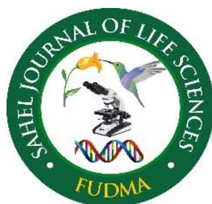

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Research Article

Safety Evaluation of Milk-clotting Enzymes Produced by *Aspergillus niger* and *Penicillium* Spp through Solid State Fermentation for Cheese Production

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ABSTRACT

Milk-clotting enzymes (MCEs) play a crucial role in cheese production, facilitating the coagulation of milk, traditionally sourced from animal rennet. Soil samples were collected from Sidi Mamman Assarakawa and Sidi Akibu farms, while wheat bran was obtained from Sokoto Central Market. Fungi were isolated using serial dilution and cultured on Potato Dextrose Agar (PDA) with chloramphenicol (0.05 g/L). A potential milk-clotting enzyme (MCE)--producing fungi were screened using Skim Milk Agar, and the potential isolates that showed clear zones were identified based on macroscopic and microscopic characteristics using lacto-phenol cotton blue staining and subjected to solid-state fermentation using wheat bran as a substrate to produce MCEs. The crude enzyme extracts were evaluated for aflatoxin B1 content using ELISA. A total of 12 fungal isolates were obtained from soil samples collected from the dairy farms. Among them, two isolates (SMA 3 and SA 4) demonstrated significant proteolytic activity, forming clear zones of 8.0 mm and 6.0 mm, respectively. The isolates were respectively identified as *Aspergillus niger* and *Penicillium* spp with Aflatoxin B1 content of 7.1 $\pm$ 0.02 from *Aspergillus niger* and 7.6 $\pm$ 0.05 from *Penicillium* spp This study indicates the safe utilization of *Aspergillus niger* and *Penicillium* spp enzymes in solid-state fermentation for cheese production in Nigeria, providing a local and economically viable alternative in the dairy industry.

Keywords: Aflatoxin B1; *Aspergillus niger*; Milk-Clotting Enzymes; *Penicillium* spp; Solid-state fermentation

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INTRODUCTION

Milk-clotting enzymes (MCEs) play a crucial role in cheese production, facilitating the coagulation of milk (Kumar *et al*., 2014). Traditionally, rennet extracted from the stomach lining of young animals has been used as the Milk-clotting enzyme. However, the increasing demand for cheese, availability of calf rennet, costs and religious considerations have led to the exploration of microbial sources (Hayam *et al.,* 2013).

The use of microbial rennet has gained popularity in recent years due to its potential to reduce cost and improve the sustainability of cheese production (Silveira *et al.,* 2005). Several successful attempts have been made to produce milk-clotting enzymes from other sources such as plants and microorganisms (Rodarte *et al.,* 2011).

Most of these enzymes are mainly produced by genera such as *Aspergillus*, *Mucor, Entothia, Rhizopus, Penicillium* and *Fusarium*. These fungi are characterized by their adaptation to solid-state fermentation (SSF) using cheap substrates, which improve the recovery of extracellular enzymes from fermentation media, solve contamination problems, and process investment costs (Sathya *et al.,* 2009).

The safety of MCEs produced by solid-state fermentation must be evaluated to ensure their suitability for use in cheese production. This study aimed to evaluate the safety of milk-clotting

enzymes (MCEs) produced by *Aspergillus niger* and *penicillium spp* by solid-state fermentation (SSF).

MATERIALS AND METHODS

Collection of Samples

Soil samples were obtained from Sidi Mamman Assarakawa farm and Sidi Akibu farm.

The soil samples were collected from the top surface and at a depth of about 10cm. A sterile spatula was used to collect 10g from each farm of the soil samples (Sathya *et al.,* 2009).

Processing of Industrial Residues

Wheat bran, obtained from Sokoto Central Market, was also sieved using a 1 mm metal sieve (Hayam *et al.,* 2013) before being used in this study.

Isolation and Identification of Fungi

Ten-fold serial dilutions were carried out (10^{-1} – 10^{-10}) and 0.2ml from a dilution factor of 10^{-3}, 10^{-5} and 10^{-8} were plated onto Potato Dextrose Agar (PDA) medium containing 0.05 g/L chloramphenicol. The plates were incubated at 30 °C for 5 to 7 days to isolate distinctive colonies (Sathya *et al.,* 2009).

Fungal identification was done based on macroscopic and microscopic characterization. (Mohanasrinivasan *et al.,* 2012). Macroscopic identification was based on characteristics of fungal growth on PDA plates; while microscopic identification was through lacto-phenol cotton blue staining technique (Mohanasrinivasan *et al.,* 2012).

Lacto-phenol Cotton Blue Staining

Lacto-phenol Cotton Blue stain (Sigma-Aldrich, Germany) was used to study the microscopic characteristics of fungi. A drop of lacto-phenol solution was placed on a clean slide. Using a needle that has been sterilized in a Bunsen burner flame, a tincture of fungal growth together with a very small layer of the agar was taken and placed on the drop of lacto-phenol solution (Mohanasrinvasan *et al*., 2012). The fungal culture was spread onto a slide by using a second needle to tease out the fungal structures. The needles were sterilized in a bunsen flame (Mohanasrinvasan *et al*., 2012).

Screening for MCEs-Producing Fungi

Primary screening for MCEs production was tested using the method of Sanna and Sayed (2001).

Protease productions were tested using the Skim Milk Agar medium for the production of a clear zone. The detection medium (Skim Milk Agar, SMA) was prepared using 10g of skim milk, 2g of agar-agar each dissolved in 150 mL distilled water, and the pH was adjusted to 6.0. All three media components were autoclaved separately to avoid coagulation and charring of milk and later mixed under sterile conditions. The plates were then subsequently inoculated with mycelia from a 5-day-old culture and incubated at 30°C for 3 to 4 days. The plates were examined for the formation of the clearing zone. The species that exhibited maximum clear zone were selected for identification (Sanna and Sayed, 2001).

Preparation of Inocula

Fungal isolates were grown on PDA and incubated at 30°c for 5 days. The isolates were scraped using 10 mL of sterile distilled water to prepare spore suspension. 1.0 mL of spore solution (10^6 spores/mL) were used according to the study by Sathya *et al*. (2009).

Medium and Cultural Condition for Solid-State Fermentation

The fermentation was carried out in 250 mL conical flask which contained 10g of wheat bran, 2g of skim milk powder. This was moistened with 10ml of mineral solution (g/L) : 2.0, KNO_3; 0.5, $MgSO_4 \cdot 7H_2O$; 1.0, K2HPO₄; 0.439, $ZnSO_4 \cdot 7H_2O$; 1.116, $FeSO_4 \cdot 7H_2O$; 0.203, $MnSO_4 \cdot 7H_2O$, and pH was adjusted to pH 5.0. The mixtures were autoclaved at 121 ^{O}C for 20 min. and allowed to cool. After sterilization, the flasks were inoculated with 1.0 ml of spore suspension and incubated at 30 °C for 120 h (Sathya *et al.,* 2009).

Extraction of Enzymes

After the fermentation period, 100mL of distilled water were added to the solid-state fermentation medium and homogenized by shaking for 40 min. Cell-free supernatant was obtained by centrifuging the extract at 10,000 rpm for 30 min. The centrifuged extract was filtered through Whatman No.1 filter paper to obtain crude enzymes (Ja'afar *et al*., 2020).

Aflatoxin B1 Analysis

The crude enzyme extracts were evaluated for aflatoxin B1 content using enzyme-linked Immunosorbent assay (ELISA) kits**.**

RESULTS

Fungal Isolation, Screening, and Identification

A total of twelve (12) fungal isolates were obtained from the two farms, Sidi Mamman Assarakawa farm (SMA) and Sidi Akibu farm (SA). These isolates were screened for their ability to produce protease activity using Skim Milk Agar. The presence of a clear zone around the fungal colony on the Skim Milk Agar medium indicated the hydrolysis of casein, which is a sign of protease enzyme production.

As shown in Table 1, only two (2) isolates demonstrated the ability to hydrolyze casein by the formation of clear zones, while the remaining ten (10) isolates showed no proteolytic activity. The two isolates that exhibited positive results were SMA 3, which produced a clear zone of 8 mm, and SA 4, which produced a clear zone of 6 mm. These two isolates were identified and confirmed as

Aspergillus niger and *Penicillium* spp respectively (Table 2).
In Table 3, the results indicate that the concentration of aflatoxin B1 produced by *Aspergillus niger* and *Penicillium* spp are 7.1 ± 0.02 and 7.6 ± 0.05 µg/L respectively.

Table 1: Proteolytic Activity of the Fungal Isolates on Skim Milk Agar Media

Isolate code	Clear zone (mm)
SMA 1	0.0
SMA 2	0.0
SMA 3	8.0
SMA 4	0.0
SMA 5	0.0
SMA 6	0.0
SA 1	0.0
SA 2	0.0
SA 3	0.0
SA 4	6.0
SA 5	0.0
SA 6	0.0

Keys: SMA=Sidi Mamman Assarakawa farm, SA= Sidi Akibu farm

Table 2: Macroscopic and Microscopic Characteristic of Potential MCEs-producing Fungi

Location/Isolate code	Colony Description	Microscopic	Fungal identity
SMA 3	Pin-like black growth	Non-branched conidiophores with bulb end carry conidia like sun rays	*Aspergillus niger*
SA 4	Green-greyish color colonies	The brush-like conidiophore carries conidia	*Penicillium* spp

Keys: SMA=Sidi Mamman Assarakawa farm, SA= Sidi Akibu farm

Table 3: Concentration of Aflatoxin B1 Produced By the Fungal Isolates

Fungal isolate	Mean Concentration±SEM (µg/L)
Aspergillus niger	7.1 ± 0.02
Penicillium spp	7.6 ± 0.05

The values are the mean of three determinations (triplicate) SEM = Standard error of the mean

DISCUSSION

In this study, a total of 12 fungal isolates were obtained from two (2) different dairy farms to identify potential isolates capable of producing milk-clotting enzymes (MCEs). Among these, only two isolates exhibited clear zones of hydrolysis on Skim Milk Agar, indicating proteolytic activity. Based on macroscopic and microscopic characteristics using lacto-phenol cotton blue staining, these isolates were identified as *Aspergillus niger* and *Penicillium* spp *Aspergillus niger* demonstrated the highest milk-clotting activity, forming a clear zone of 8 mm, while *Penicillium* spp produced a hydrolysis zone of 6 mm. These zones were formed due to the digestion of protein by multiple enzymes secreted by the fungal isolates.
The findings of this research align with previous research. For example, Abdalla *et al.* (2018) reported that *Aspergillus* spp exhibited the highest

milk-clotting enzyme activity on Skim Milk Agar compared to other fungal isolates. Similarly, Arun *et al.* (2015) screened 31 fungal isolates using Casein Agar, Skim Milk Agar, and Gelatin Agar, where six isolates exhibited enzymatic activity on the media. These positive isolates displayed distinct hydrolysis zones around their colonies, reinforcing the potential of fungal isolates in enzyme production.

Furthermore, our results agree with Agu *et al.* (2023), who isolated 14 fungi from four soil samples, among which two isolates, exhibited hydrolysis on Skim Milk Agar. In their study, *Aspergillus* spp demonstrated the highest enzymatic activity. The findings of this current study are also consistent with MohanasriniVasan *et al*. (2012), who reported the isolation and screening of milk-clotting enzymes from fungal isolates in soil samples.

However, our results contrast with the findings of Abdalla *et al*. (2018), who observed that the majority of the fungal isolates in their study exhibited milk-clotting enzyme production on Skim Milk Agar. The relatively lower hydrolysis zones observed in this study may be attributed to additional components present in the Skim Milk Agar, which could interfere with enzyme-substrate interactions or compete for enzymatic activity, potentially reducing the size of the hydrolysis zones.

The safety evaluation of the milk-clotting enzymes produced by *Aspergillus niger* and *Penicillium* spp through solid-state fermentation revealed the presence of aflatoxin B1 at levels exceeding the European Union permissible limit (4µg/L). However, these levels remain within the acceptable range established by the Standard Organization of Nigeria (20µg/L), suggesting that the crude enzymes may be considered safe after food production. Previous studies also supported the safety of enzymes derived from *Aspergillus* species, which are generally recognized as safe (GRAS) Castro *et al.* (2014). Additionally, Fazouane-Naimi *et al.* (2010) reported that the culture supernatant from *Aspergillus niger* FFB1 used for cheese production, was free from Ochratoxin A (OTA), further supporting the safety of fungal-derived enzymes in food application.

CONCLUSION

This study successfully evaluated the safety of milk-clotting enzymes (MCEs) produced by *Aspergillus niger* and *Penicillium* spp through solid-state fermentation (SSF) for cheese production. A total of 12 fungal isolates were obtained from soil samples collected from dairy farms, among which two isolates demonstrated significant proteolytic activity, forming clear zones of 8.0 mm and 6.0 mm respectively. These isolates were identified as *Aspergillus niger* and *Penicillium* spp.

The safety assessment using ELISA revealed that the aflatoxin B1content in crude enzyme was 7.1 ± 0.02 for *Aspergillus niger* and 7.6 ± 0.05 for *Penicillium* spp which falls within acceptable safety limits for food applications. These findings indicate that the enzymes produced by these fungal strains can be safely utilized in cheese production.

Based on the findings of this study, it is recommended that further research should be conducted to optimize the production process of milk-clotting enzymes from *Aspergillus niger* and *Penicillium* spp to enhance their efficiency and safety for large-scale cheese production. Additionally, periodic monitoring of aflatoxin B1 levels in enzyme extracts should be implemented to ensure compliance with food safety regulations.

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