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Comparative Milk Clotting Potentials of *Fusarium oxysporum* and *Aspergillus Niger* Fungal Strains Isolated from Fermented Brown Rice

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ABSTRACT: The objective of this paper is to investigate the comparative milk clotting potentials of *Fusarium oxysporum* and *Aspergillus niger* fungal strains isolated from fermented using casein hydrolysis test initially and subsequently subjecting the fungal strains to fermentation on five agro-industrial waste and casein for detection of MCTs and MCAs. After fermentation, enzymes extracted from *F.oxysporum* treated with CS revealed highest MA (288.8SU/ml) at 416secs, followed by enzymes extracted from RB which had (160.8 SU/ml) at 750secs; but for *A.niger* enzymes extracted from CS and RB had lower MCA units as compared to F.oxysporum, however, enzymes of A.niger extracted from SBH yielded 205SU/ml at 583secs as against (51.67) at 2427secs for *F.oxysporum* enzymes from SBH. The pellet enzymes for both of the fungal strains had low MCA and high MCTs; indicating that clotting activity was more potential with supernatant enzymes across the substrates. Although 163.3 CS and 137.0 SBH pellet enzymes from *A. niger* had a bit high MCA and low MCTs as compared to pellet enzymes of *F. oxysporum* from 51.67 CS and 49.46 SBH. Production of enzymes using agro-industrial waste is a bioprocess that is cheaper and could add value economically.

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Microbial protease is a good source of enzyme because they require limited space for their growth which is very rapid and cell cultivation is easier. Proteases or proteolytic enzymes refer to the various enzyme that digest (breakdown into smaller units) proteins. These enzymes include pancreatic proteases, chymosin, trypsin, bromelain (pineapple enzymes) papain (papaya enzyme) fungal proteases and spicata peptidase (the silk worm enzymes) (Doctormurry.com, 2015).Although protease is found in a wide variety of sources such as plants, animals and microorganisms they are however mainly produced by bacteria and fungi and such protease account for 60% of the world wide enzyme commercialization (Gupta *et al.*, 2002). Proteases can be cultured in large quantities within short time by established methods of fermentation, either submerged or solid-state fermentation. Parameters such as variation in CN ratio, presence of some sugar besides several other factors are important in the

development of the fermentation processes (Desouz *et al.,* 2015; Gupta *et al.,* 2002). Microbial proteases are extracellular in nature, hence are directly secreted into the producers thus, simplifying the downstream processing of the enzyme as compared to proteases obtained from plants and other animals (Ja'afar *et al.,* 2020).

Fungi are a group of great interest for enzyme production. A good number of fungal genera such Aspergillus, Rhizopus, Mucor, penicillium, Fusarium, cephalon, Sporium, Conidiobolus, Neurospora are known to produce acid, alkaline and neutral protease (Karuna and Ayyana, 1993). It was also observed that fungal substitute for animal proteases includes those from *Rhizopus*, *Oryzae* (Kumar *et al.,* 2005), Rhizomucor (Ja'afar *et al.,* 2020). *Aspergillus oryzae* (Otani *et al.,* 1991) *Rhizomucor miechie* and *Rhizomucorpucillus* have also gained industrial acceptance as producers of milk-clotting enzymes (Sirma *et al.,* 2010). Those proteases are active over a wide pH (4-11) and temperature in the range of 30-70°c (Ja'afar *et al.,* 2020). Production of proteases has been carried out using submerged fermentation, but conditions in solid state fermentation (SSF) lead to several potential advantages for the production of fungal enzymes (Desouza *et al.,* 2015), it has the potential of high protease yield (Pandey *et al.,* 1999) economically it uses in expensive substrate, simpler matching, low energy requirement and low waste water output (Malathi and Chakraborty 1991). Hence SSF used to study the effect of various substrates on the production of milk clotting enzymes from F.oxysporum and A.niger, because the world increase of cheese production has prompted search for rennet substitutes and microbial rennet appears to be more promising such enzymes constitute 60% of the total enzyme sale globally Mourer (2004). Consequently, the objective of this paper is to investigate the comparative milk clotting potentials of *Fusarium oxysporum* and *Aspergillus niger* fungal strains isolated from fermented brown rice.

MATERIALS AND METHODS

Preparation of Inoculum: Cultures of *F. oxysporum* and *A. niger* isolated from brown were sub cultured to obtain a pure culture from which distinct colonies were streaked on PDA slants and maintained at 30°c and used for further investigations.

Screening the Fungi for Milk Clotting Potential(casein hydrolysis): Potato dextrose agar and sterile milk were mixed in a 4:1 volume ratio at 45 ^{0}C, inserted into Petri-dishes(in triplicate) and cooled down to room temperature. Each fungus was inoculated in the center of the Petri dish and incubated for 72 hrs; hydrolysis of casein resulted in the appearance of a faded white zone around the cultures (Benlounissi *et al.,* 2012).

Solid-State Fermentation for the Screening of Substrate for Protease production from the two fungal strains: Six types of media (which included casein, wheat bran, millet bran, rice bran, banana peel powder and soya bean meal) were screened as media for the production of protease from the isolated fungi using Solid State Fermentation (SSF) method. Crude enzyme was exacted after the fermentation (purified and assayed for the acid activity). For the SSF, 5.0g of each substrate was taken in a 250ml Erlenmeyer flask separately, each was moistened with salt solutions; composition (% W/V) as follows: sodium nitrate 0.2, potassium dihydrogen phosphate 0.1, magnesium sulphate 0.05, potassium dihydrogen phosphate 0.1, magnesium sulphate 0.05, potassium chloride 0.05, ferrous sulphate concentration, and zinc sulphate concentration at pH of 7.0 were used to achieve the desired moisture content. The mixture was sterilized at 121 $^{\circ}$C at 15 min, cooled and inoculated with 1 ml of fungal spore suspension (10^{6} spores/ml) and incubated at 30 $^{\circ}$C for 72 hrs. (Ja'afar *et al.,* 2020).The procedure was used for the substrates and the two fungi separately.

Enzymes Extraction (EE): After 72 hrs of fermentation 5.0 g of the fermented material was mixed with 30 ml of 0.1ml phosphate buffer and homogenized by shaking for 30 min and filtered through cheese cloth. 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 crudeenzymes (Ja'afar *et al.,* 2020).

Purification of Extracted Enzymes: The crude enzyme extracted from the samples was purified by subjecting to ammonium sulphate precipitation. The filtrate was taken and 70% fraction of ammonium sulphate was added slowly to the supernatant. While adding the ammonium sulphate, the culture was kept in ice blocks, then, the mixture was incubated overnight in refrigerator at 4°C. On the next day the mixture was centrifuged at 12,000 rpm for 10 mins. The pellet was collected and dissolved in 1M tris HCL (Ramachandran and Arutselvi, 2013).

Assay of enzyme activity (Milk clotting activity) of Supernatant and Pellets: The milk-clotting activity (MCA) of the enzyme extracted was measured by the method described by Otani *et al,* (1991), in which a 5ml portion of the substrate (12% skim milk in

10mM$CaCl_2$) was incubated for 5min at 35 °C (using water bath) and then 0.1 ml of the enzyme extract was added. The length of time (starting from the addition of the enzyme extract to the formation of the first particles) was recorded, and the milk-clotting activity was calculated by using the formula of Kawai and Mukai (1970) as:

$$\text{SU} = \frac{2400x5xD}{Tx0.5(1)} \quad (1)$$

Where:T = is Milk – clotting times (s); D = is Dilution of the enzyme, SU = One soxhlet unit (SU) of milk – clotting activities defined as the amount of enzyme required to clot 1ml of substrate within 40 min at 35 °C.

RESULTS AND DISCUSSION

Table 1 shows result of casein hydrolysis by *A. niger* and *F. oxysporum* after 72 hours of incubation, both fungi hydrolyzed casein at certain degree of inhibition zone. Enzyme assay (both supernatant and pellet) of the two fungal strains treated with the six substrates was summarized in figures; 1, 2, 3 and 4 showcasing the milk clotting time and activity of each enzyme per substrate. Substrate treatment with F. *oxysporum* showed that CS supernatant enzyme had the highest MCA of 288.8 SU/mL (Fig. 2) at 416.3 sec (Fig 1) followed by RB supernatant enzyme with 160.8 SU/mL at 750 sec. All other substrates supernatants had less than 100 SU with MCT of more than 1000 seconds. While treatment of substrates with A. *niger*for MCA (Fig. 4) and MCT (Fig. 3) showed SBH substrates as the best supernatant enzyme with MCA of 205.9 SU/mL at 583.3 sec, while WB and BPP had 130,1 SU/mL and 129.6 SU/mL at 923.3 sec and 926.7 sec respectively. The MB supernatant enzyme had the least of 101.4 SU/mL at 1187 sec.

Table 1: Casein Hydrolysis by *Fusarium oxysporum* and *Aspergillus niger*

Sample	Casein Hydrolyses	Identified Fungi
FBR (Fi) 10^{-4}	+++	*A. niger*
FBR (A)10^{-3}	++	*F. oxysporum*
FBR (A)10^{-6}	+	*F. oxysporum*
FBR (Fi) 10^{-3}	++	*A. niger*
FBR (A) 10^{-4}	++	*F. oxysporum*
FBR (Fi) 10^{-6}	+++	*A. niger*

Key: FBR; Fermented brown rice

Figure 1 and 3 alongside 2 and 4 still indicated MCTs and MCAof the pellets enzyme extracted from the 6 substrates treated with I.O and A.N result revealed high clotting time with low clotting activities as compared to supernatant, but still pellet enzymes from A. *niger* proved to be better than F. *oxysporum* pellet with 163.3 SU/ml 753.7secs from CS and 137.0SU/ml at 876.7secs from SBH and so recorded more than 80SU/ml for RB and WB in less than 2000 seconds in contrast pellets enzymes from F. *oxysporum* had less than 60SU/ml at more than 2000secs across all substrates.

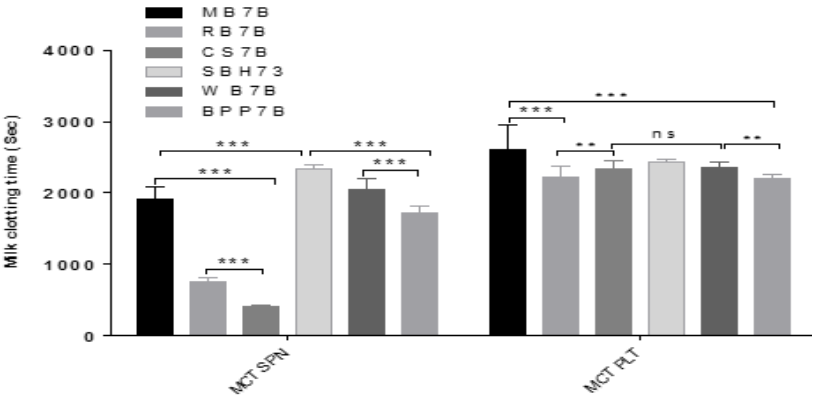

Fig 1: Effects of Various Substrates on Milk Clotting Time of Supernatant and Pellet Enzyme Produced by *F. oxysporum*
Values are mean ± SD of three replicates. Mean values with asterisk are significantly ($p<0.05$) different to one another (Two-way ANOVA followed by Bonferroni's Multiple Comparison Test). ns: non-significant ($p>0.05$); * significant ($p<0.05$); ** significant ($p<0.001$); *** significant ($p<0.0001$).

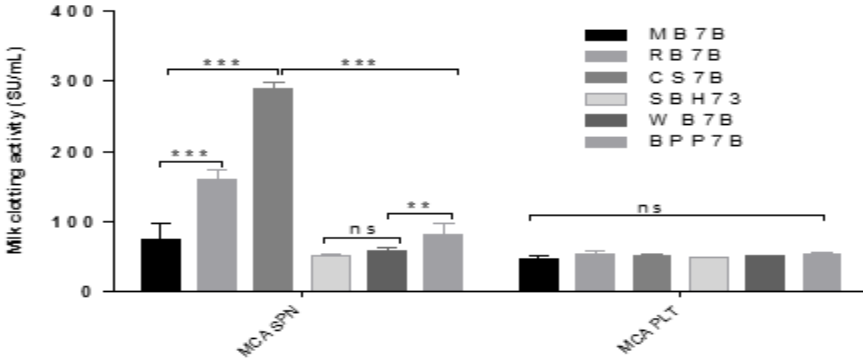

Fig 2: Effects of Various Substrates on Milk Clotting Activity of Supernatant and Pellet Enzyme Produced by *F. oxysporum*
Values are mean ± SD of three replicates. Mean values with asterisk are significantly (p<0.05) different to one another (Two-way ANOVA followed by Bonferroni's Multiple Comparison Test). ns: non-significant (p>0.05); * significant (p<0.05); ** significant (p<0.001); *** significant (p<0.0001).

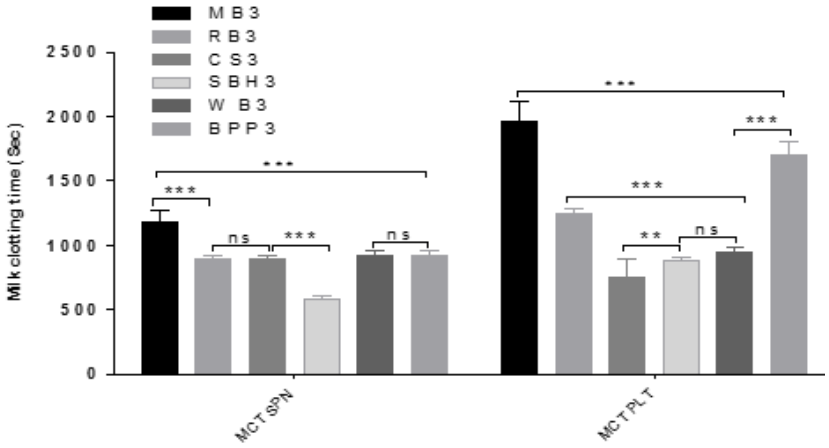

Fig 3: Effects of Various Substrates on Milk Clotting Time of Supernatant and Pellet Enzyme Produced by *A. niger*
*Values are mean ± SD of three replicates. Mean values with asterisk are significantly (p<0.05) different to one another (Two-way ANOVA followed by Bonferroni's Multiple Comparison Test). ns: non-significant (p>0.05); * significant (p<0.05); ** significant (p<0.001); *** significant (p<0.0001).*

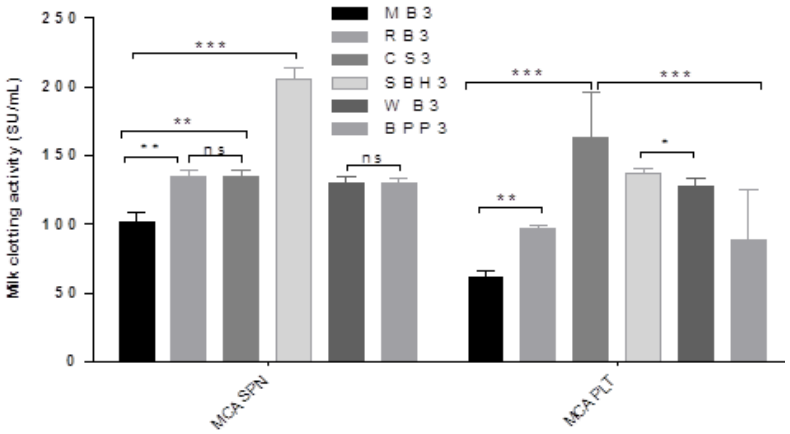

Fig 4: Effects of Various Substrates on Milk Clotting Activity of Supernatant and Pellet Enzyme Produced by *A. niger*
*Values are mean ± SD of three replicates. Mean values with asterisk are significantly (p<0.05) different to one another (Two-way ANOVA followed by Bonferroni's Multiple Comparison Test). ns: non-significant (p>0.05); * significant (p<0.05); ** significant (p<0.001); *** significant (p<0.0001).*

Result of casein hydrolysis indicated that *A. niger* best hydrolyse casein, while F. oxysporum has a moderate hydrolysing effect. This is related to the finding of Mirian *et al.*, (2011) who identified that A. ochraceus hydrolyse casein strongly and some fusarium species hydrolysed casein strongly

moderately. Similarly, Benlonussi *et al.* (2012) also isolated *A. niger*, *A. flavus* and *A. tamari* from soil and from soil and all of them with casein hydrolysing capability (by clotting milk) on enzyme activity of the two fungal strains treated with the various substrates, it is interesting to that both fungi showed remarkable MCAs on the supernatant although F. oxysporum had low MCAs and MCTs for MB, SBH and WB supernatants it was however found to have high MCAs and low MCTs with RB an CS supernatants this is in agreement with the finding of Ali and Vidhale (2013) that reported F. oxysporum protease production on RB and found maximum activity of 70 U/g after 72hrs. *A. Niger* had best result with SBH supernatant but also better than F.o across all the supernatants enzymes of the substrates with shortest clotting times and activities this is related the findings of Benlonussi *et al.*, 2012 who reported A. tamari and A. niger fermented industrial whey of cheese to clot milk within 5minutes.

Enzyme supernatants of both F. oxysporum and A. *niger* from CS and pellet from A. niger had high MCA (SU) units; Sirma*et al.*, (2010) also found Casein supplementation on wheat bran during SSSF to yield move Sus of renin therefore casein can be good supplement for milk clotting enzyme production.

Conclusion: This study demonstrates the comparative milk-clotting efficiency of enzymes produced by *Fusarium oxysporum* and *Aspergillus niger* fungal strains using various agro-industrial wastes as substrates. The findings reveal that F. *oxysporum* enzymes treated with CS exhibited the highest milk clotting activity (MCA) and the shortest milk clotting time (MCT), outperforming enzymes derived from rice bran (RB) and soybean husk (SBH). While A. niger enzymes generally had lower MCA units compared to F. *oxysporum* on CS and RB, they performed better on SBH, demonstrating variability in enzyme activity based on substrate type. Across both fungal strains, supernatant enzymes exhibited superior clotting activity relative to pellet enzymes, though A. *niger* pellet enzymes from CS and SBH displayed relatively higher MCA and shorter MCT compared to those of F. *oxysporum*. This research highlights the potential for cost-effective, sustainable enzyme production using agro-industrial wastes, offering a valuable alternative for enzyme-based applications in dairy and other industries.

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Data availability statement: Data are available upon request from the corresponding author.

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