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Koji – the only one domesticated fungi: characterization, enzymology, and use

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ABSTRACT

Koji mold (*Aspergillus oryzae*) is the only known domesticated fungal species, playing a central role in traditional East Asian fermented foods such as soy sauce, miso, and sake. This filamentous fungus is highly valued for its exceptional enzymatic capabilities, as it secretes diverse hydrolytic enzymes, including proteases and amylases. A. oryzae possesses 65 endopeptidase and 69 exopeptidase genes, supporting efficient protein degradation. In contrast, A. sojae contains 83 endopeptidase and 67 exopeptidase genes. Key enzymes include alkaline protease (optimal at pH 9.0 and 40 °C), neutral protease I (broad specificity), and acid protease (optimal at pH 3.7, 39 kDa). α-Amylase and glucoamylase production are also prominent; the former exhibits thermal stability up to 75 °C, while the latter displays optimal activity at 60 °C and has a molecular weight of between 60–70 kDa. Genomic studies reveal that A. oryzae contains three amylase genes, in contrast to only one in A. sojae, which correlates with its superior saccharification performance. Importantly, A. oryzae is genetically incapable of producing aflatoxin due to critical mutations in the aflR regulatory gene and deletions within the aflatoxin biosynthesis cluster, particularly in Group 1 and 2 strains that dominate industrial use. Koji mold is traditionally cultivated through solid-state fermentation, primarily on steamed, polished rice, under controlled conditions (30–35°C, 90% humidity). This review provides a comprehensive overview of the enzymology, genomic adaptations, and fermentation technologies associated with A. oryzae, emphasizing its unique domestication, safety profile, and industrial relevance in enzyme production and sustainable food biotechnology.

Keywords: koji, *Aspergillus oryzae,* fermentation, microbial enzymes

INTRODUCTION

Aspergillus oryzae is a filamentous microscopic fungus, belonging to the division of *Ascomycota*. It is widely used in traditional Japanese fermentation industries, as in the production of sake, soy sauce, bean curd seasoning, or vinegar. Enzymatic activity is one of the main properties for which filamentous fungi are the subject of in-depth study. Among them, *A. oryzae* stands out for its remarkable potential in enzyme secretion. Furthermore, advancements in genetic engineering have enabled its application in the field of biotechnology, particularly in the industrial production of enzymes. Notably, *A. oryzae* was first microorganism used for the commercial production of lipase enzymes used as a laundry detergent since 1988 **[1]**.

A wide variety of koji-fermented foods and beverages are produced using *A. oryzae*, including sake (Japanese rice wine), honkaku shochu (a distilled Japanese liquor), amazake (a non-alcoholic sweet rice drink), soy sauce, miso (fermented soybean paste), mirin (a sweet rice wine for seasoning), rice vinegar, salted koji, and pickles. Over centuries, koji mold has been carefully cultivated and utilized by our ancestors, significantly enriching both nutrition and culinary traditions. Recognizing its cultural and historical significance, the Scientific Conference of the Brewing Society of Japan declared koji fungi as the National Heritage in 2006. Additionally, in 2013, it was

included in UNESCO's Intangible Cultural Heritage list under the designation Washoku: Traditional Dietary Cultures of the Japanese **[2]**.

For thousands of years, *A. oryzae* has provided immense benefits, and it is likely to continue playing a significant role in the future. However, many aspects of *A. oryzae* remain mysterious, including its unique cultivation methods, species origin, and isolation techniques **[3]**.

AFLATOXIN PRODUCTION

Aspergillus oryzae and *Aspergillus sojae* do not produce aflatoxin, a known carcinogen. Numerous studies have demonstrated that aflatoxin production in these species is both undetectable and biologically inactive. Although *A. sojae* contains a 70-kb aflatoxin biosynthetic gene cluster in its genome, a nonsense mutation in the transcription factor AflR-responsible for activating gene expression within the AF biosynthetic cluster-results in the deletion of 62 amino acids at the C-terminal region, including the transcriptional activation domain. Consequently, transcriptional activation is lost, and the genes involved in aflatoxin biosynthesis remain unexpressed **[4]**.

In *A. oryzae* RIB 40, several mutations have been identified in the aflR promoter region and in three open reading frames (aflT, norA, and verA) within the homologous aflatoxin biosynthesis gene cluster when compared to *A. flavus*. These mutations include deletions, frameshifts, and base-pair substitutions. Additionally, a 1.5-kb deletion within the norB-cypA sequence has been observed, consistent with findings in other A. *oryzae* isolates and *A. flavus* S isolates **[5]**. In aflatoxin biosynthesis, norA **[6]**, nor-1 **[7]**, and norB **[8]** are believed to play key roles **[9]**. Disruption of nor-1 has been associated with a substantial decrease in aflatoxin B1 production **[10]**. However, the disruption of norA or norB does not significantly affect aflatoxin synthesis, suggesting that mutations in these genes in A. *oryzae* do not directly contribute to its inability to produce aflatoxin **[11]**.

The absence of expression in key aflatoxin biosynthesis genes, including avnA, vbs, verB, and omtA, is thought to result from reduced transcription levels of the regulatory gene aflR. However, even if aflR is expressed at low levels, it is possible that translation does not occur or that AflR is rapidly degraded. Regardless, it is clear that Group 1 strains of *A. oryzae* cannot produce aflatoxin, as avnA, verB, vbs, and omtA, all essential for aflatoxin biosynthesis, were undetectable via RT-PCR **[12]**. Notably, avnA is considered essential for the production of aflatoxin. The lack of expression of these genes in Group 1 strains confirms that A. oryzae does not produce aflatoxin. However, it is unlikely that base substitutions at putative binding sites alone account for this non-productivity. Structural analysis has yet to provide a definitive explanation **[11]**.

In approximately 40% of *A. oryzae* strains (classified as Groups 2 and 3), large portions of the aflatoxin biosynthesis gene homolog cluster, including aflR are deleted. Notably, 60% of strains originating from tane-koji (the mold starter used in sake, soy sauce, and miso production) belong to Group 2. In contrast, Group 3 strains exhibit at least partial amplification of vbs, although only a few amplification patterns have been confirmed **[11]**.

ENZYMATIC ACTIVITY OF KOJI

The primary function of koji mold in brewing is to produce enzymes that break down large molecular compounds, such as proteins and starches, in raw materials into smaller, fermentable substances like amino acids and glucose. A key distinction between *Aspergillus oryzae* and *Aspergillus sojae* lies in their production of α-amylase and endopolygalacturonase. *A. oryzae* exhibits higher α-amylase productivity, while *A. sojae* demonstrates greater endopolygalacturonase productivity **[13]**.

Due to its strong amylolytic activity, *A. oryzae* generates more glucose, thereby enhancing alcoholic fermentation in yeast. As a result, it is extensively used in the brewing of soy sauce, sake, and miso. As comparative genomic analysis has shown, *A. oryzae* RIB40 contains three amylase genes, whereas *A. sojae* NBRC4239 possesses only one. However, no significant differences in the number of protease genes have been observed between the two species **[14]**. This finding has been confirmed across various industrial strains of *A. sojae*. Research indicates that a low-affinity binding sequence to the Hap complex and the reduced copy number of the taa gene contributes to the lower amylolytic activity of *A. sojae* compared to *A. oryzae*. Additionally, differences in aromatic components between the species may influence the sensory characteristics of the final products **[15]**.

PROTEASES

Proteins present in the fermented base are initially broken down into large and small peptides and subsequently degraded into various amino acids by the proteolytic enzymes of koji mold. These enzymes are classified into two main categories:

- Endopeptidases, which hydrolyze peptide bonds within proteins and peptides, breaking them into larger peptide fragments.

- Exopeptidases, which cleave peptide bonds at the ends of proteins and peptides, producing smaller peptides such as dipeptides, tripeptides, and free amino acids **[13]**.

Endopeptidases are further categorized into six types based on the structure of their active site: serine, cysteine, aspartic, metallo-, threonine, and unclassified. Exopeptidases, on the other hand, are classified based on their mode of action, whether they act at the N- or C-terminal end of peptides. Genomic studies have identified 65 endopeptidase genes and 69 exopeptidase genes in *A. oryzae*, compared to 83 endopeptidase genes and 67 exopeptidase genes in *A. sojae* **[16]**.

In *A. oryzae*, three endopeptidases-alkaline protease, neutral protease I, and neutral protease II, along with two peptidases (leucine aminopeptidases I and II), play a crucial role in breaking down raw material proteins during soy sauce fermentation. In *A. sojae*, the same three endopeptidases are present, along with three peptidases: leucine aminopeptidases I and III, and acid carboxypeptidase IV, that contribute to protein degradation in soy sauce brewing **[15]**. Additionally, several leucine aminopeptidases and acid carboxypeptidases have been reported to facilitate the release of glutamic acid, an essential component of soy sauce flavor **[17]**.

Alkaline Protease: Alkaline proteases from *Aspergillus oryzae* are a very valuable resource for potential industrial applications, particularly in food processing and biotechnology. These enzymes exhibit optimal activity at alkaline pH levels, typically ranging from pH 8 to 10, with an optimal temperature of 40 °C, making them highly suitable for applications such as soy sauce fermentation and protein degradation in food products **[18]**, **[19]**, and **[20]**. The ability of *A. oryzae* to produce alkaline proteases is attributed to its extensive repertoire of hydrolytic enzymes, which includes a variety of proteases that function effectively under alkaline conditions **[21]**. Alkaline protease from A. oryzae is classified as a metal-ion-independent serine protease. It exhibits optimal and stable activity at pH 9.0 and 40 °C. Its relative activity increases significantly from approximately 20% at 20 °C to 100% at 40 °C, before sharply decreasing to 26% at 65 °C. The enzyme remains nearly 100% stable between 20 °C and 40 °C, but its stability declines to 40% at 65 °C. Additionally, alkaline protease activity is less affected by NaCl compared to neutral protease I. In a 3.0 M NaCl solution, alkaline protease activity is reduced by less than 12%, whereas neutral protease I activity decreases by nearly 30% **[18]**.

Neutral Protease: Neutral proteases are zinc-dependent metalloproteases that function optimally at neutral pH levels (pH 6.0 – 7.0). They are categorized into two types: neutral protease I and neutral protease II, also known as deuterolysin. Neutral protease I effectively digests a variety of proteins, particularly milk casein and soybean proteins. In contrast, neutral protease II exhibits a much narrower substrate specificity, primarily targeting basic proteins such as histones and protamines. While neutral protease I demonstrates broad substrate activity, neutral protease II is significantly more thermostable, retaining its activity even after exposure to 99 °C for 10 minutes **[22]**. However, the thermostability of neutral protease II differs among species. For instance, the neutral protease II of *A. sojae* becomes unstable after treatment at 75 °C due to autoproteolysis, whereas this effect is not observed in *A. oryzae* **[23]**. Both neutral proteases contribute to protein digestion but do not produce low-molecular-weight peptides or free amino acids **[13]**.

Acid Protease: Acid protease from *A. oryzae* has been extensively studied for its role in protein degradation. An acid protease isolated from *A. oryzae* 460 exhibits optimal activity at pH 3.7 and has a molecular weight of 39 kDa. When this purified acid protease hydrolyzes α-soybean protein, peptides consisting of 7 – 9 amino acid residues are produced, though no free amino acids are released. However, when acid protease is combined with carboxypeptidase IV at 30 °C and pH 5, glutamic acid is detected, and the overall amount of free amino acids increases **[24]**. An extracellular acid protease (47 kDa) from *A. oryzae* MTCC 5341, recognized as one of the most active strains, exhibits optimal activity in the pH range of 3.0 – 4.0 and remains stable between pH 2.5 and 6.5. Its peak activity is observed at 55 °C **[25]**.

AMYLASES

Amylases are enzymes that hydrolyze glucosidic bonds in starch, as α-amylase, β-amylase, glucoamylase, and isoamylase. In Aspergillus oryzae, the primary amylases secreted are α-amylase and glucoamylase. α-Amylases (EC 3.2.1.1) catalyze the cleavage of α-1,4 glycosidic bonds within the starch chain. However, they are unable to hydrolyze α-1,6 glycosidic bonds in the branched regions of starch, resulting in hydrolysis products such as glucose, maltose, and oligosaccharides containing α-1,6 glycosidic linkages **[26]**. Glucoamylases (EC 3.2.1.3) break down starch by cleaving α-1,4 glycosidic bonds from the non-reducing end, producing glucose **[27]**. During soy sauce fermentation, amylases play an essential role in degradation of starch in raw materials into fermentable sugars, which contribute to the color, aroma, and flavor of the final product **[28]**. The glucose produced serves as a carbon source for starter molds, yeasts, and other microorganisms, while additional sugars support the metabolism of yeast and lactic acid bacteria **[29]**. Furthermore, these sugars contribute to the sweetness of soy sauce and enhance its color through the Maillard reaction, in which they interact with amino acids released from

protein degradation **[30]**. Additionally, cellulases are enzymes that hydrolyze β-1,4 glycosidic bonds in cellulose. These include exocellulase (EC 3.2.1.91), endocellulase (EC 3.2.1.4), and cellobiase (EC 3.2.1.21) **[30]**.

α-Amylase: α-Amylase, an enzyme produced by *Aspergillus oryzae*, plays a vital role in starch hydrolysis, making it essential for wide spectrum of industrial applications, including food processing and biofuel production. The enzyme efficiently catalyzes the breakdown of starch into mono- and disaccharids and shows wide substrate specificity, making it advantageous for diverse applications **[31]**. Structural studies have identified a catalytic triad responsible for enzymatic activity, consisting of key residues that facilitate substrate binding and hydrolysis **[32]**. The isoelectric point of α-amylase ranges from 3.25 to 10.1, which is crucial for optimizing purification processes **[33]**. The thermal stability of *A. oryzae* α-amylase is a critical factor in industrial applications, particularly those requiring high-temperature processing. The enzyme retains significant activity at elevated temperatures, with optimal stability between 50 °C and 75 °C. Immobilization techniques can further enhance its thermal stability, prolong its functional lifespan and increase its resistance to denaturation. These characteristics make α-amylase particularly beneficial for industrial processes that require prolonged exposure to heat **[34]**. The production of α-amylase from A. oryzae can be optimized using various fermentation techniques, with solid-state fermentation proving especially effective. This method utilizes agricultural by-products, such as rice hulls and potato pulp, as substrates, which not only support enzyme production but also contribute to sustainable waste management **[31]**. Key factors influencing enzyme yield include temperature, moisture content, and nutrient availability **[35]**.

Glucoamylase: Glucoamylase (Gla; α-1,4-glucan glucohydrolase, EC 3.2.1.3) hydrolyzes starch into glucose and is produced in significant amounts by *A. oryzae*, making it widely used in the fermentation processes of biotechnological industry **[36]**. The enzyme exhibits high substrate specificity, primarily targeting starch, maltodextrins, and oligosaccharides **[37]**. The molecular weight of *A. oryzae* glucoamylase ranges between 60 and 70 kDa, with its activity influenced by factors such as pH and temperature. Like many glucoamylases, it is produced as a precursor that undergoes proteolytic processing to become active **[38]**. Unlike α-amylases, most glucoamylases can hydrolyze α-1,6 glycosidic bonds at amylopectin branching points, albeit at a slower rate than α-1,4 linkages. Although different microorganisms produce these enzymes, *A. oryzae* is capable of synthesizing both α-amylase and glucoamylase simultaneously **[39]**. For industrial applications, thermal stability is a key consideration. *A. oryzae* glucoamylase exhibits optimal activity at around 60 °C, with considerable stability at elevated temperatures. However, it tends to have lower thermal stability compared to glucoamylases from other sources, such as *A. niger* **[40]**, and **[41]**.

FERMENTATION OF KOJI

Solid-state fermentation is widely utilized in traditional food fermentation processes and presents opportunities for enhancing the production of both novel and existing food products and ingredients **[36]**. Recent advancements have demonstrated that SSF can achieve high yields of pure enzymes more efficiently than submerged fermentation **[42]**. Fungi play a crucial role in SSF due to their ability to develop hyphae, which enable them to effectively colonize and penetrate solid substrates **[36]**. However, during hyphal growth, fungi must adapt to concentration gradients of substrates and enzymes, substrate–air interfaces, and variations in water content and temperature within the fermentation environment **[43]**. These factors influence enzyme production, making SSF a unique and efficient approach for microbial fermentation.

TECHNOLOGY

Preparation of substrate

For optimal growth of fungal mycelium, steamed rice is typically used. Rice mustn´t be boiled, but gelatinization of starch is crucial for successful development. Steaming is the best way to achieve it. Because steaming cooks the rice grains from the inside, but the surface remains dry, the grains will stay separated during inoculation and solid-state fermentation, creating a substrate with gaps for mycelium and air. It is possible to use any other grains; crucial is a carbohydrate source and nitrogen source in them, but grains must be polished. Bran creates a barrier for mycelium growth. If it is necessary to use grains with brans, it is better to mill it to beter degradation **[44]**.

The substrate must be cooled to a temperature of less than 37°C. For better and more homogeneous inoculation, spores are first mixed with a small amount of substrate or rice flour and then carefully added to the substrate, thoroughly mixed **[45]**.

Figure 1 Koji spores homogenized in small amount of substrate for better and more homogenous inoculation.

Figure 1 Substrate with Koji spores (green powder) homogenously inoculated, before mixing.

Koji fermentation

Historically, Koji originated from East Asia, where the weather is warm and humid. In these conditions, Koji can grow spontaneously. For stable and standard production, controlled conditions are used. The temperature is 30-35°C and the humidity is 90%. Koji prefers stable air without fan or other air moving **[46]**. Koji must be mixed at least once a day; a better option is every 12 hours. During the first 12-24 hours, Koji is fermented "in a heap," which helps form spores with better thermal regulation during the young stages of growth. When Koji starts to smell sweet and produce heat, it is transferred to boxes with a maximum level of Koji rice at 5cm. Technological Koji needs 2 days for growing, if we want to produce spores. A longer period of 3-4 days and a lower level of Koji are used because Spores are produced only on the surface **[45]**.

Figure 2 First 24 hours of Koji fermentation "in a heap".

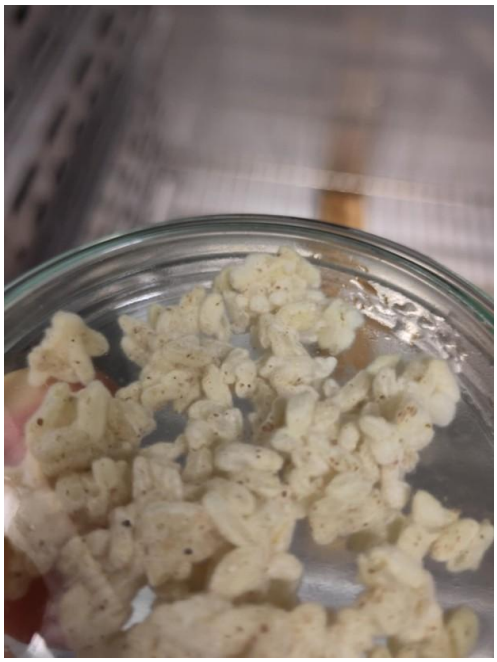

Figure 3 Well-grown white mycelium of Koji after 24 hours, transferred for spores production.

Figure 4 Well-grown Koji in terminal stadium of spores production, prepared for careful drying process.

Moromi fermentation

Moromi is name for liquid fermentation of Koji-based products. There are different types of Moromi fermentation. For example, the same name is used for soy sauce and sake production, but the processes are different. The connected element is a liquid base; both processes begin with mixing koji with water. Water creates optimal conditions for enzymes to degrade and forms microorganisms for fermentation **[47]**. The length of this process varies depending on the product, substrate, or temperature. Saccharification of starch from rice is possible within 24 hours at 60°C, but complete degradation of protein in soy sauce into amino acids requires months, sometimes years.

Figure 5 Moromi fermentation of soy sauce.

CONCLUSION

Koji mold (*Aspergillus oryzae*) serves as a cornerstone of traditional Japanese fermentation and a key contributor to modern industrial biotechnology. Its unique enzymatic profile and safety make it an indispensable tool in food production. Genomic analyses have revealed that A. oryzae possesses 65 endopeptidase and 69 exopeptidase genes, enabling efficient protein degradation during fermentation. Additionally, the mold expresses three amylase genes, including α-amylase and glucoamylase, which are essential for starch hydrolysis and glucose production. These enzymes display high thermal stability, with α-amylase remaining active up to 75 °C and glucoamylase showing optimal activity at around 60 °C. Koji mold does not produce carcinogenic aflatoxins, unlike some related Aspergillus species. In A. oryzae RIB 40 and other industrial strains, critical mutations in the aflR regulatory gene, deletions in genes such as norA, verA, and a 1.5-kb deletion in the norB-cypA region render the aflatoxin biosynthetic pathway inactive. RT-PCR analyses have confirmed the absence of expression for essential aflatoxin biosynthesis genes (avnA, verB, vbs, omtA) in Group 1 strains, which are commonly used in food fermentation. Moreover, over 60% of strains derived from tane-koji (mold starter) belong to Groups 2 and 3, where large portions of the aflatoxin gene cluster are either deleted or non-functional. Koji is cultivated under controlled solid-state fermentation conditions, typically at 30–35 °C and 90% humidity, using steamed polished rice as substrate. These optimized conditions promote mycelial growth and enzyme production. Its industrial relevance continues to expand beyond traditional foods like miso, soy sauce, and sake to applications in enzyme manufacturing, waste valorization, and sustainable bioprocessing. As research continues to uncover new genetic traits and regulatory mechanisms, A. oryzae remains a model organism in microbial biotechnology. Its long-standing cultural heritage, combined with its proven safety and enzymatic efficiency, ensures that koji will continue to play a central role in both artisanal fermentation and innovative industrial processes.

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