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Preliminary Studies on the Bioactivity of Secondary Metabolites From *Aureobasidium pullulans* and *Emericela rugulosa*

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Abstract: In the course of searching for bioactive fungal metabolites, crude extracts of *Aureobasidium pullulans* and *Emericela rugulosa* were screened *in vitro* against eleven human pathogens. The production medium changed from slightly basic (pH 7.21) to slightly acidic (pH 6.72) as fermentation progressed till the 12th day. The wet biomass of the fungal mycelia as well as the dry weights of the crude extracts increased while the amounts of glucose in the growth medium reduced with a corresponding increase in incubation period. Contact bioautographic assay showed that the extracts exhibited broad-spectrum antimicrobial potency. Activity against some human pathogenic organisms including Gram-positive bacteria (*Bacillus subtilis*, *Staphylococcus aureus), Gram-negative bacteria (Escherichia coli, Klebsiella pneumoniae*, *Pseudomonas aeruginosa, Salmonella typhi)* and fungal strains (*Candida albicans, Trichophyton concentrum, T. mentagrophytes, T. rubrum* and *T. torulans)* is discussed. Bioactivity of the extracts, as depicted by zones of inhibition in agar well diffusion assay, was found to increase as the fermentation days increased, irrespective of the fungal biosource of the metabolites. In most cases, *A. pullulans* grew faster and caused higher inhibition than *E. rugulosa*.

Key words: *Aureobasidium pullulans, Emericella rugulosa,* antibacterial, antifungal, fungal metabolites, bioactivity

INTRODUCTION

In the developing countries large numbers of the world's population are unable to afford pharmaceutical drugs and they continue to use their own systems of indigenous medicine that are mainly plant-based (Phillipson, 2001). The question is whether such anti-infective properties are produced by the plant itself or as a consequence of a mutualistic relationship with beneficial organisms in plant tissue. There is a basic supposition that any plant possessing clinical effectiveness must contain an active principle which can completely replace the plant extract. But many reports have shown that in a microbe-plant relationship, endophytes contribute substances that possess various types of bioactivity, such as antimicrobial and pesticidal (Rodriguez *et al*., 2000; Filip *et al*., 2003; Taechowisan *et al*., 2005). Hence, the interest for endophytic fungi as potential producers of novel biologically active products has increased in the last decade. Endophytes are microorganisms that live in the intercellular spaces of stems, petioles, roots and leaves of plants causing no discernible manifestation of their presence and have typically gone unnoticed (Calvo *et al*., 2002; Radu and Kqueen, 2002).

Biologically active secondary metabolites may be involved in defining the endophyte-host plant relationship. The nature and biological role of endophytic fungi with their host plant is variable. Endophytic fungi are known to have mutualistic relations to their hosts, often protecting plants against herbivory, insect attack or tissue invading pathogens (Vinton *et al*., 2001; Cannon, 2002; Filip *et al*., 2003). In some instances, the endophyte may survive as a latent pathogen, causing

quiescent infections for a long period and induce symptoms only when physiological or ecological conditions favor virulence. For instance, while *Phoma medicaginis* can exist as a prolonged asymptomatic infection of its host plant (*Medicago* spp.), this fungus produces detectable levels of the toxin brefeldin A only during and after the switch from the endophytic to the saprobic phase upon host death (Weber *et al*., 2006).

A large number of bioactive and structurally diverse fungal metabolites have been isolated and characterized and some of these have been used for the development of valuable pharmaceuticals and pesticides. Of the estimated 1.5 million species of fungi recorded worldwide, approximately 4,000 secondary metabolites of fungal origin are known to possess biological activities (Dreyfuss and Chapela, 1994; Strobel and Daisy, 2003); the vast majority coming from the species of *Penicillium, Aspergillus*, *Acremonium and Fusarium* (Schulz *et al.,* 2002).

In the course of bio-prospecting for microbial endophytes and natural products in early 2004 the author isolated a fungal endophyte, *Emericella rugulosa* (Thom and Raper) C.R.Benjamin from pawpaw leaves/petioles as well as *Aureobasidium pullulans* (de Bary) Arnaud from the rhizoplane of *C. papaya* growing in Southwestern Nigeria. The present investigation has thus aimed to provide information on the microfermentation, isolation and biological properties of secondary metabolites produced by *A. pullulans and E. rugulosa.* But elsewhere, species of *Emericella* have been the source of a variety of natural products, mainly sesquiterpenes for example, prenylated xanthones astellatol, variecolin, varitriol, varioxirane, varixanthone, ergosterol, terrecin and shamixanthone (Malmstrom *et al*., 2002) from *E. variecolor*. Other compounds isolated include secoemestrin from *E. folveolata* (Ooike *et al*., 1996), falconensins from *E. falconensis* (Ogasawa and Kawai, 1998), an antibacterial depside guisinol and unguisin from *E. unguis* (Nielsen *et al*., 1999). Two natural products, evariquinone and isoemericellin were recently isolated from *E. variecolor* (Bringmann *et al*., 2003). In addition, *Aureobasidium pullulans* is mainly saprobic on various tropical fruits. Due to the production of melanin, it is popularly known as black yeast. The fungus is industrially important because of its ability to produce a polysaccharide pullulan. *A. pullulans* has also been implicated in the biological control of some post-harvest diseases of fresh fruits and vegetables (Lima *et al*., 1997; Castoria *et al*., 2001).

MATERIALS AND METHODS

Microorganisms

Aureobasidium pullulans and *Emericella rugulosa* were respectively isolated from the rhizoplane and leaves/petioles of apparently healthy pawpaw plant growing in the southwestern part of Nigeria, as described by Rodriguez and Samuel (1999). The taxonomic identity of the fungi was confirmed by Dr R. A. Samson of Centraalbureau voor Schimmel cultures, Utrecht, The Netherlands. The antibiotic assays were performed using *Bacillus subtilis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Staphylococcus aureus*, *Candida albicans*, *Trichophyton concentrum*, *T. mentagrophytes*, *T. rubrum* and *T. tonsurans.* All the pathogenic organisms were obtained from Medical Microbiology Laboratory, Obafemi Awolowo University Teaching Hospital, Ile-Ife, Nigeria. The bacteria and fungal stock cultures were maintained on Mueller Hilton Agar and Sabouraud Dextrose Agar, respectively.

Culture Condition and Processing

Ten fungal inoculum's discs were aseptically transferred into each of the twelve 1 L Erlenmeyer flasks containing 250 mL of fermentation medium. The medium consisted of yeast extract, 0.4%; malt extract, 1.0% and glucose, 0.4%; pH 7.21. All the flasks were incubated at 25°C in a rotary shaker (130 rpm) for up to 12 days. During incubation, a flask was removed at random every 24 h and the

fermented medium was processed (Radu and Kqueen, 2002). The broth cultures were passed through a 0.45 μm filter paper under vacuum to separate the mycelia from culture broth. The wet mycelia were weighed, triturated in a domestic grinder and extracted with ethyl acetate (1:1$^{w/v}$). The pH value of the broth was noted after which it was extracted with equal volume of ethyl acetate (1:1). In each case, the organic layer was dried over anhydrous Na_2SO_4 and concentrated *in vacuo* to dryness in pre weighed vials. Sample extracts were periodically analyzed by Thin-layer chromatography to ensure that the desired metabolites had been produced (Furnado *et al*., 2005).

Changes in Concentration of Reducing Sugars

Changes in the level of maltose and glucose in the culture broth, relative to period of fermentation, was monitored as described by Petrini *et al*. (1992). Standard curve of pure glucose was constructed at 100-1000 μg mL^{-1}. Then, culture filtrate was diluted 5- and 15-fold with distilled water and each dilution was used for the estimation of reducing sugars using 3, 5-initro salicylic acid (DNSA). The amount of reducing sugars present in the filtrate was calculated using the standard curve of glucose.

Contact Bioautographic Assay

TLC analysis of each extract was done on pre-coated silica gel (60A K6F) plates. Each extract was applied and the layers developed with petroleum ether: EtOAc (1:1). Inocula of the test pathogenic organisms were prepared from stock broth cultures and suspensions were adjusted to 0.5 McFarland turbidity standard. Chromatograms were inverted onto pre-inoculated agar plates and incubated overnight at 37°C (for bacteria) and 27°C (in case of fungi). Subsequently, bioautograms were sprayed with an aqueous solution of methylthiazolyltetrazolium chloride (MTT; 2.5 mg mL^{-1}) and incubated for another 30 min. Yellow coloured inhibition zones were observed against a purple background (depicting microbial growth) on the TLC plates (Saxena *et al*., 1995).

Antibiotic Agar-Well Diffusion Assay

The antimicrobial activity was determined by the agar well diffusion method (Perez *et al*., 1990). Agar plates were inoculated with 0.5 McFarland standard broth cultures of the test organisms. The dried extract (1 mg) was solubilised in 1 mL of dimethyl sulphoxide (DMSO). Five millimeter diameter wells were punched into the agar and filled with 30 μL of each extract dilution and the solvent blanks. Treated plates were incubated at the appropriate temperatures for 24 h, after which zones of inhibition were measured in mm using electronic calipers.

RESULTS AND DISCUSSION

The influence of fermentation period on the pH of the growth medium as well as on the yields of fungal mycelia and crude extracts is represented in Table 1 and 2. The production medium changed from slightly basic (pH 7.21, 7.22) to slightly acidic (pH 6.69, 6.72) as fermentation progressed till day 12, irrespective of the fungal species cultivated. The wet mass of the fungal mycelia and the dry weights of the crude extracts increased with a corresponding increase in the incubation period. However, such increases in yields of fungal extracts were usually remarkable during the first 8 days of incubation. Thereafter, there was little or no increase in mycelial growth; probably due to the exhaustion of available nutrients in the growth medium. *Emericela rugulosa* did not grow until the third day whereas the growth of *Aureobasidium pullulans* was observed 24 h after incubation. Whenever growth occurred, the wet biomass of *A. Pullulans* mycelia was usually 3 to 5 times greater than that of *E. rugulosa*. The amounts of glucose in the culture medium reduced with increased incubation period. This means that more of the glucose and other nutrients are needed to cope with the increased growth of the fungi as fermentation continued until a period (days 9-12) at which nutrients became a limiting factor (Table 1 and 2).

Table 1: Effect of incubation period on wet mass of *A. pullulans* mycelia, dry weights of crude extracts and pH of growth medium (initial volume of growth medium = 1 L)

Days	Final pH	Glucose conc (g L^{-1})	Wet wt. of mycelia (g)	Dry wt. of cf (mg)	Extract my (mg)
0	7.21	3.85	0.00	0.00	0.00
1	7.19	54.80	54.80	16.31	11.32
2	7.11	87.21	87.21	70.14	28.14
3	6.92	196.40	196.40	101.62	64.26
4	6.92	244.80	244.80	152.21	85.46
5	6.86	315.20	315.20	243.63	175.12
6	6.82	338.02	338.02	261.12	199.36
7	6.79	339.63	339.63	258.24	199.36
8	6.78	342.82	342.82	258.24	200.16
9	6.78	343.61	343.61	258.51	199.22
0	6.74	344.42	344.42	258.46	198.48
11	6.73	344.43	344.43	257.63	196.57
12	6.72	345.21	345.21	258.15	192.24

cf = culture filtrate extract, my = mycelial extract, Each value is a mean of 5 replicates

Table 2: Effect of incubation period on wet mass of *E. rugulosa* mycelia, dry weights of crude extracts and pH of culture medium (initial volume of growth medium = 1L)

Days	Final pH	Glucose conc (g L^{-1})	Wet wt. of mycelia (g)	Dry wt. of cf (mg)	Extract my (mg)
0	7.22	3.89	0.00	0.00	0.00
1	7.22	3.89	0.00	0.00	0.00
2	7.21	389	0.00	0.00	0.00
3	7.13	3.69	25.12	5.42	8.22
4	7.04	3.35	37.91	10.11	21.12
5	7.01	2.37	51.33	19.64	33.51
6	6.97	2.21	68.75	36.42	48.46
7	6.91	1.62	85.81	51.75	63.92
8	6.82	1.07	106.11	70.37	88.55
9	6.79	0.52	119.56	79.81	105.74
10	6.76	0.15	119.52	79.92	106.31
11	6.71	0.07	119.73	79.92	106.31
12	6.69	0.05	119.73	80.11	106.52

cf = culture filtrate extract, my = mycelial extract, Each value is a mean of five replicates

Antibacterial and antifungal assays using contact bioautographic overly showed that the extracts exhibited broad-spectrum antimicrobial activities i.e., to both Gram-positive and Gram-negative bacteria as well as to some dermatophytes. For instance, there were yellow zones on the thin-layer chromatoplates of *A. pullulans* extract inoculated with *Bacillus subtilis* (Gram-positive), *Escherichia coli*, *Pseudomonas aeruginosa* (Gram-negative), *Trichophyton concentrum*, *T. mentagrophytes*, *T. rubrum* and *Candida albicans* (dermatophytes). This indicates that compounds inhibitory to the growth of these organisms are present in the yellow spots as induced by the fungal metabolites. However, all the TLC plates treated with A. pullulans extract and inoculated with *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Salmonella typhi* and *Trichophyton torullans* chaged to purple colour, indicating that the growth of these organisms was not inhibited by the extract. Similarly, crude extract of *E. rugulosa* was inhibitory to *E. coli*, *S. aureus*, *S. typhi*, *P. aeruginosa*, *T. mentagrophytes*, *T. rubrum* and *T. torulans*, but not to *B. subtilis*, *K. pneumoniae*, *T. concentrum* and *Candida albicans*. The blind control (dimethyl sulphoxide) did not inhibit any of the microorganisms tested.

The above results prompted investigations into the actual zones of inhibition against all the test organisms that were susceptible to the fungal metabolites. Extracts tested for antibiotic activities in the agar well diffusion assay revealed that bioactivity of the extract, as depicted by the zones of inhibition, was found to increase as the fermentation days increased, irrespective of the fungal biosource of the metabolites (Table 3 and 4). Possible explanation for this observation could be that higher concentrations of the bioactive secondary metabolites were produced by the growing fungus as the incubation period increased. The maximum zones of inhibition were usually induced by extracts

obtained on the 8th and 9th days of fermentation. Comparison of the susceptibility of all the bacterial and fungal biotests to the various extracts indicated that *T. mentagrophytes* and *T. rubrum* appeared to be the most sensitive organisms to metabolites from *A. pullulans* and *E. rugulosa*, respectively.

The toxicity of the fungal metabolites herein screened to *P. aeruginosa* is of biological interest. Some strains causing septicemia and pneumonia in cystic fibrosis and immunocompromised patients are becoming difficult to treat with available antimicrobial agents (Lory, 1990). Due to multi-resistance of this bacterium, there is a lack of active antibiotics effective against it, resulting in an increase in nosocomial infections and high mortality. Sequel to these facts, fungi as *A. pullulans* and *E. rugulosa* effective against *P. aeruginosa*, are proper candidates to search for new principles. Also, the fact that extracts from these fungi exhibited activities against some of the microorganisms implicated in the pathogenesis of skin diseases (yeast such as *Candida albicans* and Dermatophytes such as *Trichophyton concentrum, T. torullans, T. mentagrophytes* and *T. rubrum*) provides some scientific basis for the utilization of substances from these two fungi for the treatment of skin diseases.

Bioassay-directed fractionation of the extracts is in progress to isolate and identify the compounds responsible for the antimicrobial activities, as they may represent novel therapeutic agents.

Table 3: Effect of fermentation period (days) on the antimicrobial activity of *A. pullulans extracts* using agar diffusion assay

	Zones of inhibition (mm)						
Days	*Pa*	*Bs*	*Ec*	*Tc*	*Tm*	*Tr*	*Ca*
1	0	0	0	0	0	0	0
2	o	3.5a	5.1a	2.3a	3.1a	3.5a	0
3	2.5a	7.7b	7.5a	4.5a	6.3a	5.5a	2.2a
4	5.3a	10.1c	12.5b	7.5b	8.5b	8.4b	3.1a
5	7.2b	12.5c	16.5b	11.5b	0.3c	11.5b	3.9a
6	10.5c	15.5c	19.3c	18.7c	19.5c	15.5c	4.7a
7	12.1c	15.5c	18.5	19.5c	22.3c	18.5c	6.4b
8	12.5c	15.2c	18.1c	20.2c	21.5c	18.5c	6.9b
9	11.2c	14.7c	17.4c	18.6c	21.5c	17.5c	8.1b
10	10.5c	12.5c	16.5b	17.5c	20.5c	17.2c	7.9b
11	10.3c	12.1c	13.5b	17.1c	20.1c	15.5c	7.6b
12	10.1c	10.3c	12.5b	16.4c	17.5c	15.1c	7.6b

Bs = Bacillus subtilis, Ca =Candida albicans, Ec = Escherichia coli, Tc = Trichophyton concentrum, Pa = Pseudomonas aeruginosa, Tm = Trichophyton mentagrophyte, Tr = Trichophyton rubrum.
Each value is a mean of 5 replicates, Mean values with the same letter(s) are not significantly different

Table 4: Effect of fermentation period on the antimicrobial efficacy of *E. rugulosa* crude extract using agar diffusion assay

	Zones of inhibition (mm)					
Days	*Ec*	*Sa*	*Pa*	*St*	*Tr*	*Tt*
1	0	0	0	0	0	0
2	0	0	0	0	0	0
3	0	0	0	0	0	0
4	2.21	3.32	0	2.41	3.15	0
5	2.82	3.85	2.34	3.27	3.93	2.11
6	3.71	4.43	2.72	3.93	4.87	2.93
7	4.15	5.27	3.23	4.35	5.72	3.67
8	4.22	5.52	3.71	4.31	6.15	3.62
9	4.91	6.11	4.27	4.84	7.31	4.35
10	4.91	6.11	4.31	4.92	7.31	4.62
11	4.8	5.84	4.13	4.65	7.1 9	4.55
12	4.7	5.69	3.95	4.42	6.85	4.58
	ns	ns	ns	ns	s	ns

St = Salmonella typhi, Pa = Pseudomonas aeruginosa, Ec = Escherichia coli, Tr = Ttrichophyton rubrum, Sa = Staphylococcus aureus, Tt = Trichophyton tonsurans.
Each value is a mean of 5 replicates, s = significant, ns = not significant

The results obtained in the present study have indicated that the potential value of investigating metabolite production by fungi, including endophytes from tropical regions could be demonstrated. Therefore, there is the strong need to intensify this study towards the Nigerian mycobiota as a source of bioactive compounds.

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