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Influence of carbon and nitrogen sources on the spore yield of *Trichoderma harzianum* in fed-batch culture

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Abstract

The influence of carbon and nitrogen sources on the spore yield of *T. harzianum* in fed-batch culture was investigated. *T. harzianum* was cultivated in liquid culture media under the effect of different carbon and nitrogen sources at different concentration using a fed-batch process. It was observed that among the various carbon sources studied, glucose (2.5 g l^{-1}) gave the highest spore yield of 2.81±0.14 while starch (15.0 g l^{-1}) gave the lowest spore yield (0.22±0.17). The effect of nitrogen sources revealed that 1.0 g l^{-1} of casein enhanced the highest spore yield (2.88±0.02) while the lowest spore yield (0.23±0.02) was recorded in medium containing soy meal (9.0 g l^{-1}) preparation. The results revealed that *T. harzianum* has the ability to utilize various carbon and nitrogen compounds and produce high spore yield at low carbon and nitrogen concentration. This can be considered during industrial production of *T. harzianum* spores for biocontrol.

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Introduction

Cowpea, a drought tolerant and warm weather crop is one of the most important food legume crops in the semi-arid tropics covering Asia, Africa, Southern Europe and Central and South America. It has the useful ability to fix atmospheric nitrogen through its root nodules (Singh, 2003). It can be used for various purposes such as food crop, cash crop and animal feed (Singh *et al.,* 1997). Cowpea is susceptible to a variety of pests and pathogens which can cause damage to crop at all stages of growth (Summerfield and Roberts, 1985). In countries like Nigeria, *Rhizoctonia* species, *Phythium* species and *Fusarium* species are of great economic importance and they cause great loses due to seed decay and seedling damping off (Singh and Rachie, 1985). *Rhizoctonia solani,* a soil-borne pathogen causes stem canker, storage rot, aerial blight and seedling damping-off diseases in cowpea (Carisse *et al.,* 2001). *R. solani* is posing serious threats to cowpea cultivation and the damage occurs at any time during the growing season and the pathogen attacks mostly young seedlings (Dorrance *et al.,* 2001).

Trichoderma specie wide spread application has been lately exploited and reported against several soil-borne phytopathogenic fungi (Baker and Cook, 1974). *Trichoderma harzianum* has shown potential to control diseases caused by *R. solani* (Askew and Laing, 1994). Compared to most fungal biological control agents (BCAs), *Trichoderma* spp. can be effectively used as spores (especially conidia), which are more active as BCAs, more tolerant to adverse environmental conditions during product formulation and field use and are produced faster in abundance in contrast to mycelial and chlamydospore forms as microbial propagules (Amsellem *et al.,* 1999, Verma *et al.,* 2005).

In general, both conidia and mycelia can be produced in either solid-state or liquid fermentation.

However, liquid fermentation appears to be a more suitable method over solid state fermentation due to its labour, scale-up, process control, productivity compatibility with pre-existing large scale facilities (Molla *et al.,* 2004). Commercial production of conidia typically relies on manipulation of nutrients and substrate to promote conidiation in *Trichoderma* spp. The carbon and nitrogen status and the C:N ratio in addition to ambient pH are the main nutritional factors influencing conidiation in *Trichoderma* spp. (Aube and Gagnon, 1969, Monga, 2001, Gao *et al.,* 2007).

However, there are little report on the studies regarding culture conditions like fed-batch culture in a fermentor that allows the control of substrate concentration (carbon, nitrogen) and spore yield of *T. harzianum*, a biocontrol agent. Thus there is a need to study the above listed parameters to induce sporulation by *T. harzianum* for industrial and biotechnological use as biocontrol agent on large scale. This paper therefore reports the influence of different carbon and nitrogen sources on the spore yield of *T. harzianum* in fed-batch culture.

Materials and methods

Fungal isolate and inoculum

T. harzianum used in this study was obtained from The Culture Collection Centre of The Department of Microbiology University of Ibadan, Nigeria. Conidial inoculum was prepared according to Nahar *et al.* (2008). The conidial concentration was measured using a haemocytometer and adjusted to 10^5spores ml^{-1} and thereafter used for inoculation.

Fed-batch culture

Fed-batch culture was prepared as described by Cascino *et al.* (1990). The fed-batch contained (g l^{-1}) ammonium chloride (2.0), sodium potassium tartrate (2.0), $MgSO_4.7H_2O$ (4.0), K_2HPO_4 (14.0), $CaCl_2$ (0.2), KH_2PO_4 (4.0), yeast extract (4.5), trace element (2.0 mL), [$ZnSO_4.7H_2O$ (0.0014),

$FeSO_4.7H_2O$ (0.005), $MnSO_4$ (0.0016), $CoCl_2$ (0.002)], glucose (7.5), $NaNO_3$ (6.0), and corn steep liquor (5.0) was adjusted to pH 5.5 using citrate buffer. The composition of the fed-batch medium has been reported by Al-Taweil *et al.* (2009). For cultivations in fed-batch vessel, 50 mL of basal medium were inoculated with 1 ml of the spore suspension and incubated at ambient temperature under static condition for 7 days. During incubation, at every 12 hour interval, 4 ml of yeast extract (0.05 mg ml^{-1}) which is the limiting nutrient was fed into the basal medium.

Influence of carbon sources on the spore yield of T. harzianum

Five carbon sources including Monosaccharide (Glucose), Sugar alcohols (Mannitol), Polysaccharides (Starch, Wheat bran and Rice bran) were used for the study. The carbon sources were supplemented at the rate of 2.5, 5.0, 7.5, 10.0 and 15.0 g l^{-1}, which replaced the carbon source in the basal medium. After which the liquid medium was sterilized and inoculated with 1mL spore suspension of *T. harzianum*. Cultures were incubated at ambient room temperature for 7 days after which the spore yield was quantified.

Influence of nitrogen sources on the spore yield of T. harzianum

Five nitrogen compounds including inorganic amino acids ($NaNO_3$, NH_4SO_4) and complex organic sources (Peptone, Soy meal preparation and Casein) were used for this study. The nitrogen sources were supplemented at the rate of 1.0, 3.0, 5.0, 7.0 and 9.0 g l^{-1}, which replaced the nitrogen source in the basal medium. Thereafter, the liquid medium was sterilized and inoculated with 1mL spore suspension of *T. harzianum*. Cultures were incubated at ambient room temperature for 7 days after which the spore yield was determined.

Measurement of spore yield

The spore yield was measured using a spectrophotometer (Perkin Elmer Lambda 25 UV) at the end of the fed-batch cultivation. For cultivations in fed-batch vessels the spores were harvested by filtering samples through a sterilized double-layer muslin cloth so as to separate the harvestable spores from the mycelium in the liquid medium. The filtered suspension was kept on Rotary Flask Shaker (MAC, MSW-301) for 2 minutes, after which 3 ml of the suspension was poured into a cuvette. The equipment was calibrated with 3 ml of blank solution (Waghunde *et al.,* 2010).

Results

Influence of carbon sources on the spore yield of *T. harzianum*

Results (Fig. 1.) indicated that *T. harzianum* utilized all the different sources of carbon and attained its highest spore yield (2.81±0.14) in glucose at a concentration of 2.5 g l^{-1} followed by starch (2.5g l^{-1}) with a spore yield of 2.42±0.02. The lowest spore yield (0.22±0.17) was observed when 15.0 g l^{-1} of starch was the carbon source.

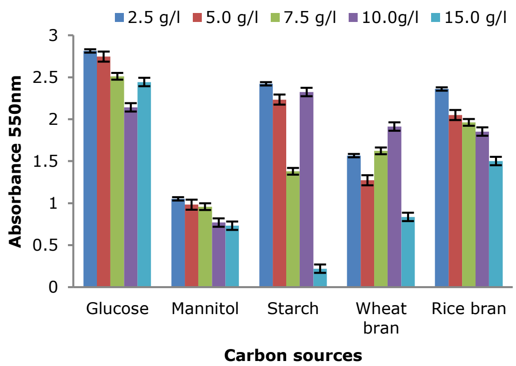

Fig. 1. Influence of different carbon sources on spore yield of *T. harzianum*. Values are means of three replicates; *error bars* represent standard deviation obtained during the fed-batch cultivation.

Influence of nitrogen sources on the spore yield of *T. harzianum*

The results of the effect of different nitrogen sources on spore yield of *T. harzianum* in fed-batch experiments are presented in Fig. 2. The highest spore yield (2.88±0.02) was observed in medium containing casein (1.0 g l^{-1}) followed by NH_4SO_4 (5.0 g l^{-1}) which gave a spore yield of 2.84±0.05. The lowest spore yield (0.23±0.02) was recorded when soy meal preparation (9.0 g l^{-1}) was the nitrogen source.

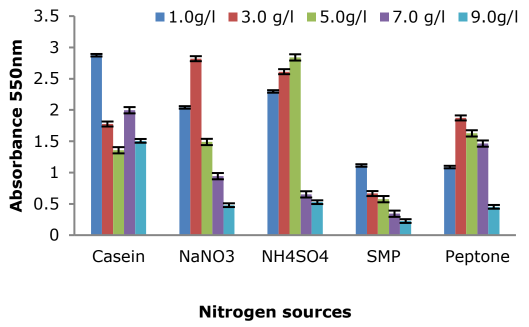

Fig. 2. Influence of different nitrogen sources on spore yield of *T. harzianum.* Values are means of three replicates; *error bars* represent standard deviation obtained during the fed-batch cultivation.

Discussion

In this present study, significant spore yield was observed among the various carbon sources investigated in the fed-batch culture. This finding confirms the competitive nature of *Trichoderma* spp. as described by Eric (2005). Glucose was the most suitable carbon source that stimulated the highest spore yield followed by starch and rice bran in the fed-batch cultures. Furthermore, glucose being a monosaccharide and a simpler metabolite explains the reason for maximum spore yield compared to the other carbon sources studied. However, the present findings are in accordance with the findings of Said (2007) who reported that spores produced by *T. harzianum* were significantly affected by glucose concentration and CN ratio of the culture media. In addition, Lewis and Papavizas (1983) had earlier reported that glucose and soluble starch promotes the sporulation of *Trichoderm*a spp. Wheat bran and rice bran, a low cost source of carbon and nitrogen were shown to also enhance sporulation. Bakri *et al.* (2003) reported that wheat bran contains adequate amount of nutrients that benefits mycelium growth and spore yield; earlier studies (Ibrahim and Low, 1993, Sharma *et al.,* 2002) revealed that rice is a suitable media for the mass production of deuteromycete fungi. Maximum spore yield was recorded at 2.5g l^{-1} concentration in almost all the carbon sources, increasing concentration of the carbon sources brought a significant decrease in spore yield of *T. harzianum*. The results agreed with previous reports that fungi produce more spores under low nutrient conditions (Xiao and Sitton, 2004).

In the case of effect of nitrogen source, casein, $NH_4SO_4$and $NaNO_3$ gave optimum spore yield in the fed-batch culture at 1.5 g l^{-1}. Lower amounts of nitrogen favoured sporulation and this is in agreement with previous investigations (Aube and Gagnon, 1969; Gao *et al.,* 2007). Maximum spore yield was observed in *T. harzianum* when casein was the sole nitrogen source in fed-batch culture, this may be attributed to the peptides and amino acids present in casein. Among the nitrogen sources tested, ammonium sulphate was the most suitable inorganic nitrogen source in terms of sporulation and it was confirmed that significant sporulation resulted in *Trichoderma* spp. when cultivated in media containing ammonium sulphate (Onilude *et al.,* 2012; Shirsole and Mishra, 2014; Rai and Tewari, 2016), as a result of its ability to alter the pH of the liquid medium to a slightly acidic pH. It was interesting to note that *T. harzianum* preferred ammonium forms of nitrogen for sporulation which is appropriate in selecting *T. harzianum* as biocontrol agent.

Conclusion

Trchoderma spp. are known to have good biocontrol activity in the environment and, industrial production of this fungus involves bulk spore production. Culturing *T. harzianum* in a fed-batch culture containing different concentrations of carbon and nitrogen sources showed that spore production can be stimulated at low concentration of carbon and nitrogen source. Therefore, this should be considered in industrial production of *T. harzianum* spores for biocontrol use.

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