Studies on the Nutritional Requirements of an Ochratoxin A-Degrading *Rhizopus* sp.

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Abstract: Studies were conducted on the carbon, nitrogen, carbon/nitrogen ratio (C:N), and vitamin requirements of a recently isolated *Rhizopus* sp. that is capable of degrading ochratoxin A *in vitro*. The results obtained showed that glucose supported the best growth of 57.0 mg followed by fructose (51.7 mg) while the poorest growth (1.16 mg) was supported by lactose. Urea was the most utilized of all the nitrogen sources investigated, producing a mycelial dry weight of 90.0 mg while DL-citrulline supported the poorest mycelia growth of 13.0 mg. Results on the effect of various carbon to nitrogen ratio revealed that a C:N ratio of 3:1 produced the best mycelia weight of 28.0 mg while a C:N ratio of 1:2 produced the poorest mycelia weight of 1.7 mg. Among the vitamins studied, pyridoxine was the most utilized with a mycelial dry weight of 60.0 mg followed by cobalamine (43.3 mg) while riboflavin and biotin stimulated the poorest growth (35.0 mg) each.
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Introduction

Ochratoxin A (OTA) is a 7-carboxy-5-chloro-8-hydroxy-3, 4-dihydro- 3*R*-methylisocoumarin compound, linked through its 7-carboxy group to L-*â*-phenylalanine by an amide bond (Rigot *et al*., 2006), produced by several species of *Peniciliium* and *Aspergillus* as their secondary metabolite (Khoury and Atoui, 2010). It is frequently found contaminating a wide array of food and feed commodities such as rice and rice products (Gonzalez *et al*., 2006), coffee (Pittet *et al*., 1996), beer and wine (Visconti *et al*., 1999). OTA is a nephrotoxin whose principal target organ is the kidney (Ribelin, 1978) and epidemiological studies have reported its potential implication in the human fatal disease known as Balkan Endemic Nephropathy (BEN) (Pfohl-Leszkowicz, 2009). OTA has also been experimentally shown to be teratogenic, a potent renal carcinogen, immunosuppressive, an enzyme inhibitor and has effects on lipid peroxidation, it is listed as a possible carcinogen of group 2B by the International Agency for Research on Cancer (IARC, 1993).

The prevention of OTA contamination in the field is the main goal of agricultural and food industries, however, the contamination of commodities with *Aspergillus*, and *Penicillium* sp. and possibly ochratoxins is unavoidable under certain environmental conditions (Varga *et al*., 2005), hence certain decontamination/detoxification procedures have been suggested in order to reduce to the barest minimum, problems associated with exposure to OTA contamination. Such strategies are in three categories: physical, chemical and biological. Physical and chemical decontamination strategies involve the use of different absorbent and chemicals which bind with the mycotoixns and make them unavailable to animals and humans. Biological detoxification, on the other hand, involves the use of microorganisms and (or) their enzymes and this has led to the isolation and screening of various microorganisms that can degrade mycotoxin (Hult *et al*., 1976; Cheng and Draughon, 1994; Bejaouii *et al*., 2006; Baptista *et al*., 2004; Fuchs *et al*., 2008; Mateo *et al*., 2010).

In this study we report the nutritional requirements of an OTA-degrading *Rhizopus sp*. The studies of the nutritional requirements will acts as additional information which can be employed to improve the use of this *Rhizopus* sp. in the decontamination of OTA contaminated food and feed commodities.

Materials and Methods

Microorganism

A recently OTA- degrading *Rhizopus* sp. isolated from spoilt 'Ori' (Garuba, unpublished data) obtained from the culture collection centre of the Department of Biological Sciences, Bowen University Iwo, was used in this study. The organism was maintained on Potato Dextrose Agar slants supplemented with chloramphenicol 50 ppm at 4 ^{0}C. This study was carried out at the Microbiology Laboratory, Department of Biological Science, Bowen University Iwo, between January and December 2010.

Inoculum preparation

Inoculum used in this study was prepared using the method of Nahar *et al*. (2008).

Effect of different carbon sources

Different carbon sources used were Arabinose, Fructose, Galactose, Glucose, Mannose, Sorbose, Rhamnose, Xylose, Lactose, Maltose, Mellibiose, Raffinose, Starchyose, Sucrose, Cellibiose, Inositol, Mannitol Sorbitol, Cellulose, Dextrin and Soluble starch. Sterile basal medium, containing (g l^{-1}) yeast extract (2.0), KH_2PO_4 (1.0), $MgSO_4.7H_20$ (0.5), was dispensed in 30 ml amount into Erlenmeyer's flask and sterilized at 121 ^{0}C for 15 min and allowed to cool. The media were later supplemented with 0.8% (w/v) of each of the sterile carbon sources and inoculated with 1 ml of the inoculum (containing 20 X 10^{10} spores) of the *Rhizopus* sp. Incubation was done at 35 ^{0}C for 120 h. A control without any carbon source was also set up. The mycelia were harvested by filtration using a pre-weighted filter paper and then dried to constant weight in an oven at 80 ^{0}C to obtain the dry weight of the mycelia. Each treatment was done in triplicates.

Effect of different nitrogen sources

The utilization of different nitrogen sources by the organism was determined using 0.1% of different nitrogen sources in a basal medium containing (g l^{-1}) $MgSO_4.7H_20$ (0.5), KH_2PO_4 (0.5) and glucose (10). The nitrogen sources used: $NaNO_3$, KNO_3, $Ca(NO_3)_2$, NH_4NO_3, $(NH_4)_2SO_4$, L-Aspartic acid, L-Asparagine, DL-Citrulline, D-Cysteine, L-Glutamine, L-Glutamic acid, L-Histidine, L-Arginine, DL-Leucine, DL-Methionine, L-Tryptophan, DL-Valine, Casine, Malt extract, Peptone, Urea and Yeast extract were sterilized by millipore filtration. A control experiment, made up of basal medium and glucose without any nitrogen source was also set up. Set up were inoculated with 1 ml of the inoculum (containing 20 X10^{10} spores) of the *Rhizopus* sp. Incubation was done at 35 ^{0}C for 120 h and the mycelia were harvested and dried to a constant weight as described previously.

Effect of different C:N

The effect of different C:N on the mycelia growth of the organism was studied by varying the different concentration of the best utilized carbon and nitrogen sources in a basal medium containing (g l^{-1}) KH_2PO_4 (0.05), $MgSO_4.7H_20$ (0.05), KNO_3 (1.55). Set ups were inoculated with 1 ml of the inoculum (containing 20 X10^{10} spores) of *Rhizopus* sp. and incubated at 35 ^{0}C for 120 h. Mycelia were harvested and dried as described above.

Effect of different vitamins

The vitamins used were Ascorbic acid, Biotin, Cobalamine, Folic acid, Nicotinic acid, pyridoxine, Riboflavin, Thiamine. These were supplemented (at a concentration of 500 µg l^{-1}) in a basal medium containing (g l^{-1}) fructose (10.0), peptone (1.0), $MgS0_4$. $7H_20$ (0.5), KH_2P0_4 (0.05). A basal medium containing all the vitamins served as control 1, while a basal medium without any vitamins served as control 2. A 30 ml quantity of the medium supplemented with each of the vitamins was inoculated with 1 ml of the inoculum (containing 20 X10^{10} spores) of the *Rhizopus* sp and incubated as described above. The vitamins were sterilized by millipore filtration and special care was taken to avoid the destruction of riboflavin by strong light.

Statistical analysis

Results obtained in this study were subjected to analysis of variance using ANOVA and separation of means was carried out by Duncan's Multiple Range Test (Duncan, 1955).

Results

The results of the effect of various carbon sources on the vegetative growth of *Rhizopus sp.* are presented in table 1. The results indicated that this species of *Rhizopus* is able to utilize all the carbon sources investigated in this study. However, glucose was found to stimulate the best mycelial growth of 57.0 mg when incorporated into the basal medium followed by fructose (51.8 mg) while the lowest mycelial weight of 1.1 mg was recorded with lactose as the carbon source.

Table 2 shows the results of the effect of nitrogen sources on the vegetative growth of *Rhizopus* sp. The results revealed that the organism was able to utilize all the nitrogen sources investigated in this study. Of all the inorganic nitrogen sources investigated, ammonium sulphate was the most utilized, producing a mycelia weight of 60.0 mg while the lowest weight was recorded by potassium nitrate. Of the amino acids investigated, L-arginine produced the highest mycelia weight of 65.0 mg while DL-citruline produced the lowest mycelia weight of 13.0 mg. Urea was found to support the best growth of all the complex nitrogen sources (and the overall best) with a mycelial weight of 90.0 mg while the poorest growth (28.5 mg) was obtained in a medium containing casine.

Among the various C:N investigated in this study a C:N of 3:1 was found to stimulate the best growth (28.0 mg), followed by ratios 3:2 and 2:5 with growths 25.0 mg and 17.0 mg respectively while a ratio of 1:2 supported the least growth of the organism (table 3).

Among the vitamins investigated in this study, pyridoxine stimulated the best growth of 60.0 mg, followed by cobalamine and both ascorbic acid and thiamine with growths of 43.3 mg (table 4) and 41.7 mg respectively. Riboflavin supported the poorest growth (34.0 mg) among all the vitamin sources investigated.

Table 1: Effect of carbon sources on the vegetative growth (mg) of *Rhizopus sp.*

Carbon Compounds	Mycelia weight (mg)
Monosaccharides	
Arabinose	22.7±1.4530[a]
Fructose	51.8±0.7126[bcd]
Galactose	11.7±1.6667[a]
Glucose	57.0±1.5507[a]
Mannose	30.3±2.4841[a]
Sorbose	26.0±3.0551[abc]
Rhamnose	2.60±0.8819[a]
Xylose	40.0±1.7735[b]
Oligosaccharides	
Lactose	1.16±3.3333[ab]
Maltose	13.3+3.1798[b]
Mellibiose	17.6±0.8819[ab]
Raffinose	10.3±1.2019[a]
Starchyose	20.8±2.9627[c]
Sucrose	50.4±1.4874[c]
Cellibiose	15.7±0.6667[bc]
Sugar alcohols	
Inositol	21.0±2.0817[bc]
Mannitol	3.7±0.2019[a]
Sorbitol	21.8±0.4096[b]
Polysaccharides	
Cellulose	10.0±0.000[a]
Dextrin	2.0±1.6000[a]
Soluble starch	1.6±0.3333[a]
Control	1.0±0.1667[a]

Data are means of three replicates ± SEM. Values followed by the same letters are not significantly different by Duncan's multiple range test ($P = 0.01$).

Table 2: Effect of nitrogen sources on the vegetative growth (mg) of *Rhizopus sp.*

Nitrogen sources	Mycelia dry weight (mg)
Inorganic nitrogen	
$NaNO_3$	38.0±6.0093[a]
KNO_3	15.0±2.8868[a]
$Ca(NO_3)_2$	25.0±2.8868[b]
NH_4NO_3	60.0+0.4096[a]
$(NH_4)_2SO_4$	37.0±0.000b[c]
Amino acids	
L-Aspartic acid	45.0+0.000[b]
L-Asparagine	23.0±63596[a]
DL-Citrulline	13.0±0.333[a]
D-Cysteine	35.0±0.6667[de]
L-Glutamine	45.0±0000[d]
L-Glutamic acid	43.0±1.6606[b]
L-Histidine	30.0±0.0000[a]
L-Arginine	65.0±2.8868[b]
DL-Leucine	15.0±2.8868[c]
DL-Methionine	30.0±0.0000[a]
L-Tryptophan	15.0±0.6667[a]
DL-Valine	30.0±5.7735[ab]
Complex nitrogen	
Casine	28.5±1.6667[abc]
Malt extract	42.0±0.4096[d]
Peptone	37.0±3.3330[b]
Urea	90.0±5.7735[bc]
Yeast extract	52.0±4.4096[cd]
Control	3.7±0.0000[e]

Data are means of three replicates ± SEM. Values followed by the same letters are not significantly different by Duncan's multiple range test ($P = 0.01$).

Table 3: Effect of C:N ratio on the vegetative growth of *Rhizopus sp.*

Carbon:Nitrogen	Mycelia dry weight(mg)
1:1	8.0±3.333[a]
1:2	1.7±1.6667[a]
1:3	5.0±2.8868[a]
1:4	15.0±2.8867[b]
1:5	8.0±1.6667a
2:1	6.7±1.6667a
2:3	13.0±3.3333[abc]
2:5	17.0±3.3333[ab]
3:1	28.0±2.8868[b]
3:2	25.0±0.333[a]
3:4	8.7±4.4096a
3:5	3.6±1.3333[a]
4:1	6.7±1.6667[ab]
4:3	8.0±1.6667[a]
4:5	11.6±4.4096[abc]
5:1	6.7±1.6667[a]
5:2	4.0±3.0000[a]
5:3	15.0±2.8868[a]
5:4	8.0±1.6667[ab]
Control Basal medium	1.5±0.0000[bc]

Data are means of three replicates ± SEM. Values followed by the same letters are not significantly different by Duncan's multiple range test ($P = 0.01$).

Table 4: Effect of vitamins on vegetative growth of *Rhizopus sp.*

Vitamins	Mycelia dry weight(mg)
Ascorbic acid	41.7+7.265[b]
Biotin	35.0+5.000[ab]
Cobalamine	43.7±0.667[bc]
Folic acid	36.7±6.667[a]
Nicotinic acid	38.3±1.667[c]
Pyridoxine	60.0±2.887[c]
Riboflavin	34.0±2.887[ab]
Thiamine	41.7±4.410[ab]
Basal medium + all vitamins (control 1)	50.0±5.774[a]
Basal medium only (control 2)	36.7±3.333[b]

Data are means of three replicates ± SEM. Values followed by the same letters are not significantly different by Duncan's multiple range test ($P = 0.01$).

Discussion

All the carbon sources studied in this work supported the growth of *Rhizopus* sp. This observation has also been reported for various filamentous fungi by various researchers (Nout and Rabouts 1990; Rehms and Barz, 1995; Amadioha, 1998). Glucose supporting the highest mycelial growth (57.0 mg) could be as a result of the ease with which it is broken down and ease of oxidation in generating cellular energy within the cells (Schlegel, 2002). Fructose which supported mycelial growth (51.7 mg) next to glucose has also been reported by Garraway and Evan (1984). Griffin (1994) also observed that the best carbon source after glucose is fructose. This could be as a result of the fact fructose is an isomer of glucose and can be chemically converted to glucose during cellular respiration (Moat *et al*., 2002). The poor utilization of lactose (a disaccharide) by this specie of *Rhizopus* could be as a result of the *Rhizopus* sp.'s inability to produce adequate enzyme that is necessary for the breakdown of lactose sugar (Seyis and Aksoz, 2004).

Nitrogen is needed for the synthesis of amino acids, purines, pyrimidines, some carbohydrates and lipids, enzyme cofactors and other substances by the cells (Zang *et al*., 2007). The utilization of all the nitrogen sources investigated in this study by the *Rhizopus* fungus is in agreement with earlier reports of Oso (1974), Olutiola (1976), and Medwid and Grant (1984) from studies on the utilization of nitrogen sources by fungi. The preferential utilization of ammonium sulphate compared to all the inorganic nitrogen sources by this organism is in agreement with the report of Prescott *et al*. (1996) and this could be due to its relatively ease of incorporation into organic material compared to nitrate and nitrite salts, which must first be reduced to ammonium before the nitrogen can be converted to an organic form (Prescott *et al*., 2008). The preferential utilization of L-arginine by this *Rhizopus* sp. could be as a result of its ease of transport across the fungal cell membrane (Griffin, 1994). Urea was observed to be the most utilized of all the complex nitrogen sources studied in this work. This is in agreement with the report of Nahar *et al*. (2008). The preferential utilization of urea could possibly be attributed to its hydrolysis to ammonia (by urease) (Raimbault, 1998) which is easily and directly incorporated into organic material (Prescott *et al*., 1996). Gbolagade *et al*. (2006) also suggested that complex nitrogen sources supporting better growth in higher fungi might be due to the fact that these complex nitrogen compounds contain combined amino acids and carbohydrate which also will support fungal growth.

The ratio of carbon to nitrogen of 3:1 which supported the highest mycelia growth is different from that obtained for other higher fungi (Engelkes *et al*., 1997; Li and Liu, 2010). Gbolagade *et al*. (2006) suggests that the variation could be as a result of the difference in the carbon to nitrogen ratio of different organisms.

Pyridoxine was found to stimulate the highest growth of all the vitamins investigated in this study. This is in support of the reports of Fasidi and Olorunmaiye (1994), Jonathan and Fasidi (2001), Jonathan *et al*. (2004) which observed similar utilization of pyridoxine in higher fungi. Jonathan *et al*. (2004) suggested that pyridoxine supporting the best growth could be attributed to its conversion to functional phosphate which is important in the synthesis of tryptophan which is an amino acid needed for growth.

In conclusion, it is clear from this work that for the cultivation of this OTA-degrading *Rhizopus* sp. on synthetic medium, glucose or fructose will be the appropriate carbon source and urea as the nitrogen source in the C:N ratio of 3:1. Vitamins such as pyridoxine, ascorbic acid and cobalamine also need to be incorporated into the synthetic medium for optimum growth of the organisms. Since the contamination of food and feed commodities by OTA-producing strains in certain areas seems to be inevitable, information on the nutritional requirements of this Ochratoxin A-degrading *Rhizopus* sp. can be employed to improve the use of this organism in the decontamination and detoxification of OTA-contaminated food and feed commodities hence, reducing the problems associated with the exposure of humans and animals to OTA-

contaminated food and feed commodities in these areas.

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