Effect of inoculum size on solid state fermentation of pearl millet (*Pennisetum glaucum*) by *Rhizopus oligosporus*

Egbune E. O.[1]*, Orhonigbe I.[1], Adheigu R. O.[1], Oniyan U. P.[1] and Tonukari N. J.[1]

Department of Biochemistry, Faculty of Science, Delta State University, Abraka, Nigeria.
*Corresponding author. E-mail: egbuneegoamaka@gmail.com.

Accepted 16th June, 2021

In this present study the effects of inoculum size on Solid-State Fermentation of pearl millet (*Pennisetum glaucum*) by the fungus *Rhizopus oligosporus* was investigated. The biochemical parameters determined in samples fermented with *R. oligosporus* at different inoculum size (5 - 30%) and the unfermented control (0%) include amylase activity, total phenol, soluble protein and glucose levels. The 1,1-diphenyl-2-prycrilhydrazil radical scavenging activity model (DPPH) was employed for the antioxidant study. This study established that solid state fermentation of pearl millet (*P. glaucum*) by *R. oligosporus* statistically ($p<0.05$) increased amylase activity, total phenol, soluble protein and glucose levels compared with that present in the unfermented sample. The nutritional properties of the *R. oligosporus* fermented pearl millet have thus established the fact that it could be a potential and improved source of components for feed formulations.

Key words: Solid-state Fermentation, Pearl millet, *Rhizopus oligosporus*, soluble proteins, antioxidants.

INTRODUCTION

Historically, solid state fermentation has been used to cultivate microorganisms for food processing around the world. Solid state fermentation (SSF) is a process involving the use of little or no free water in the growth of microorganisms. However, in recent times, SSF has been utilized in the production of biomolecules and important products for various industries ranging from pharmaceutical, food and biochemical to textile industries (Pandey, 2003; Soccol and Vandenberghe, 2003). Over the years, great strides have been made in developing more economic technologies to improve the production of biomolecules from plants, especially cereals. Fermentation procedure has appeared to be quickly emerging technology employed by scientists, industrialists and researcher to enhance the nutritional status of food and foodstuff (Aganbi et al., 2020). SSF procedures are secure, renewable and depend on using suitable and specific microbes (Salar et al., 2012; Salar and Purewal, 2017). Notwithstanding, a diversity of microorganisms are accessed that can modulate the bioactive compounds of cereal grains, microorganisms that belong to Aspergillus family are getting more focus among scientists. Prior researches on SSF technology show the advantages of using starter cultures *Aspergillus awamori* on pearl millet and wheat cultivars (Sandhu et al., 2016; Bhanja et al., 2009; Salar et al., 2016) and *Aspergillus candidus* on rice (Yen *et al.*, 2003). Meanwhile, choosing the appropriate microorganism and substrate (in them) is a singular important determinant of the quantity of bioactive compounds that will be derived from the raw material (Pandey, 2003).

The quality of meal standard is explained by nutritional information and the existence of bioactive compounds applying helpful result on the total wellbeing of humans (Cassano et al., 2008). Cereals grains consist of many health improving constituents like minerals, vitamins and phytochemicals made up of phenolic compound. Maize, rice and wheat have been widely investigated to examine their bioactive

prospective and the phenolic content. With the exception of these cereals, millets are priceless. Pearl millet (*Pennisetum glaucum*) happens to be among the staple minor millets in the Indian region and many of the African nations. These small-scale sown grains with nut brown to reddish brown colored seed coat seat polyphenol 0.09 to 2.44 mg chlorogenic equivalent/100 mg (Ramachandra et al., 1977). Biofunctionality of pearl millets like hypoglycemic, hypocholesterolemic and anti-ulcerative attributes are known due to its polyphenolic contents (Hegde et al., 2002; Kumari and Sumathi, 2002; Chethan and Malleshi, 2007). Phenolic compounds exist all over the millet diversities and are joined to different cell wall constituents across glycosidic linkages, such as protein and arabinoxylans. Bound phenolics are found in cell walls and are chemically linked to cell wall components, mainly polysaccharides. Free phenolics exist in bound or in the free form are housed in vacuoles. Pearl millet seed coat precisely accomodate bioactive phenolics in wall bound form. During the 10 years, filamentous fungi are applied to accelerate the content of phenolic compounds in different food products, thereby increasing their nutritional composition by fermentation processes (Aganbi et al., 2020). The enhancement of cereals through the process of fermentation gives a broad range for advancement of food which offers health benefits.

MATERIALS AND METHODS

Collection and preparation of plant material

Pearl millets (*P. glaucum*) were bought from Abraka market, Delta state and was identified and authenticated in the Department of Botany, University of Benin Edo State Nigeria (Voucher number: UBH-P398). The samples were pulverized using a commercial grinding machine (SM-1 Retsch GmbH 5667 HAAN, West Germany) and stored at room temperature.

Starter organism

The *R. oligosporus* strains were obtained from Harmony Path. Ltd. laboratory located at Songhai in Amukpe, Sapele Delta State. Solid state fermentation was carried out at pH 6 in petri dishes using 50 mM phosphate and citrate buffers at room temperature for 72 h.

Substrate preparation for solid state fermentation

R. oligosporus at different inoculum size (5, 10, 15, 20, 25, and 30%) were homogenized in 15 ml of prepared 50 mM acetate buffers at pH 6 in seven different petri dishes and was labeled according to the corresponding inoculum size. Exactly 7 g of the ground pearl millet flour was added to each of the petri dishes, and then the contents of each petri dish were mixed thoroughly. The petri dishes were then covered and the pearl millet was left to ferment for 72 h. An unfermented control (0%) (Containing dried and grounded pearl millet, devoid of any presence of molds; with buffer only, and without any cells) was prepared alongside the test samples. After fermentation, about 6 g of the mixture were taken from each of the petri dish from the different inoculum size and then 40 ml of distilled water were added to the mixture and homogenized using a mortar and pestle, 10 ml of the mixture were collected into a test tube then centrifuged for 10 min to separate the residue from the supernatant. Then the supernatant were stored in a universal container at room temperature and were used as the crude extract or sample for the various assays which were carried out in triplicates.

Total amylase activity

α-amylase activities was carried out by the procedure reported by Nouadri et al. (2010). The reaction mixture contains 0.5 ml of 1% (w/v) soluble starch in 0.1 M phosphate buffer (pH 6.5). Also, 0.2% (w/v) standard maltose solution in the range of 0 to 2 mg/ml was prepared such that the final volume for the assay is 1 ml. The mixture was allowed to warm at 30°C for 30 min. The reaction was stopped by the addition 2 ml of 3, 5-Dinitrosalicylic acid reagent followed by boiling for 5 mins to develop colour. The absorbance of the standard and samples was read at 540 nm. One unit (U) of amylase activity was described as the amount of enzyme that released 1 µg of maltose (as reducing sugar equivalent) under the assay conditions per ml per min.

Total soluble proteins

This was done following to the procedure described by Gornall et al. (1949) employing bovine serum albumin as the standard. Series of protein standards ranging in concentration from 0.5 to 10 mg/ml was prepared such that the final volume for the assay is 0.5 ml. The various samples were also prepared in a series of test tubes such that the final volume is 0.5 ml. Distilled water was used as blank. Thereafter, 2.5 ml of Biuret reagent was added to each sample and standard, vortexed and allowed to react for 30 min. After the incubation time has elapsed, the spectrophotomer was zeroed using blank and the absorbance of the standard and samples was read at 540 nm. A graph of absorbance of standards was plotted against concentration (mg/ml) to obtain a standard calibration curve of bovine serum albumin. The amount of protein present in the sample can then be estimated directly from the standard graph.

Total phenol content

This was done following to the procedure described by Singleton and Rossi (1965) using catechin as the standard. 1 ml of Folin C reagent was added to 1 ml of the sample. After 3 min, 1 ml of saturated Na_2CO_3 solution was added and the solution was made up to 10 ml with distilled water. The reaction mixture was kept in the dark for 90 min. The absorbance was read at 725 nml.

Test for glucose

This was done following the procedure described by the Randox glucose kit following the manufacturer instructions. 20 µl of standard and 20 µl of the various samples was pipetted into a series of test tubes labeled in triplicates, and then 2 ml of the reconstituted glucose working reagent was added to each test tube. Distilled water was used as blank. The contents of the test tubes were mixed and incubated for 25 mins at 15-25°C. After the incubation time has elapsed, the spectrophotomer was zeroed using blank and the absorbance of the standard and samples was read at 500 nm. Glucose concentration (mg/dl) was then calculated using the formula:

$$Glucose\ Conc. = \frac{Abs\ sample}{Abs\ standard} \times Standard\ conc.\ that\ is, 1.02\ mg/ml$$

Antioxidant Inhibition of 2,2-diphenyl-1-picrylhydrazyl (DPPH) Radical

This was done following the procedure described by Hatano et al. (1988). To 0.3 ml of the extract, 2.7 ml methanolic solution of DPPH radical (6 × 10−5 mol/L) was added. The mixture was shaken vigorously and left to stand for 60 min in the dark until stable absorption values could be obtained. The reduction of the DPPH radical was determined by measuring the absorption at 517 nm.

$$\%\ RSA = \frac{A_{DPPH} - A}{A_{DPPH}} 100$$

where, %RSA = Percentage of DPPH discoloration; ADPPH = absorbance of DPPH solution; AS = absorbance of the solution when the sample was added at a particular level.

Statistical analysis

All data were subjected to statistical analysis. Values were reported as Mean ± Standard deviation and the experimental results were analyzed using analysis of variance (ANOVA) and also a Fischer test of least significance (LSD) was carried out to compare the various group means. The results were considered significant at p-values of less than 0.05, that is, at 95% confidence level ($p<0.05$).

RESULTS AND DISCUSSION

The results of the effect of inoculum size on amylase activity during solid -state fermentation of pearl millet by the fungus *R. oligosporus* are expressed as µg of maltose liberated by the enzyme. Fermentation resulted in an outstanding increase ($p < 0.05$) in amylase activity from 200.02 ± 14.04 (ug/g/min) in the unfermented control (0%) to 308.92 ± 7.46 (ug/g/min) at 25% inoculum size after 72 h. An increase in amylase activity was also observed at other inoculum size (Figure 1). Chandrasekar and Shahidi (2010) reported that during fermentation, α-amylase activity increased due to enzyme secretion by the

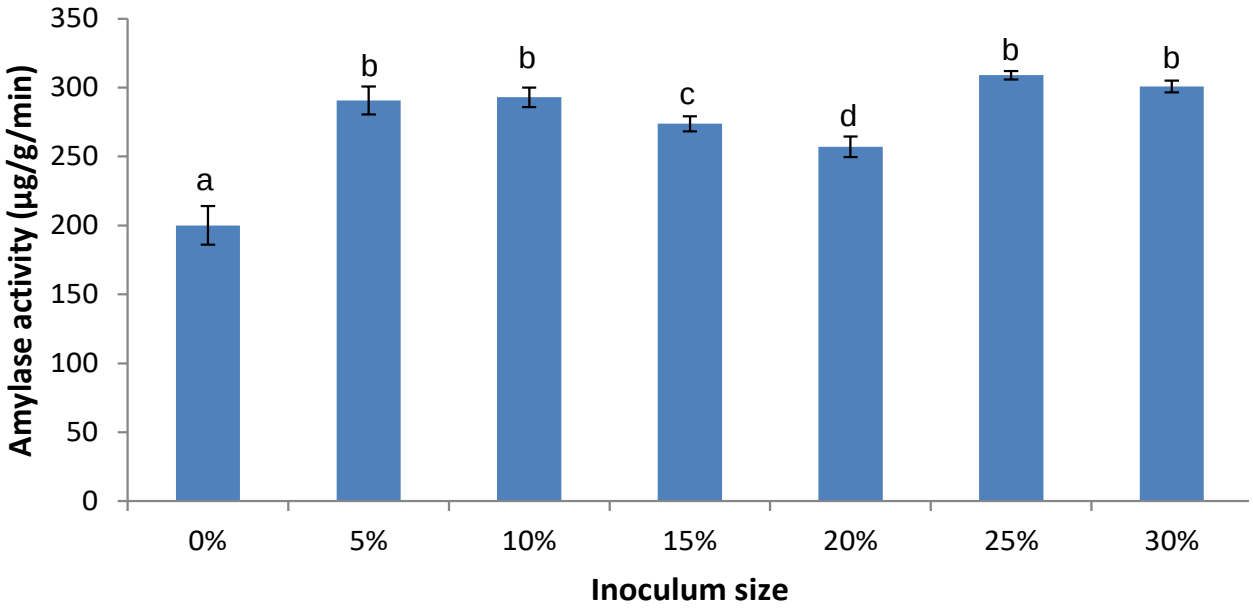

Figure 1. Effect of inoculum size on the amylase activities. Different alphabets on the bars signify statistically different result ($p < 0.05$).

microorganism, which is in line with this study. Amylase increased the fermentable and reducing sugars of flour and thereby enhanced the Maillard reaction to accentuate the bread flavor and colour and also it hinders the amylopectin recrystallization (Goesaert et al., 2009).

Fermentation resulted in a significant increase ($p<0.05$) in soluble protein from 105.48 ± 0.36 (mg g^{-1}) in the unfermented control (0%) to 169.13 ± 1.01 (mg g^{-1}) at sample fermented at 10% inoculum. An increase in soluble proteins was also observed at all inoculum size fermented (Figure 2).

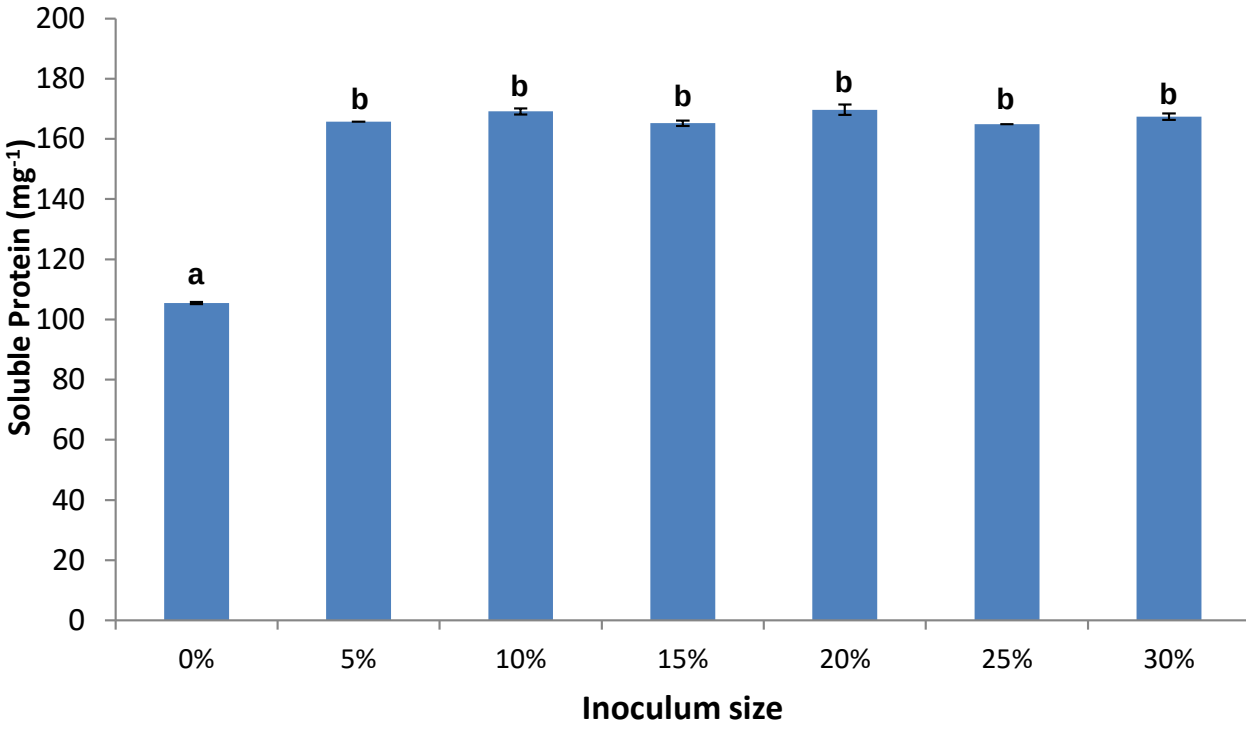

Figure 2. Effect of inoculum size on the levels of soluble proteins. Different alphabets on the bars signify statistically different result ($p < 0.05$).

Fermentation of pearl millet using the fungus *R. oligosporus* caused degradation and solubilization of fungal protein which was noticed as the increase in crude protein (CP) content (Belewu and Belewu, 2005). Rise in crude protein (CP) could be as a result of the hydrolysis of starch to glucose followed by its use by same organism as a source of carbon to synthesize fungal biomass rich in protein (Bender, 1970). Our results are in lined with the findings of Belewu and Okhawere (1998) who noted that the colonization of lignocellulosic waste by the fungi results in increase in their nutritional value probably caused by increase of the fungal biomass/reproduction in fungal treated straw.

Fermentation resulted in a significant increase ($p < 0.05$) in total phenol content (TPC) from 169 ± 0.36 (µg/ml) in the unfermented control (0%) to 233 ± 1.01 (µg/ml) at sample fermented at 30% inoculum (Figure 3). An increase in total phenol content was also noted at all inoculum size fermented at different inoculum size (202 ± 0.8; 224 ± 1.1; 215 ± 0.8; 230 ± 0.4; 214 ± 0.4) µg/ml. Increase in phenol from 169 ± 0.36 (µg/ml) in the unfermented control (0%) to 233 ± 1.01 (µg/ml) at sample fermented at 30% inoculum is similar with the work of Zhao et al. (2017).

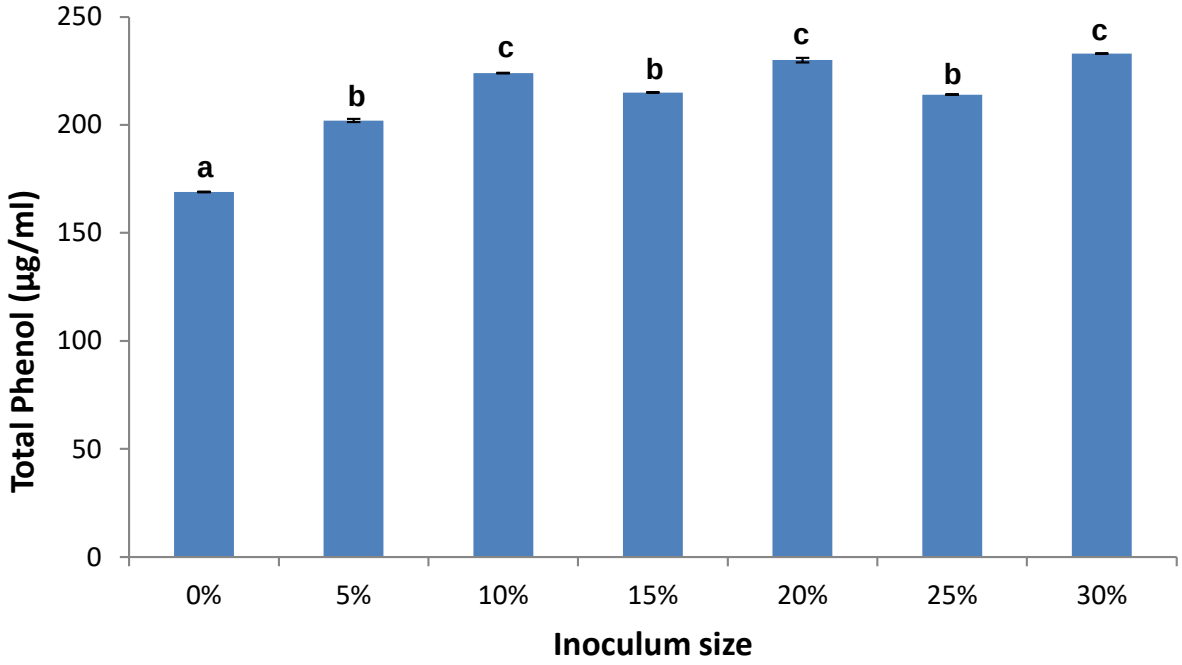

Figure 3. Effect of inoculum size on total phenol content. Different alphabets on the bars signify statistically different result ($p < 0.05$).

Phenolic compounds are secondary metabolites that have health-promoting benefits such as anticancer activities and antioxidant (Balasundram et al., 2006). Thus, SSF may support the conversion of bound to free phenolics, thus, improving their bioavailability (Dey et al., 2016). The increase in TPC may be validated by the proteolytic activities of fungi leading to the release of bound to free TPC.

Fermentation resulted in a significant increase ($p < 0.05$) in glucose level from 0.93 ± 0.36 (mg g^{-1}) in the unfermented control (0%) to 2.97 ± 1.01 (mg g^{-1}) at sample fermented at 10% inoculum. An increase in levels of glucose was also observed at all inoculum size fermented (Figure 4). From the results, it is clear that solid state fermentation increases glucose concentration at the different inoculum size considered. This increase in the glucose in the pearl millet during the fermentation with *R. oligosporus* agrees with earlier report on *R. oligosporus* fermentation of maize (*Zea mays*) offal (Anigboro et al., 2020). This increase in glucose concentration can be attributed to the ability of the microorganism to secrete extracellular enzymes, which degrade the complex polysaccharides to glucose during SSF,

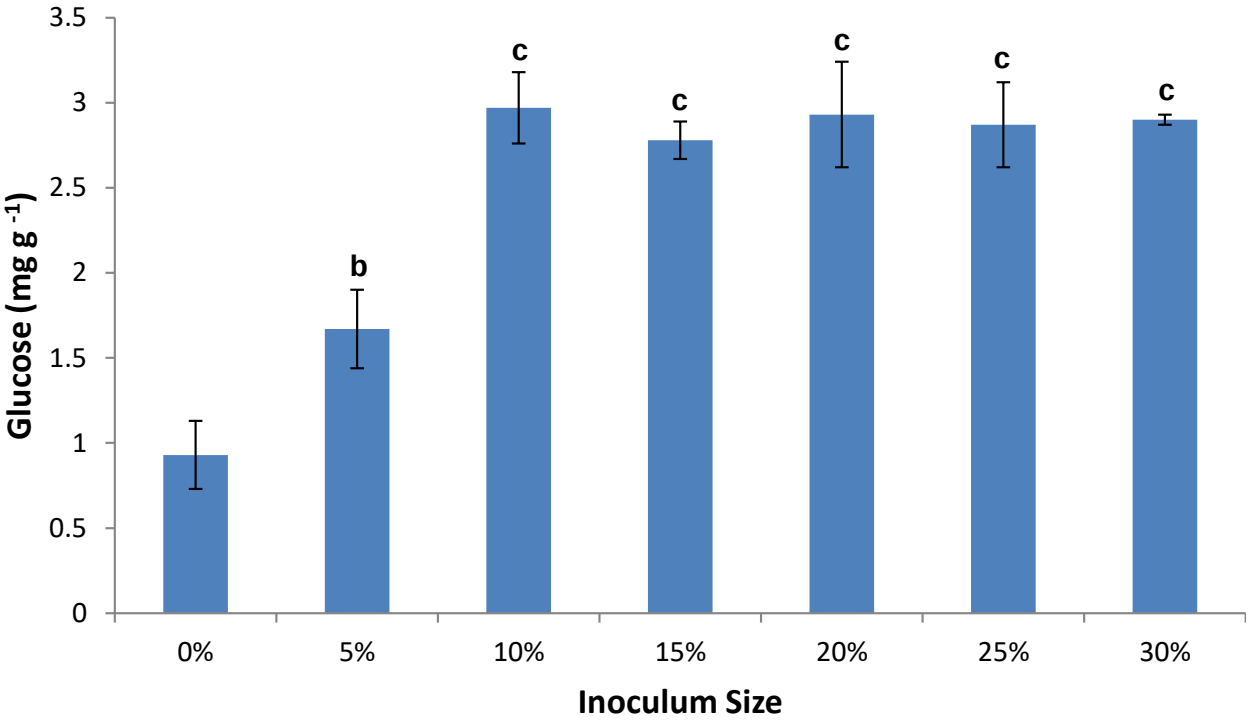

Figure 4. Effect of inoculum size on the levels of glucose. Different alphabets on the bars signify statistically different result ($p < 0.05$).

this is could be explained by the favorable state of the fermentation procedure at pH 6 (Tonukari et al., 2016). This indicates that moderately acidic and basic medium could accelerate the production or synthesis of glucose as the enzyme amylase performs excellently at pH of 6. This report is in agreement with the report of Oyarekua et al. (2013).

From the results, DPPH free radical scavenging capacity increased significantly ($p<0.05$) from 44 ± 0.08% in the unfermented control to 61 ± 0.4% at sample fermented at 10% inoculum. An increase in antioxidant activity was also observed at all inoculum size fermented (Figure 5). The result of the scavenging activity assays indicates that the fermented pearl millet was potentially active at all the inoculum sizes tested. This suggests that the fermented samples contains compounds that are capable of donating hydrogen to free radicals (chemical species with lone pair electrons) thereby reducing such radicals or reactive species to their inert states and this could help to prevent free radical-induced cellular damage in individuals that consume this pearl millet as part of their regular diets (Tonukari et al., 2015). DPPH is employed to evaluate the free radicals scavenging activity of natural antioxidants. Free radicals are inevitable metabolites in aerobic organisms during respiration (Viña et al., 2020). They are highly reactive and very unstable to be able to oxidize lipids, proteins, DNA and other biomolecules, resulting in oxidative damage in human body (Ali et al., 2020). Antioxidants are believed to play a major role in the body's defense system against free radicals. Therefore, it is very paramount to examine their free radical-scavenging activity in order to estimate repercussion in oxidative stress in living beings. Many of fermented products, such as natto, tempeh and fermented soymilk and cowpea, have been described to possess high free radical-scavenging activity (Dey et al., 2016).

Conclusion

SSF by the fungus *R. oligosporus* influenced the amylase activity, soluble protein, phenol content, glucose, and antioxidant activities inhibition of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical of pearl millet. SSF increased the health value mainly the soluble protein content, this study reveals that millet improved by SSF fermentation will be an antioxidant rich and healthy food additive when compared to unfermented millet.

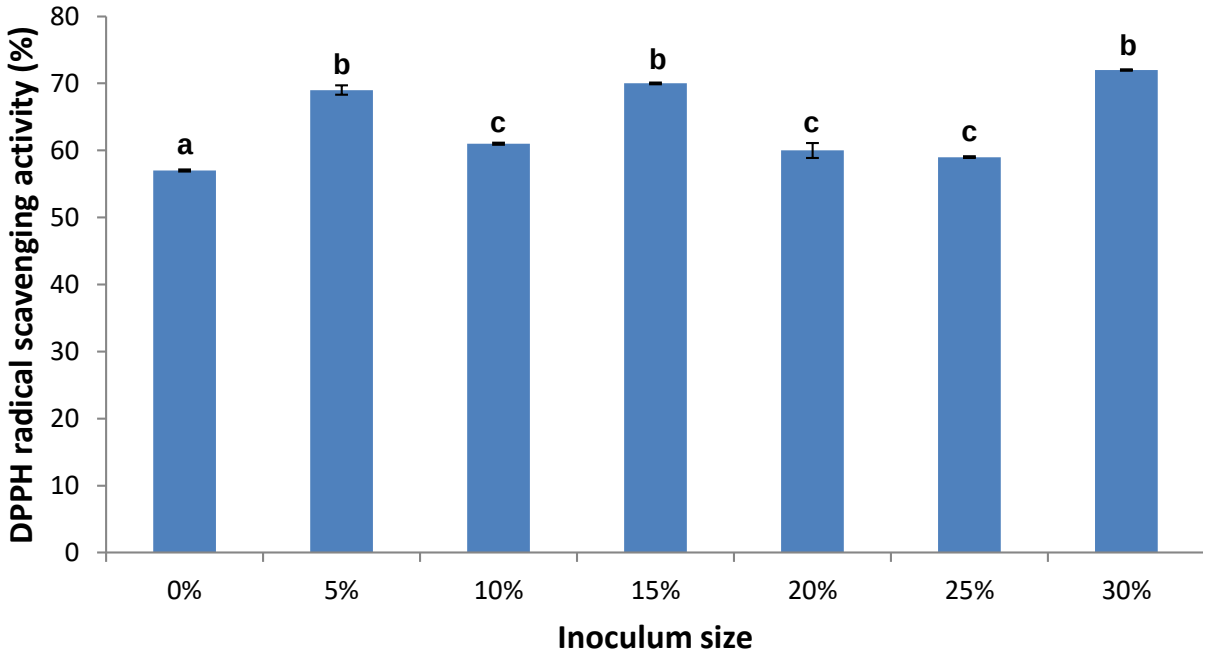

Figure 5. %DPPH radical scavenging activity. Different alphabets on the bars signify statistically different result ($p < 0.05$).

The observation from the work above is relevant to expedite optimization for pearl millet solid-state bioprocessing strategy. The functional properties and nutritional features of the solid-state fermented pearl millet showed that they could be acceptable components for feed formulations.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

REFERENCES

Aganbi, E., Anigboro, A. A. and Tonukari N. J., (2020). Changes in Glucose, Amylase And Soluble Proteins Levels In Solid State Fermented Yam (*Dioscorea Spp*.) Peels By *Rhizopus oligosporus*. *Nigerian Journal of Science and Environment*, Vol.18 (1).

Ali, S. S., Ahsan, H., Zia, M. K., Siddiqui, T., and Khan, F. H. (2020). Understanding oxidants and antioxidants: Classical team with new players. *Journal of Food Biochemistry*, doi:10.1111/jfbc.13145

Anigboro, A. A., Aganbi, E. and Tonukari, N. J. (2020). Solid State Fermentation of Maize (Zea mays) Offal by *Rhizopus oligosporus* under Acidic and Basic Conditions, *Journal of Scientific Research*. **12**(4): 751-756.

Balasundram, N., Sundram, K. and Samman, S. (2006). Phenolic compounds in plants and agri-industrial by-products: Antioxidant activity, occurrence, and potential uses. Food Chemistry, **99**(1): 191–203.

Belewu M. A. and Belewu, K. Y. (2005). Cultivation of mushroom (Volvariella volvacea) on banana leaves. *African Journal of Biotechnology* 4 (1) : 1401–03.

Belewu, M. A. and Okhawere, O. C. (1988). Evaluation of Feeding Fungi Treated Rice Husk to Ram. Proceeding of the 25th Annual Conference and Silver Jubilee of the Nigerian Society Animal Production 21–24th March 1998, pp 321–22.

Bender, P.F. (1970). Under Utilized Resources as Animal Feedstuffs. Pp100 National Academy Press Washington D C.

Bhanja, T., Kumari, A., and Banerjee, R. (2009). Enrichment of phenolics and free radical scavenging property of wheat koji prepared with two filamentous fungi. *Bioresource Technology*, **100**: 2861-2866.

Cassano A, Donato L, Conidi C. and Drioli E. (2008). Recovery of bioactive compounds

in kiwifruit juice by ultrafiltration. *Innovative Food Science and Emerging Technologies,* **9**:556–562

Chandrasekara, A. and Shahidi, F. (2010). Content of insoluble bound phenolics in millets and their contribution to antioxidant capacity. *Journal of Agricultural and Food Chemistry.* **58**(11): 6706–6714.

Chethan, S. and Malleshi NG (2007). finger millet polyphenols: characterization and their nutraceutical potential. *America Journal of Food Technology*, **2**:582–592

Dey, T. B., Chakraborty, S., Jain, K. K., Sharma, A. and Kuhad, R. C. (2016). Antioxidant phenolics and their microbial production by submerged and solid-state fermentation process: A review. *Trends in Food Science & Technology*, **53**: 60–74.

Goesaert, H., Brijs, K., Veraverbeke, W., Courtin, C., Gebruers, K. and Delcour, J. 2005. Wheat flour constituents: how they impact bread quality, and how to impact their functionality. *Trends in Food Science & Technology,* **16**(1):12-30.

Goesaert, H., Slade, L., Levine, H. and Delcour, J.A. (2009). Amylases and bread firming–an integrated view. Journal of Cereal Science, 50 (3):345-352.

Gornall, A. G., Bardawill, C. J., and David, M. M. (1949). Determination of serum proteins by means of the biuret reaction. *Journal of Biological Chemistry.* **177:**751-756.

Hatano, T., Kagawa, H., Yasuhara, T. and Okuda, T. (1988). Two New Flavonoids and Other Constituents in Licore Root: Their Relative Astringency and Radical Scavenging Affects. Chemical & Pharmaceutical Bulletin, 36, 2090-2097.

Hegde, P.S., Chandrakasan, G. and Chandra, T.S. (2002). Inhibition of collagen glycation and crosslinking in vitro by methanolic extracts of finger millet (Eleusine coracana) and Kodo millet (Paspalum scrobiculatum). *Joural of Nutritional Biochemistry,* **13**:517–521

Kumari, L.P. and Sumathi, S. (2002). Effect of consumption of finger millet on hyperglycemia in non-insulin dependent diabetes mellitus (NIDDM) subjects. *Plant Foods Hum Nutr,* **57**:205–213

Noudri, T., Meraihi, Z., Shahrazes, D. D. and Leila, B. (2010). Purification and characterization of amylase isolated from Penicillium camemberti PL21. *African Journal of Biochemistry Research* 4 (6): 155-162.

Oyarekua, M.A., Akinyele IO, Treche S and AF Eleyinmi (2013). Amylolactic Acid Fermentation of Maize/Cowpea 'Ogi' Int. Journal of Food Processing & Preservation. **32**: 286-305.

Pandey, A. (2003). Optimization of concomitant production of cellulase and xylanase from Rhizopus oryzae SN5 through EVOP-factorial design technique and application in Sorghum Stover based bioethanol production. *Renewable Energy,* **98**:51---56.

Ramachandra, G., Virupaksha, T.K. and Shadaksharaswamy, M. (1977). Relationship between tannin levels and in vitro protein digestibility in finger millet (Eleusine coracana Gaertn). *Journal of Agricultural and Food Chemistry,* **25**:1101–1104 3.

Salar, R. K. and Purewal, S. S. (2017). Phenolic content, antioxidant potential and DNA damage protection of pearll millet (Pennisetum glaucum) cultivars of North Indian region. *Journal of Food Measurement and Characterization,* **11**: 126-133.

Salar, R. K., Certik, M. and Brezova, V. (2012). Modulation of Phenolic content and Antioxidant activity of Maize by Solid State Fermentation with Thamnidium elegans CCF-1456. *Biotechnology and Bioprocess Engineering,* **17**: 109-116.

Salar, R. K., Purewal, S. S. and Bhatti, M. S. (2016). Optimization of extraction conditions and enhancement of phenolic content and antioxidant activity of pearll millet fermented with Aspergillus

awamori MTCC-548. *Resource-Efficient Technologies,* **2**: 148---157.

Sandhu, K. S. Punia, S. and Kaur, M. (2016). Effect of duration of solid state fermentation by Aspergillus awamori Nakazawa on antioxidant properties of wheat cultivars. *LWT- Food Science and Technology*, **71**: 323-328

Segura, M.E.M. and Rosell, C.M. (2011). Chemical composition and starch digestibility of different gluten free breads. *Plant Foods for Human Nutrition,* **66** (3):224- 230.

Singleton, V.L. and Rossi, J.A. (1965) Colorimetry of Total Phenolics with Phosphomolybdic-Phosphotungstic Acid Reagents. *American Journal of Enology and Viticulture*, 16, 144-158.

Soccol, C. R. and Vandenberghe, L. P. S. (2003). Overview of solidstate fermentation in Brazil. *Biochemical Engineering Journal,* **13**: 205-218.

Tonukari, N. J., Avwioroko, O. J., Ezedom, T. and Anigboro, A. A. (2015). Effect of Preservation on Two Different Varieties of Vernonia amygdalina Del. (Bitter) Leaves. *Food and Nutrition Sciences*, 6, 623-632.

Tonukari, N. J., Egbune, E. O., Avwioroko, O. J., Aganbi, E., Orororo, O. C., and Anigboro, A. A. (2016). A novel pig feed formulation containing Aspergillus niger CSA35 pretreated-cassava peels and its effect on growth and selected biochemical parameters of pigs. *African Journal of Biotechnology*. 15(19), 776-785.

Vina, jose, Olaso, G., Arc-Chagnaud, C., de la Rosa, A., and Gomez-Cabrera, M. C. (2020). Modulating oxidant levels to promote healthy ageing. *Antioxidants & Redox Signaling*, doi:10.1089/ars.2020.8036.

Yen, G. C., Chi, C. Y. and Su, S.W. (2003). Antioxidant activity and active compounds of rice koji fermented with Aspergillus candidus. *Food Chemistry*, **83**: 49- 56

Zhao, H. M., Guo, X. N. and Zhu, K. X. (2017). Impact of solid-state fermentation on nutritional, physical and flavor properties of wheat bran. *Food Chemistry,* **217**: 28–36