ISSN: 0975-833X

Available online at http://www.journalcra.com

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

International Journal of Current Research
Vol. 13, Issue, 10, pp.19214-19217, October, 2021

DOI: https://doi.org/10.24941/ijcr.42083.10.2021

RESEARCH ARTICLE

SCREENING OF EXOPOLYSACCHARIDE PRODUCTION AND EVALUATION OF BIOFILM FORMATION CAPACITY IN ASPERGILLUS STRAINS ISOLATED FROM STORED PEANUT SEEDS

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ARTICLE INFO

Article History:
Received 17th July, 2021
Received in revised form
20th August, 2021
Accepted 14th September, 2021
Published online 30th October, 2021

Key Words:

Peanut, Mould,
Adaptation,
Food Safety, Health.

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ABSTRACT

The preservation and conservation of foodstuffs are steps that ensure better food safety. Indeed, in our countries, the storage of fruits, vegetables and seeds maintain temperature and humidity conditions favorable to the emergence of several microorganisms including molds. These moulds develop mechanisms that allow them to adapt to the support and the food stored. In order to assess the food safety of stored peanut seeds, peanut samples were analyzed for molds in their microbiological quality. Four strains of moulds of the genus *Aspergillus* isolated were analysed in their capacity of production of exopolysaccharides and the formation of biofilms. The production of exopolysaccharides was detected by colorimetric methods by culturing the moulds on Sabouraud medium with Congo Red added. Screening of biofilms was carried out on microplate wells by staining with crystal violet of the culture. Quantification of biofilm production was done by following the kinetics by spectrophotometric method. The results obtained from the phenotypic screening showed exopolysaccharide production in all strains. The kinetics of biofilm formation showed a better quantity in the sucrose supplemented medium. The adaptive mechanisms developed by molds in food can be the cause of the formation of toxic compounds that constitute a public health problem, hence the need to take $preventive measures for the conditions of food preservation and storage.

Citation: Joël Prince BIDOUNGA, Rachel MOYEN, Christoffer MOUNKALA. *"*Screening of exopolysaccharide production and evaluation of biofilm formation capacity in Aspergillus strains isolated from stored peanut seeds*", 2021. International Journal of Current Research, 13, (10), 19214-19217.*

INTRODUCTION

The peanut, like all oilseeds, is a source of materials necessary for the maintenance of basic metabolism. It is produced and consumed in all regions of the world in different forms (Mejrhit *et al*.,2015). The periodic production of this oilseed requires conservation methods to make the food available throughout the year, including drying the pods. In Africa in general and in Congo in particular, these pods are kept in warehouses that are often poorly maintained and subject to abiotic factors that favour microbial contamination, the most representative of which are fungal (Boli *et al*.,2013). Indeed, food molds of the genera *Aspergillus, Fusarium* and *Penicillium* are often involved in toxi-infections (Zamblé, 2020). They can form microbial communities that allow them to adapt to environmental conditions in order to exert their pathogenicity (Diakité *et al*.,2017; Adebayo-Tayo, 2006; Karthikeyan, 2015). This pathogenicity is linked to their ability to produce exopolysaccharides and to the formation of biofilms[7]. Several studies have addressed the issue of biofilm formation by foodborne pathogens, however, little work has been done on those formed by molds (Zamblé, 2020; Cortesao, 2020; Uzundag, 2020). Indeed, biofilms contribute to the virulence of microorganisms by providing antimicrobial resistance; an ability to evade host immunity and other environmental stresses[10]. Thus, they are a food safety hazard and a real public health problem.

MATERIALS AND METHODS

Biological materials: The study was conducted with four strains of *Aspergillus* previously isolated from stored peanut seeds as described below

Method

Phenotypic detection of exopolysaccharides: It was done according to the modified method described by Chibi[11]. Isolated strains were previously reactivated by culture on Sabouraud agar medium and incubated at 37°C for 7 days.

Spore collection from each culture was performed by flooding with sterile physiological water and transferring to tubes. After shaking the tubes with a vortex, the optical density of the suspension equivalent to 10^8 spores/ml was read with a spectrophotometer at 623nm. 0.10ml of the suspension was plated on Sabouraud agar medium supplemented with Congo Red and incubated at 37°C for 24 hours. The presence of exopolysaccharide was demonstrated by the appearance of a slime or black viscous layer formed as a result of the reactions between Congo Red organic dye and exopolysaccharide. A better visualization of the exoployssaccharide production was done by streaking the black colonies on the same medium.

In vitro biofilm formation: The formation of biofilms was demonstrated by the microplate method described by Fan[12]. It consists in preparing in each microplate well mixtures containing 100µl of physiological water, 100µl of Sabouraud broth and 100µl of each fungal spore suspension of concentration 10^8 spores/ml. The same experiment was performed in another plate using Sabouraud broth supplemented with 2% sucrose. The mixtures were incubated at 37°C for 48h. After incubation, the microplate wells were washed 3 times with 200µl of sterile physiological water to remove free cells; then stained with crystal violet for 30 minutes, followed by washing with PBS (0.1N) solution and drying at room temperature. Biofilm formation in the wells after crystal violet staining was revealed by blue staining, the intensity of which was proportional to the mass of biofilm formed.

Biofilm formation kinetics: The kinetics of biofilm formation was performed by the method described by Siqueira[13], following the technique of Fan[12].Microplates of previously formed biofilms of 24h, 48h to 72h were flooded with 95° alcohol. The different suspensions obtained were transferred into tubes using a micropipette and then shaken with a vortex. The quantification of biofilms was done by reading the optical density of the homogeneous solution obtained with the help of a zuzi spectrophotometer at 623nm.

RESULTS

Phenotypic detection of exopolysaccharides : The results obtained showed that all tested Aspergillus strains produce exopolysaccharades on Sabouraud agar supplemented with Congo Red as shown in (Figure 1).

In vitro biofilm formation : The results of biofilm formation in the wells were represented in the following figure (Figure 2a, 2b).

Biofilm formation kinetics

Evolution of biofilm formation on Sabouraud broth without sucrose supplementation : The evolution of biofilm formation of *Aspergillus* strains on Sabouraud broth without sucrose supplementation was monitored in (Figure 3a, 3b, 3c, 3d).

Evolution of biofilm formation on Sabouraud broth with sucrose supplement: The evolution of biofilm formation of *Aspergillus* strains on Sabouraud broth with sucrose supplementation was monitored in (Figure 4a, 4b, 4c, 4d).

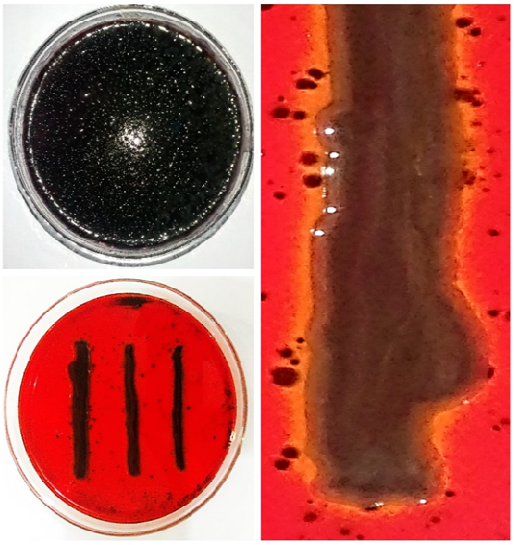

Figure 1: (A) 24h culture on Sabouraud Congo Red agar (SRC) of exopolysaccharide-producing moulds (*Aspergillus sp6*); (B) Parallel streaks of 24h colonies on Sabouraud Congo Red agar (SRC) coated with exopolysaccharides (*Aspergillus sp6*); (C) Viscous layer of exopolysaccharides interacting with Congo Red giving a black pigmentation (*Aspergillus sp6*)

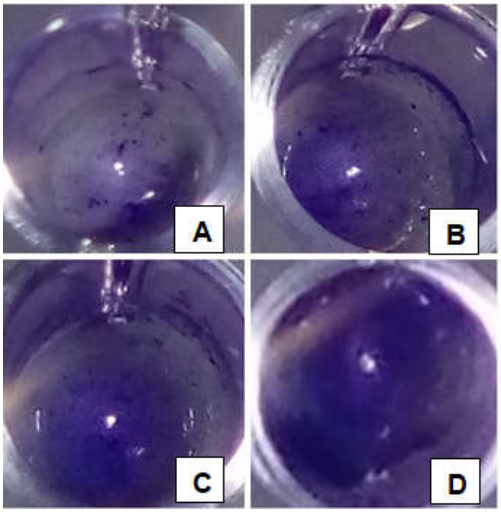

Figure 2a: 48h biofilm formation on Sabouraud broth titration microplate without sucrose supplementation; (A) *Aspergillus sp1;* (B) *Aspergillus sp6* (C) *Aspergillus sp9*, (D) *Aspergillus sp12*

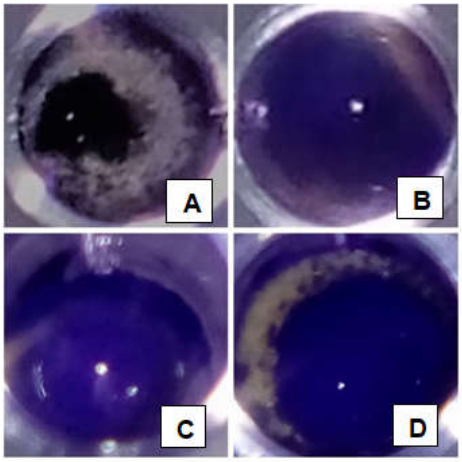

Figure 2b. 48h biofilm formation on Sabouraud broth titration microplate with sucrose suppl ement; (A) *Aspergillus sp1;* (B) *Aspergillus sp6* (C) *Aspergillus sp9*, (D) *Aspergillus sp12*

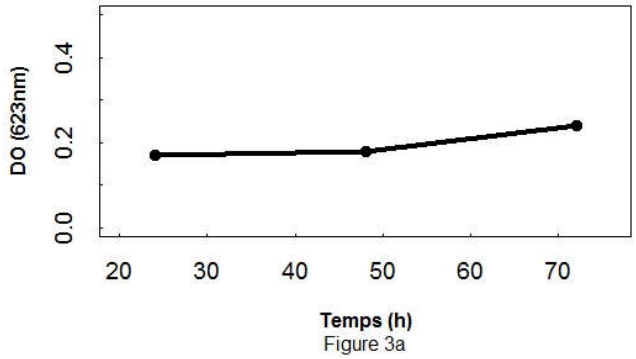

Figure 3a. Biofilm formation curve in *Aspergillus sp1* on Sabouraud broth without sucrose supplementation

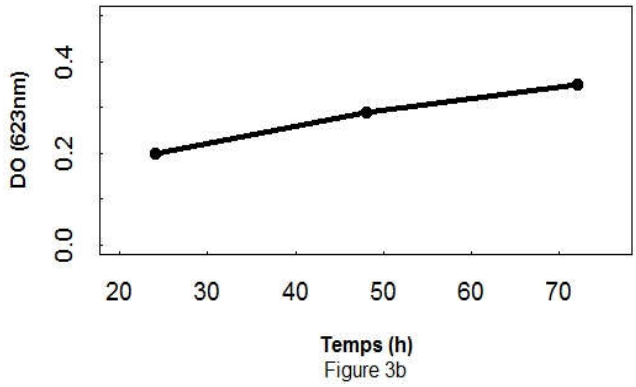

Figure 3b. Biofilm formation curve for *Aspergillus sp6* on Sabouraud broth without sucrose supplementation

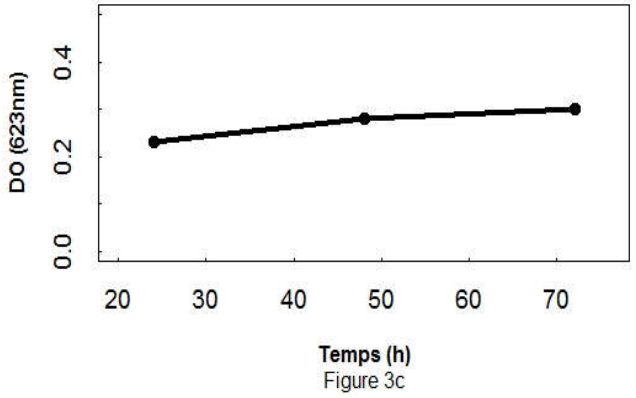

Figure 3c. Biofilm formation curve in *Aspergillus sp9* on Sabouraud broth without sucrose supplementation

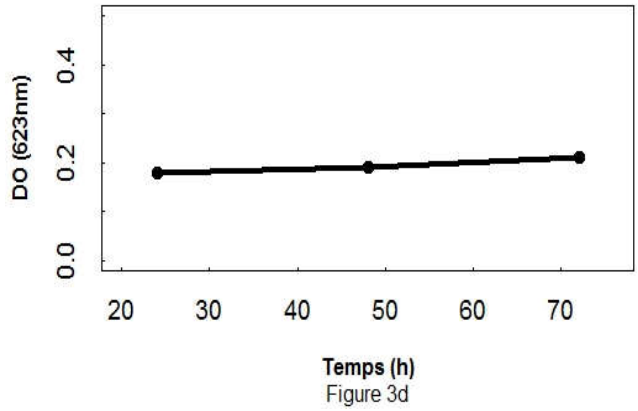

Figure 3d. Biofilm formation curve in *Aspergillus sp12* on Sabouraud broth without sucrose supplementation

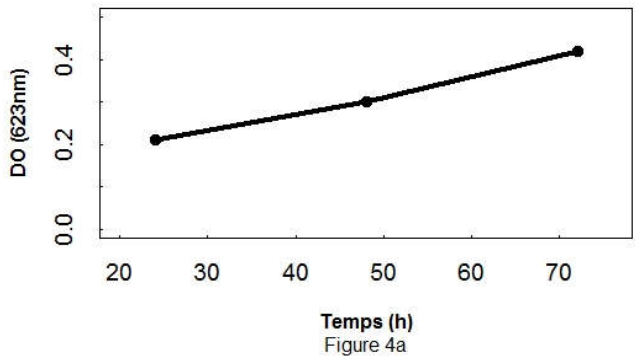

Figure 4a. Biofilm formation curve in *Aspergillus sp1* on Sabouraud broth with sucrose supplementation

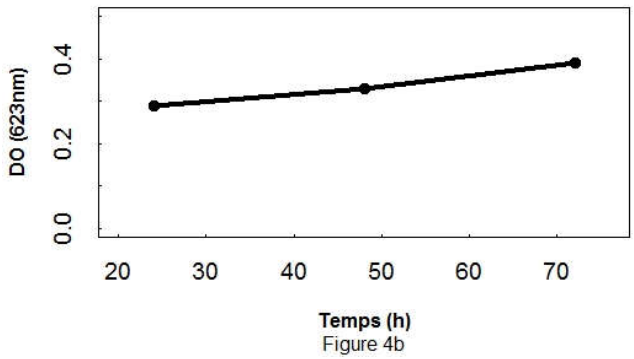

Figure 4b. Biofilm formation curve in *Aspergillus sp6* on Sabouraud broth with sucrose supplementation

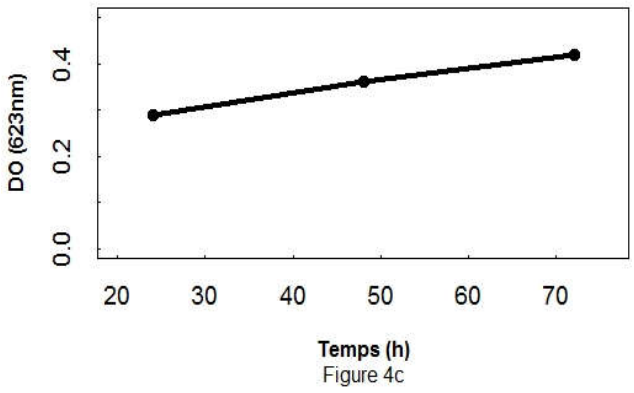

Figure 4c. Biofilm formation curve in *Aspergillus sp9* on Sabouraud broth with sucrose supplementation

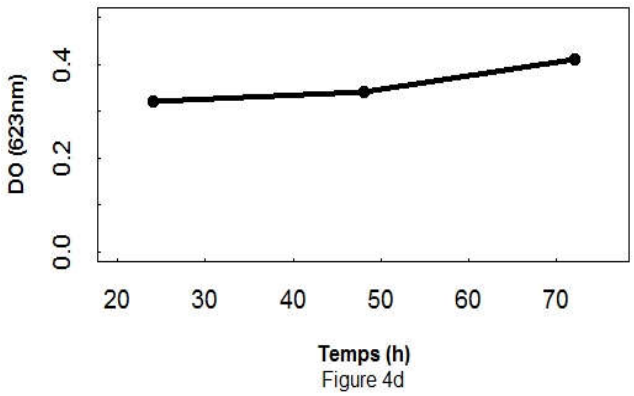

Figure 4d. Biofilm formation curve in *Aspergillus sp12* on Sabouraud broth with sucrose supplementation

Mould	Section
Aspergillus sp1	*Flavi*
Aspergillus sp6	*Flavi*
Aspergillus sp9	*Nigri*
Aspergillus sp12	*Nigri*

DISCUSSION

Phenotypic screening of exopolysaccharide synthesis, showed that all *Aspergillus* strains produce exopolysaccharides by the appearance of a black pigmented slime layer, these results were similar to those found by Sheikh and others (Sheikh, 2020) regarding the same characteristics. The addition of Congo Red to the medium interacts with the exopolysaccharides and gives a black pigment to the slime layer, this method is very effective due to the properties that this organic dye possesses, this appreciation has also been proven by Kaiser and others (Kaiser *et al*.,2012; Ferreira *et al*., 2014). The totality of the *Aspergillus* strains were able to form biofilms, these results are in agreement with the results of Siqueira and others (Siqueira, 2012; Kaur, 2013; Mitchell, 2016), who showed that molds have the ability to form biofilms. Monitoring of the kinetics showed that biofilm production was significant with time. Indeed after 72h the production was maximal and these results are similar to those found by Mitchell and others (Siqueira *et al*.,2020; Ammek,. 2019)**;** who showed that the constitutive biomass of biofilm evolves with time and depends on the constituents of the medium. It was also favoured by the nutrients present. Figure 2b shows a high production of biofilms in the wells of *Aspergillus sp6* and *Aspergillus sp9* strains revealed by a strong intensity of the staining which is proportional to the biomass contrary to figure 2a. Thus the addition of 2% sucrose as a carbon source to the medium influences the production of biofilms and demonstrates that the capacity to produce biofilms is dependent on the microorganism. These results corroborate those found by Ferreira and others16 ; who showed that biofilm formation is strongly influenced by the nutritional requirements of the growth medium.

Conclusion

The results obtained on the tested moulds of the genus *Aspergillus* showed that the latter could synthesize exopolysaccharides constituting a precursor of biofilm formation, and also form biofilms. This ability to form biofilms was amplified by the addition of sucrose in the growth medium. These results showed the ability of molds to form biofilms depending on the substrate present in their environment. These parameters are essential determinants in their pathogenicity and adaptation to both substrates and the environment.

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