Sakuradani, E. Advances in the production of various polyunsaturated fatty acids through oleaginous fungus Mortierella alpina breeding. Biosci. Biotechnol. Biochem. 2010, 74, 908–917. [CrossRef] [PubMed]
[89.] Hao, G.; Chen, H.; Gu, Z.; Zhang, H.; Chen, W.; Chen, Y.Q. Metabolic engineering of Mortierella alpina for enhanced arachidonic acid production through the NADPH-supplying strategy. Appl. Environ. Microbiol. 2016, 82, 3280–3288. [CrossRef]
[90.] Takeno, S.; Sakuradani, E.; Tomi, A.; Inohara-Ochiai, M.; Kawashima, H.; Shimizu, S. Transformation of oil-producing fungus,Mortierella alpina 1S-4, using Zeocin, and application to arachidonic acid production. J. Biosci. Bioeng. 2005, 100, 617–622. [CrossRef] [PubMed]
[91.] Jin, M.J.; Huang, H.; Xiao, A.H.; Gao, Z.; Liu, X.; Peng, C. Enhancing arachidonic acid production by Mortierella alpina ME-1 using improved mycelium aging technology. Bioprocess. Biosyst. Eng. 2009, 32, 117–122. [CrossRef]
[92.] Huang, M.; Chen, H.; Tang, X.; Lu, H.; Zhao, J.; Zhang, H.; Chen, Y.; Chen, W. Two-stage pH control combined with oxygen enriched air strategies for the highly efficient production of EPA by Mortierella alpina CCFM698 with fed-batch fermentation. Bioprocess. Biosyst. Eng. 2020, 43, 1725– 1733. [CrossRef]
[93.] Takeno, S.; Sakuradani, E.; Tomi, A.; Inohara-Ochiai, M.; Kawashima, H.; Ashikari, T.; Shimizu, S. Improvement of the fatty acid composition of an oil-producing filamentous fungus, Mortierella alpina 1S-4, through RNA interference with Δ12-desaturase gene expression. Appl. Environ. Microbiol. 2005, 71, 4992–4997. [CrossRef]
[94.] Tai, M.; Stephanopoulos, G. Engineering the push and pull of lipid biosynthesis in oleaginous yeast Yarrowia lipolytica for biofuel production. Metab. Eng. 2013, 15, 1–9. [CrossRef]
[95.] Ferreira, R.; Teixeira, P.G.; Gossing, M.; David, F.; Siewers, V.; Nielsen, J. Metabolic engineering of Saccharomyces cerevisiae for overproduction of triacylglycerols. Metab. Eng. Commun. 2018, 6, 22–27. [CrossRef]
[96.] Leber, C.; Polson, B.; Fernandez-Moya, R.; Da Silva, N.A. Overproduction and secretion of free fatty acids through disrupted neutral lipid recycle in Saccharomyces cerevisiae. Metab. Eng. 2015, 28, 54–62. [CrossRef]
[97.] Zhou, Y.J.; Buijs, N.A.; Zhu, Z.; Qin, J.; Siewers, V.; Nielsen, J. Production of fatty acid-derived oleochemicals and biofuels by synthetic yeast cell factories. Nat. Commun. 2016, 7, 11709. [CrossRef] [PubMed]
[98.] Jiang, W.; Li, C.; Li, Y.; Peng, H. Metabolic Engineering Strategies for Improved Lipid Production and Cellular Physiological Responses in Yeast Saccharomyces cerevisiae. J. Fungi 2022, 8, 427
[99.] Tsuchiya, K.; Tada, S.; Gomi, K.; Kitamoto, K.; Kumagai, C.; Jigami, Y.; Tamura, G. High level expression of the synthetic human lysozyme gene in

Aspergillus oryzae. Appl. Microbiol. Biotechnol. 1992, 38, 109–114. [CrossRef] [PubMed]

[100.] Tsuchiya, K ; Gomi, K.; Kitamoto, K.; Kumagai, C.; Tamura, G. Secretion of calf chymosin from the filamentous fungus Aspergillus oryzae. Appl. Microbiol. Biotechnol. 1993, 40, 327–332. [CrossRef]

[101.] Nakajima, K.; Asakura, T.; Maruyama, J.; Morita, Y.; Oike, H.; Shimizu-Ibuka, A.; Misaka, T.; Sorimachi, H.; Arai, S.; Kitamoto, K.; et al. Extracellular production of neoculin, a sweet-tasting heterodimeric protein with taste-modifying activity, by Aspergillus oryzae. Appl. Environ. Microbiol. 2006, 72, 3716–3723. [CrossRef]

[102.] Ito, K.; Asakura, T.; Morita, Y.; Nakajima, K.; Koizumi, A.; Shimizu-Ibuka, A.; Masuda, K.; Ishiguro, M.; Terada, T.; Maruyama, J.; et al. Microbial production of sensory-active miraculin. Biochem. Biophys. Res. Commun. 2007, 360, 407–411. [CrossRef] [PubMed]

[103.] Chen, X.A.; Ishida, N.; Todaka, N.; Nakamura, R.; Maruyama, J. Takahashi, H.; Kitamoto, K. Promotion of efficient saccharification of crystalline cellulose by Aspergillus fumigatus Swo1. Appl. Environ. Microbiol. 2010, 76, 2556–2561. [CrossRef] [PubMed]

[104.] Yoon, J.; Aishan, T.; Maruyama, J.; Kitamoto, K. Enhanced production and secretion of heterologous proteins by the filamentous fungus Aspergillus oryzae via disruption of vacuolar protein sorting receptor gene Aovps10. Appl. Environ. Microbiol. 2010, 76, 5718–5727. [CrossRef]

[105.] Yoon, J.; Kikuma, T.; Maruyama, J.; Kitamoto, K. Enhanced production of bovine chymosin by autophagy deficiency in the filamentous fungus Aspergillus oryzae. PLoS ONE 2013, 8, e62512. [CrossRef]

[106.] Hoang, H.D.; Maruyama, J.; Kitamoto, K. Modulating endoplasmic reticulum-Golgi cargo receptors for improving secretion of carrier-fused heterologous proteins in the filamentous fungus Aspergillus oryzae. Appl. Environ. Microbiol. 2015, 81, 533–543. [CrossRef]

[107.] Yoon, J.; Kimura, S.; Maruyama, J.; Kitamoto, K. Construction of quintuple protease gene disruptant for heterologous protein production in Aspergillus oryzae. Appl. Microbiol. Biotechnol. 2009, 82, 691–701. [CrossRef]

[108.] Yoon, J.; Maruyama, J.; Kitamoto, K. Disruption of ten protease genes in the filamentous fungus Aspergillus oryzae highly improves production of heterologous proteins. Appl. Microbiol. Biotechnol. 2011, 89, 747–759. [CrossRef] [PubMed]

[109.] Maruyama, J.; Kitamoto, K. Multiple gene disruptions by marker recycling with highly efficient gene-targeting background (DeltaligD) in Aspergillus oryzae. Biotechnol. Lett. 2008, 30, 1811–1817. [CrossRef]

[110.] Maruyama, J.; Kitamoto, K. Targeted gene disruption in koji mold Aspergillus oryzae. Methods Mol. Biol. 2011, 765, 447–456. [PubMed]

[111.] Fleissner, A.; Dersch, P. Expression and export: Recombinant protein production systems for Aspergillus. Appl. Microbiol. Biotechnol. 2010, 87, 1255–1270. [CrossRef] [PubMed]

[112.] Fan, J.; Zhang, Z.; Long, C.; He, B.; Hu, Z.; Jiang, C.; Zeng, B. Identification and functional characterization of glycerol dehydrogenase reveal the role in kojic acid synthesis in Aspergillus oryzae. World, J. Microbiol. Biotechnol. 2020, 36, 136. [CrossRef] [PubMed]

[113.] Huynh, H.H.; Morita, N.; Sakamoto, T.; Katayama, T.; Miyakawa, T.; Tanokura, M.; Chiba, Y.; Shinkura, R.; Maruyama, J. Functional production of human antibody by the filamentous fungus Aspergillus oryzae. Fungal Biol. Biotechnol. 2020, 7, 7. [CrossRef]

[114.] Jin, F.J.; Maruyama, J.; Juvvadi, P.R.; Arioka, M.; Kitamoto, K. Development of a novel quadruple auxotrophic host transformation system by argB gene disruption using adeA gene and exploiting adenine auxotrophy in Aspergillus oryzae. FEMS Microbiol. Lett. 2004, 239, 79–85. [CrossRef]

[115.] Oikawa, H. Reconstitution of biosynthetic machinery of fungal natural products in heterologous hosts. Biosci. Biotechnol. Biochem. 2020, 84, 433–444. [CrossRef] [PubMed]

[116.] Promdonkoy P, Tirasophon W, Roongsawang N, Eurwilaichitr L, Tana Pongpipat S. Methanol Inducible promoter of thermotolerant methylotrophic yeast Ogataea thermomethanolica BCC16875 potential for production of heterologous protein at high temperatures. Curr Microbiol. 2014; 69:143-8.

[117.] Kruawan W, Puseenam A, Phithakrotchanakoon C, Tana Pongpipat S, Roongsawang N (2021) Modulation of heterologous protein secretion in the thermotolerant methylotrophic yeast Ogataea thermomethanolica TBRC 656 by CRISPR-Cas9 system. PLoS ONE 16(9): e0258005.
https://doi.org/10.1371/journal.pone.0258005

[118.] Cong, L.; Ran, F.A.; Cox, D.; Lin, S.; Barretto, R.; Habib, N.; Hsu, P.D.; Wu, X.; Jiang, W.; Marraffini, L.A.; et al. Multiplex Genome Engineering Using CRISPR/Cas Systems. Science 2013, 339, 819–823. [CrossRef] [PubMed]

[119.] Qi, L.S.; Larson, M.H.; Gilbert, L.A.; Doudna, J.A.; Weissman, J.S.; Arkin, A.P.; Lim, W.A. Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. Cell 2013, 152, 1173–1183. [CrossRef] [PubMed]

[120.] Gilbert, L.A.; Larson, M.H.; Morsut, L.; Liu, Z.; Brar, G.A.; Torres, S.E.; Stern-Ginossar, N.; Brandman, O.; Whitehead, E.H.; Doudna, J.A.; et al. CRISPR-Mediated Modular RNA-Guided Regulation of Transcription in Eukaryotes. Cell 2013, 154, 442–451. [CrossRef] [PubMed]

[121.] Farzadfard, F.; Perli, S.D.; Lu, T.K. Tunable and multifunctional eukaryotic transcription factors based on CRISPR/Cas. ACS Synth. Biol. 2013, 2, 604–613. [CrossRef] [PubMed]

[122.] Chavez, A.; Scheiman, J.; Vora, S.; Pruitt, B.W.; Tuttle, M.; Iyer, E.; Lin, S.; Kiani, S.; Guzman, C.D.; Wiegand, D.J.; et al. Highly efficient Cas9-mediated transcriptional programming. Nat. Methods 2015, 12, 326–328. [CrossRef] [PubMed]

[123.] Bao, Z.; HamediRad, M.; Xue, P.; Xiao, H.; Tasan, I.; Chao, R.; Liang, J.; Zhao, H. Genome-scale engineering of Saccharomyces cerevisiae with single-

nucleotide precision. Nat. Biotechnol. 2018, 36, 505–508. [CrossRef]

[124.] Deaner, M.; Alper, H.S. Systematic testing of enzyme perturbation sensitivities via graded dCas9 modulation in Saccharomyces cerevisiae. Metab. Eng. 2017, 40, 14–22. [CrossRef]

[125.] Lian, J.; Schultz, C.; Cao, M.; HamediRad, M.; Zhao, H. Multi-functional genome-wide CRISPR system for high throughput genotype-phenotype mapping. Nat. Commun. 2019, 10, 5794. [CrossRef]

[126.] Hall, M.C.; Matson, S.W. Helicase motifs: The engine that powers DNA unwinding. Mol. Microbiol. 1999, 34, 867–877. [CrossRef]

[127.] Gheinani, A.H.; Jahromi, N.H.; Feuk-Lagerstedt, E.; Taherzadeh, M.J. RNA silencing of lactate dehydrogenase gene in Rhizopus oryzae. J. RNAi Gene Silenc. 2011, 7, 443–448 [CrCrossRef].