Journal of Applied Biology & Biotechnology Vol. 1 (02), pp. 018-023, July- Aug, 2013
Available online at http://www.jabonline.in
DOI: 10.7324/JABB.2013.1204


# Effect of Wild and Mutant Strain of *Lasiodiplodia Pseudotheobromae* Mass Produced on Rice Bran as a Potential Bioherbicide Agents for Weeds under Solid state Fermentation.

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**ARTICLE INFO**

*Article history:*
Received on:02 /08/2013
Revised on:10/08/2013
Accepted on: 15/08/2013
Available online:
30/08/2013

***Key words:***
Agro-industrial wastes, conidia production, solid state fermentation, Lasiodiplodia pseudotheobromae, Rice bran.

**ABSTRACT**

Local available agro-industrial waste from rice bran was evaluated in the laboratory for use as substrate for mass culturing of *Lasiodiplodia pseudotheobromae*. Boiling water and *Anona senegalensis* extracts were added to the substrate under solid state fermentation(SSF). The effects of lengthy exposure to Ultraviolet (UV) radiations (1h 30min,1hours, 30 minutes) on the conidia production were assessed after 14days with 12h photoperiod at 25-30°C. The laboratory studies showed that rice bran with boiling water in nylon bag inoculated with the mutant strain of *Lasiodiplodia pseudotheobromae* exposed for 1hour 30 minutes had the highest number of conidia with $3.80 \times 10^9$ cfu.g$^{-1}$ followed *by Lasiodiplodia pseudotheobromae* exposed to 30 minutes U.V. radiation with conidia number of $2.08 \times 10^9$ cfu.g$^{-1}$ while the wild type had a conidia of $1.66 \times 10^9$ cfu.g$^{-1}$ after 14days with 12hours photoperiod at 25-30ºC. Therefore, agro-industrial wastes from rice bran could be suitable for mass production of bioformulated products of *Lasiodiplodia pseudotheobromae* which are used for the control of weeds in Nigeria.

## 1.INTRODUCTION

The *Botryosphaeriaceae* is a diverse group of fungi that accommodates numerous species spread over many anamorph genera, the best known of which are *Diplodia*, *Lasiodiplodia*, *Neofusicoccum*, *Pseudofusicoccum*, *Dothiorella* and Sphaeropsis[1]. Members of the *Botryosphaeriaceae* have a worldwide distribution and occur on a large variety of plant hosts including monocotyledons, dicotyledons, gymnosperms and angiosperms, on which they are found as saprophytes, parasites, and endophytes[2,3].It has long been recognized that species of the *Botryosphaeriaceae* are important pathogens of several plants[3]. Infected plants can exhibit a multiplicity of symptoms such as die back, canker, blight and rot on all above ground plant organs[4,5].

*Lasiodiplodia pseudotheobromae* emerged from a recent separation of cryptic species originally identified as *L.* theobromae[6].The species is known from Africa, Europe and Latin America, where it has been described from forest and fruit trees. Growing evidence suggests that *Lasiodiplodia pseudotheobromae*, like *Lasiodiplodia theobromae*, has a worldwide distribution and a wide host range[7,8,9].

Weed control strategies using microbial agents have received considerable attention in recent years due to the mounting expense for registration of chemical herbicides, ban on the use of chemical herbicides and public demands for reduced chemical uses[10,11,12]. The bioherbicide strategy is a microbial approach being used to control weeds in agronomic crops (Templeton, 1982). This strategy involves treating weed infested crops which are highly aggressive to specific pathogens of the targeted weed. There are worldwide resurgence of interest in the use of indigenous ecofriendly and host specific fungal pathogens as herbicides (mycoherbicides) and a significant advance in mass production and fermentation of some of them have been observed [14,15,16,17]. However, only few organisms have been commercially produced for large scale field applications [18, 19]. Non availability of low-cost mass production technology is one of the major hindrance in their application [20]. This may be achieved by selecting a suitable substrate that is simple in composition, cheaper in price and available in large quantities and developing a production procedure that is easy to apply with minimum labour. Fungi are considered advantageous over other microorganisms because they are capable of developing epidemics, infection does not require a damaged or compromised host and spores are relatively stable [21].

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Commercial production request low-cost, easily available production technology which can be achieved by solid substrate formation technology [22]. Solid substrate fermentation (SSF) technology is culturing of fungi on solid agro waste is extensively used in Biotechnology for production of organisms itself and their by products [23, 24, 25, 15, 26, 18].

A variety of substrates have been employed by several workers for the better production of conidia [19, 27, 28].

The aim of this work was to determine the effect of wild and mutant strain of *Lasiodiplodia pseudotheobromae* mass produced on rice bran under solid state fermentation**.**

## MATERIALS AND METHODS

### Source and maintainance of Fungal Isolates

Fungal strains were isolated from small chlorotic and necrotic lesions on leaves of different weeds collected from Ogbomoso and Ilorin environment. The weed leaves were surfaced sterilized for 2 minute in 0.5% sodium hypochlorite, rinsed in sterile distilled water, and placed on Difco PDA(Potatoes Dextrose Agar) plates at 24$^{o}$C with 12hours light, for 3-7days . Fungal identification was carried out according to the procedure described by Samson and Van Reenen-Hoekstra (1982). Cultural and microscopic morphology was used to confirm *Lasiodiplodia pseudotheobromae* isolates. Koch's postulates was applied to establish the pathogenic status of pure isolates of *Lasiodiplodia pseudotheobromae* on weed from which they where isolated. Purified *Lasiodiplodia pseudotheobromae* isolates were then preserved by storing a hyphal fragment and spore suspension in a 1:1 skim milk (10% v/v) to glycerol (40% w/v) solution and then stored at 4$^{o}$C . Isolates were revived by thawing a vial containing the fungus to room temperature. The content was aseptically spread on the surface of 15-cm diameter petri dishes containing Difco potato dextrose agar (PDA). The plates were then incubated at room temperature with natural light for 1–2 weeks.

### Exposure of *Lasiodiplodia pseudotheobromae* to UV light to induce Mutation.

This experiment was carried out in order to see whether by mutation we can improve the Amount of phytotoxic metabolites in the medium and increase in yield. This was carried out by preparation of fresh PDA plate to grow the organisms .After the growth of the organisms, cork borer was used to obtain several mycelia plugs from the culture into a sterile PDA plate. The sterile plate containing several mycelia plugs were placed under UV lamp at 300nm wavelength at a distance of 30cm to the plates. At different time interval (30, 60, and 90 minutes), 5 mycelia plugs were withdrawn and used as inoculants for potatoes dextrose medium on the rice bran . The mycelia plugs from the domesticated type culture serve as the control.

### Mass production of fungi

Mass production of fungi propagates from modified method of Siddiqui and Bajiwa[29]. Isolated strains of *Lasiodiplodia pseudotheobromae* were cultured on rice bran. The rice bran was added to boiling water containing 1% *Anona senegalensis* extract for 5 minutes. The substrates was treated with *Anona senegalensis* extract and the substrates for one treatment. Sterilized boiled water was added to rice bran substrate, to form another treatment for 5 minutes and it was packed into heat resistant nylon bags (250g/bag) with the mouth tied, with rubber band and steamed for 4 hour 30 minutes . This was inoculated with the spores of the wild type and mutant of *Lasiodiplodia pseudotheobromae* and incubated in the dark for 14days. In other instances, equal volume of substrate and equal volume of water (w/v) in bottles were steamed and inoculated with spores of *Lasiodiplodia pseudotheobromae*.

### Spores Suspension Preparation

Spores were harvested from one week old culture of the isolates. A suspension of spores was obtained by dilution of spores in 10 ml of sterile distilled water. The desired concentration of spores in the suspension was obtained by counting the number of cells in the suspension using heamocytometer and subsequent continuous dilution of the suspension until a desired spore concentration was obtained [30,31].

### Spore harvesting and drying

The spores were harvested on the 14days after the inoculation. To harvest the spores as powder it was necessary to dry the fungus to reduce moisture content and allow the spores to separate from the rice substrata. The spores harvested following this procedure can be preserved for a long time without loss of germinative power or pathogenicity[32].Treatments were selected at random to be dried at each harvest time. To dry the cultures the plastic bags were opened in a room with a temperature of 25 ± 4$^{o}$C and an average relative humidity of 55 ±7 % and allowed to air dry. Harvesting was done both mechanically for 20 minutes. The mechanical harvest involved the use of a shaker Ro-Tap Sieve Shaker (W.S. Tyler Inc., www.wstyler.com/) that uses horizontal circular motion and vertical taping motion to stratify and screen the particles. The samples were then put through 3 sieves as described above. The spore powder that was collected after sieving was weighed and kept in separate sterile vials for further assessments) and stored for later use inside a sealed silica gel vacuum desiccators and kept in a cool room under the conditions described above.

### Treatments of rice bran with the spores of *Lasiodiplodia pseudotheobromae*

T1= Spores of *Lasiodiplodia pseudotheobromae* +rice bran+ boiled *Anona senegalises* in bottle.

T2= Spores of *Lasiodiplodia pseudotheobromae* +rice bran+ boiled *Anona senegalises* in nylon.

T3= Spores of *Lasiodiplodia pseudotheobromae* +rice bran+ boiled water in nylon.

T4= Spores of *Lasiodiplodia pseudotheobromae* +rice bran+ boiled water in bottle.

**Assessment of fungal growth and sporulation on substrates.**

After 14 days, bags and bottles were opened and One gram of conidiated substrates were mixed with 9 ml of distilled and sterilized water. Ten flasks which each contained 100 ml of the mixture of conidiated substrates were agitated in a rotary shaker at 121 rpm for 1hr. The mixture of conidiated substrates were filtered through three layers of cheese cloth. The number of conidia was determined by a Haemocytometer [33] and calculated as conidia/g of substrate. All experiments were performed in duplicates, statistically evaluated by excel and SPSS (version 17) and results were presented as mean ± SEM. (standard error of mean).

## RESULTS AND DISCUSSION

**Fig. 1** shows the effect of rice bran with boiling water in nylon bag after 14 days of inoculation with the mutant and wild type of *Lasiodiplodia pseudotheobromae*. *Lasiodiplodia pseudotheobromae* exposed to 1 hour 30 minutes had the highest with conidia number of $2.10 \times 10^9$ cfu /g while the wild type of *Lasiodiplodia pseudotheobromae* had the lowest number of conidia with $1.66 \times 10^9$ cfu/g. There was no growth on the rice bran substrates serving as the control.

**Fig.2** shows the effect of rice bran with boiled *Anona senegalises* in nylon bag after 14days of inoculation with the mutant and wild type of *Lasiodiplodia pseudotheobromae* . *Lasiodiplodia pseudotheobromae* exposed to 1hour 30minutes had the highest number of conidia with $3.08 \times 10^9$ cfu/g while the addition of the wild type of *Lasiodiplodia pseudotheobromae* to rice bran had the lowest with conidia number of $1.82 \times 10^9$ cfu/g. There was no growth on the rice bran serving as the control.

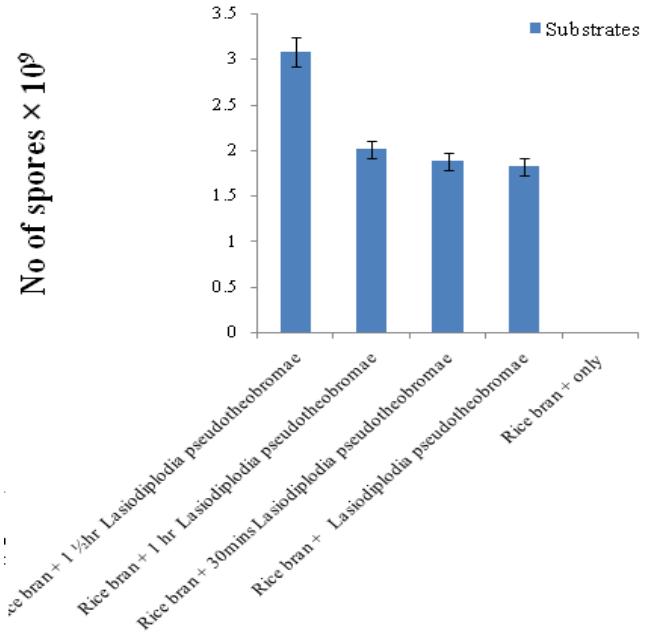


**Fig. 1:** Effect of spores produced on rice bran from *Lasiodiplodia pseudotheobromae* with boiled water in nylon bag after 14days. Error bar= standard error of observed values

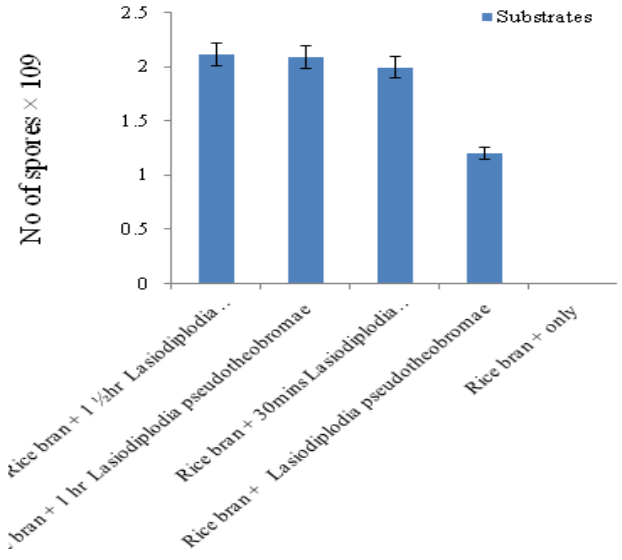


**Fig. 2:** Effect of spores produced on rice bran from *Lasiodiplodia pseudotheobromae* with boiled *Anona senegalises* in in bottle after 14days . Error bar= standard error of observed values.

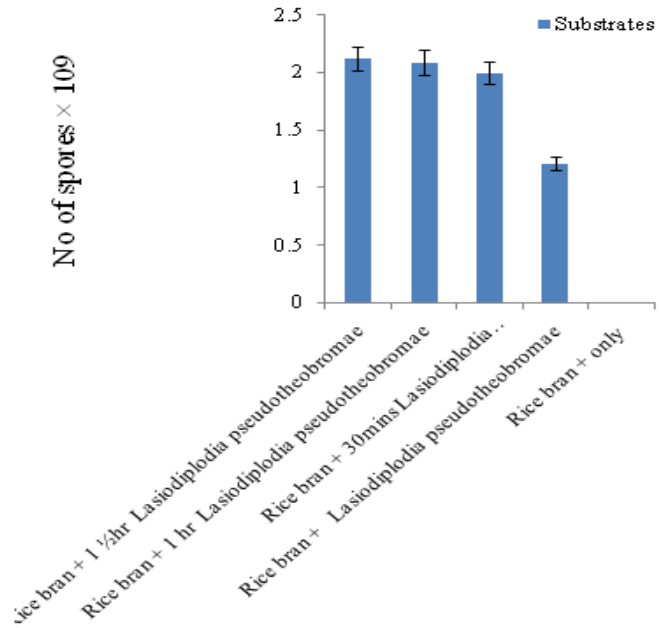


**Fig. 3:** Effect of spores produced on rice bran from *Lasiodiplodia pseudotheobromae* with boiled water in bottle after 14days. Error bar= standard error of observed values

**Fig.3**. shows the effect of rice bran with boiling water in bottle after 14 days inoculation with the mutant and wild type of Lasiodiplodia pseudotheobromae. Lasiodiplodia pseudo-theobromae exposed to 1 hour 30 minutes had the highest number of conidia with $2.12 \times 10^9$ cfu/g while the wild type of Lasiodiplodia pseudotheobromae added to rice bran that had the lowest with conidia number of $1.21 \times 10^9$ cfu/g. There was no growth on the rice bran serving as the control.

**Fig.4**. shows the effect of rice bran with boiled Anona senegalises in bottle after 14 days inoculation with the mutant and

wild type of Lasiodiplodia pseudotheobromae .Lasiodiplodia pseudotheobromae exposed to 1 hour 30 minutes had the highest number of conidia with $2.04 \times 10^9$ cfu/g while the wild type of Lasiodiplodia pseudotheobromae added to rice bran had the lowest number of conidia of $1.01 \times 10^9$ cfu/g. There was no growth on the rice bran serving as the control.

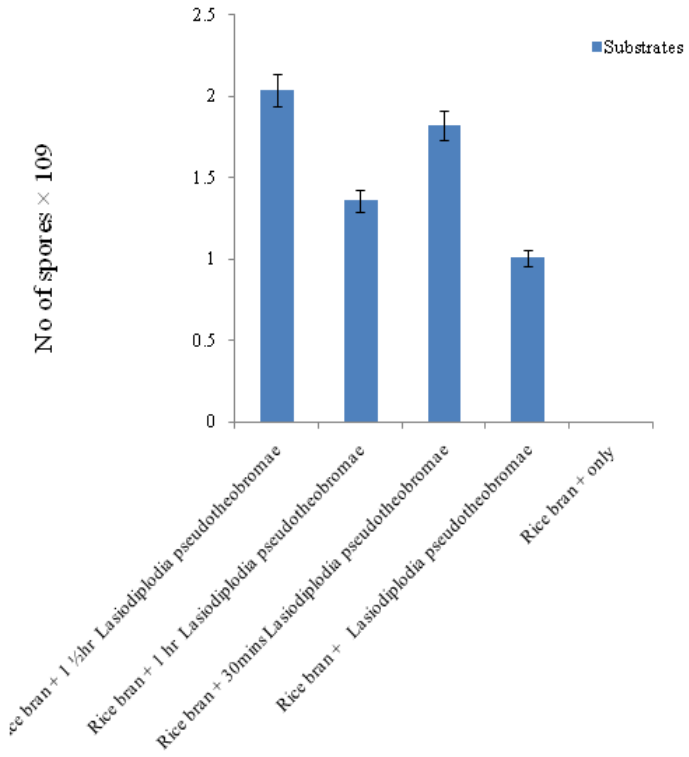


**Fig. 4:** Effect of spores produced on rice bran from *Lasiodiplodia pseudotheobromae* with boiled *Anona senegalises* in bottle after 14days. Error bar= standard error of observed values

For commercialization of bioherbicides, the method of inoculum production should be inexpensive and yield sufficient biomass containing viable, highly virulent propagules which ideally can stored within dry formulations. Submersed was found to be more economical method of production of fungal biocontrol agents compared to solid fermentation [34]. The selection of a suitable medium that enables good growth is the first step in optimizing inoculum production.

Pandey *et* al.[26], reported that agro-industrial residues, which are generally used as substrates for Solid state fermentation offer potential advantages for the filamentous fungi, which are generally capable of penetrating into the hardest of these solid substrates, aided by the tip of the mycelium. The hyphal mode of fungal growth and their good tolerance to osmotic pressure conditions make fungi efficient and competitive in natural microflora for bioconversion of solid substrates[31].

Rice bran and sorghum were been used because they are readily available and cheap. Cheap culture medium is required in order to increase the cost-benefit ratio. Hence, several sources were tested for mass multiplication. Low cost sources of nutrients like millets such as sorghum, jhangora, and pearl millet were assessed for their utility in terms of conidial yield of the test fungus. Some of these grains are inexpensive, easily available and act as best nutritive media for the mass multiplication of many micro and macro organisms [26]. The mutant and the wild strain of *Lasiodiplodia pseudotheobromae* used in this studies was grown on rice bran which form a solid state fermentation. Solid-state fermentation is the process of growing microorganisms on a solid material without any free water and is more suitable for the production process involving fungi than bacteria [35]. Solid substrate fermentation is a similar process, however in this form the solid material serves as the nutrient source as well as the supporting material [26]. Unfortunately in the literature, these two processes are often used interchangeably. In bioherbicide research, the majority of researchers have used solid substrate fermentation, especially in production of fungal pathogens.

Solid-state fermentation is a cost-effective system for sporulation of fungi [36]. An important aspect of the initial phase of bioherbicide is culturing of organism on artificial media followed by optimization for spore production. Spore production on agar media can only provide sufficient inoculum for laboratory and small plot field trials.

The various agro-industrial wastes substrates evaluated using polyethylene nylon bag was found to had the highest number of conidia yield was recorded on rice bran ($3.08 \pm 0.009 \times 10^9$ conidia/g of substrate) which was exposed to 1 hour 30 minutes Ultraviolent ray. Rice bran is being studied as a substrate for solid-state fermentation (SSF) processes, in order to increase nutrient availability through changes arising from microorganisms' metabolic activity which results in interesting compounds [37,38].

Previously, Ibrahim and low[39] found rice as a suitable media for the mass culture of *B. bassiana*. This cereal was also used for the mass production of other deuteromycete fungi. Other researchers have used extracts from the colonized grain as a bioherbicide. Hoagland *et al*.[40] used rice grain to grow *Myrothecium erraria* to control kudzu in a range of crops. They found that extracts from the rice grains were more efficacious than inoculums produced from agar when applied to soil. Rice bran is obtained from grain milling process, representing 5–8% of the total grain. Among the nutrients, bran contains minerals such as iron, phosphorus and magnesium, between 11% and 13% crude protein, approximately 11.5% of fibers and it is considered a very good source of oil because it may contain 20% of its weight in oil [41,42].

Mutation was also induced on *Lasiodiplodia pseudotheobromae* by exposing the wild type of the fungus to 1 hours 30 minutes ,1 hours,30 minutes of Ultraviolent ray so as to produced the different type of mutant strains . It was observed that Mutant strain of *Lasiodiplodia pseudotheobromae* grown on Rice bran also had the largest number of conidia as shown in figure 1. Mutation induction has become an established tool in strain improvement to supplement existing strains and to improve species in certain specific traits. Therefore, several approaches

including chemical mutation ,UV irradiation and genetic engineering to obtain high yield strains have been given a priority in the last decades [43].

Traditionally, strain development meant a laborious approach with regard to identification of superior isolates from a mutagen treated conidia and vegetative hyphae with various mutagens to search for improved mutant among the surviving progeny has now been recognize as the best mean to secure strains of improved potency [44]. Generally, mutant strain of *Lasiodiplodia pseudotheobromae* exposed to 1 hour 30minutes which was stored in nylon bags had the largest mass of conidia than the conidia stored in bottled glass container containing rice bran . For example, rice bran with boiled *Anona senegalises* in nylon bag after 14days had ($3.08\pm0.006 \times 10^9$ conidia/g of substrate) which was exposed to 1 hour30minutes compared to rice bran inoculated for 1 hour 30minutes with mutant strain of *Lasiodiplodia pseudotheobromae* added to boiled *Anona senegalises* in bottle after 14days which had ($2.04\pm0.006 \times 10^9$ conidia/g of substrate). This may be due to the fact that rice bran has a large surface area and uniform distribution of *Lasiodiplodia pseudotheobromae* on the substrate used. Aregger [45] compared the production of *B. brongniartii* conidia on barley using Erlenmeyer flasks and autoclavable polyamide bags. He suggested that the enormous surface and the granular structure of the medium could be controlled more easily and the clumps better crushed in bags than in other containers. Mccoy *et al*.[45] successfully produced conidia of *M. anisopliae* in sealed plastic bag of rice for control of rhinoceros beetle, *Oryctes rhinoceros*, a serious pest of coconut palms. Due to the relatively low cost, high availability and easy manipulation, autoclavable plastic bags can be recommended for simple and fast mass production. Furthermore our studies indicate that large quantities of conidia were produced faster on rice than other kinds of grain. This may be due to the smaller size of rice grains which provides higher ratio of surface per volume. White-rice had more surface area and space for fungus growth than broken-milled rice while corn and sorghum grains were covered by caryopsis which might protect it from fungus penetration. Also, Because polyethylene bags can retain temperature and allow the passage of oxygen which stimulate the growth and sporulation of fungal strains during fermentation ,it make it a fermentation container than glass bottle. Nutrients availability in the two substrates used also enhances the better performance of both the mutants and the wild type strain of *Lasiodiplodia pseudotheobromae* used during the course of this study. The rice bran had a higher nitrogen contents. Higher nitrogen has been shown to be necessary for mycelial growth of fungi [47,48] which may be a reason for maximum production of biomass and spores on rice bran .

Addition of *Anona senegalises* plant extract might be responsible for the increase in spores of the wild type of *Lasiodiplodia pseudotheobromae* in sorghum because *Anona senegalises* is serving as a good substrate for the wild type of *Lasiodiplodia pseudotheobromae* inside a bottle. Aregger [45] reported that development and sporulation rates can be enhanced by the addition of water, plants extracts and oil and the length of the incubation period. In addition, supplementation of substrate with additives may accelerate fungal growth and sporulation.

## CONCLUSION

The spores of *Lasiodiplodia pseudotheobromae* has been mass produced on locally available agro- industrial wastes that is very cheap, in providing alternative to costly and import dependent culture media, and thus of immense biotechnological economic benefit to our nation. The mutant strain produced more spores when compared to the wild strain on rice bran.

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**How to cite this article:**

Adetunji C. O., Oloke J.K. Effect of Wild and Mutant Strain of *Lasiodiplodia Pseudotheobromae* Mass Produced on Rice Bran as a Potential Bioherbicide Agents for Weeds under Solid state Fermentation. J App Biol Biotech. 2013; 1 (02): 018-023.