ISSN 0001-3714

Revista de Microbiologia

SBM

Sociedade Brasileira de Microbiologia

São Paulo — Brasil

Volume 25 Número 2 Abr. - Jun. 1994

FICHA CATALOGRÁFICA

Preparada pela Biblioteca do
Instituto de Ciências Biomédicas da Universidade de São Paulo

Revista de Microbiologia/Sociedade Brasileira de Microbiologia.
— Vol. 25, nº 2 (abr/jun 1994)
— São Paulo: SBM, [1970] -
v.:il; 27 cm

Trimestral
1970 - 1994, 2-25
ISBN 0001-3714

1. Microbiologia I. Sociedade Brasileira de Microbiologia

NLM-QW4

SCT/PR CNPq FINEP

Revista de Microbiologia

Publicação da Sociedade Brasileira de Microbiologia
São Paulo — Brasil

Filiado à IUMS — INTERNATIONAL UNION OF MICROBIOLOGICAL SOCIETIES

Diretora Executiva: MARIATHEREZINHAMARTINS

Assistente da Direção Executiva: Bernadette D. G. M. Franco

Diretores Associados: Caio Marcio Figueiredo Mendes
Claudete Rodrigues de Paula
Maria Lucia B. O. Rácz

Conselho Editorial

Adauto Ivo Milanez
Alcides Serzedello
Allen Norton Hagler
Ana Clara G. Schenberg
Andrejus Korolkovas
Antonio Fernando Pestana de Castro
Aramis Augusto Pinto
Arlete Emily Cury
Astrea Mennuci Giesbrecht
Augusto Cezar Montelli
Carlos da Silva Lacaz
Carmo Elias A. Melles
Celeste Fava Netto
Claudete Rodrigues Paula
Daison Olzany Silva
Edmar Chartone de Souza
Ernesto Hofer
Flavio Alterthum
Galba Maria Campos Takaki
Hermann Gonçalves Schatzmayr
Homero Fonseca
Ises de Almeida Abrahanson
João Lúcio de Azevedo
Johanna Dobereiner
José Alberto Neves Candeias
Leda Cristina Santana Mendonça Hagler
Leon Rabinovitch
Luiz Rachid Trabulsi
Marcelo Magalhães
Maria Aparecida Shikanai Yasuda
Maria Regina Fernandes Toledo
Mauro Faber de Freitas Leilão
Milton de Uzeda
Myrna Sabino
Moacyr Alcojarado Rebello
Paulo Hideki Yasuda
Paulo Suyoshi Minami
Romain Rolland Golgher
Sebastião Timo Iaria
Sérgio Olavo Pinto da Costa
Sérgio Eduardo Longo Fracallanza
Silvio Arruda Vasconcelos
Sumie Hoshino Shimizu
Vera Lucia Garcia Calich
Waldemar Francisco
Walderez Gambale
Willibaldo Schmidell Netto
Zoilo Pires Camargo

Secretária Executiva: Nancy Yuri Kawakosi de Amo

Os artigos publicados na Revista de Microbiologia são indexados em: Current Contents (USA); CNRS - Centre National de la Recherche Scientifique (França); Chemical Abstracts Service(USA); Cambridge Scientific Abstract (USA); Commonwealth Mycological Institute (England): Hamdard National Foundation (Paquistão); IMLA - Index Medicus Latino Americano (Brasil); Institut Nautchnoi Informatsii (URSS); Periodica (México); Sumarios Correntes Brasileiros; UMI - University Microfilms International (USA).

Auxílio Financeiro: Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP); Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq); FINEP.

Produzido pela **TEC art Editora:** fone (011)542-6897.

SOCIEDADE BRASILEIRA DE MICROBIOLOGIA
AV. Prof. Lineu Prestes, 1374
05508-900 - São Paulo - S.P.
Brasil
Fone (011) 813-4392
Tel. / FAX 813-9647

REVISTA DE MICROBIOLOGIA - SBM
Av. Prof. Lineu Prestes, 1374
Cid. Universitária - USP
05508-900 — São Paulo/SP

Sociedade Brasileira de Microbiologia

Filiada a IUMS – INTERNATIONAL UNION OF MICROBIOLOGICAL SOCIETIES

Diretoria

Presidente
Dra. Maria Therezinha Martins
Inst. de Ciências Biomédicas II – USP
Dept.º de Microbiologia
Av. Prof. Lineu Prestes, 1.374
05508-900 – São Paulo – SP

Vice-Presidente
Dr. Sérgio E. L. Fracallanza
Inst. de Microbiologia – UFRJ
Centro de Ciências da Saúde – Bl.1
Ilha do Fundão
21944-000 – Rio de Janeiro – RJ

1ª Secretária
Dra. Mariza Landgraf
Fac. de Ciências Farmacêuticas – USP
Av. Prof. Lineu Prestes, 580
Cidade Universitária – USP
05508-900 – São Paulo – SP

2ª Secretária
Dra. Maria de Fátima Borges Pavan
Fac. de Ciências Farmacêuticas - USP
Av. Prof. Lineu Prestes, 580
Cidade Universitária - USP
05508-900 - São Paulo - SP

1º Tesoureiro
Dr. Benedito Côrrea
Inst. de Ciências Biomédicas II – USP
Deptº de Microbiologia
Av. Prof. Lineu Prestes, 1.374
05508-900 – São Paulo – SP

2º Tesoureiro
Dra. Petra S. Sanchez
Divisão de Análises Microbiol.
CETESB
Av. Prof. Frederico Hermann Jr., 345
05489-900 - São Paulo - SP

Conselho Fiscal

Dr. Allen N. Haggler
Dr. Daison Olzani Silva
Dr. João Ruy Jardim Freire

Coordenadores de Programas da SBM

Microbiologia Industrial (Antonio M.F.L.J. Bonomi – IPT/USP-SP); (Margaretti Simões – IPT/USP-SP)
Micologia (Walderez Gambale – ICBII/USP-SP); (Claudete R. Paula ICBII/USP-SP)
Biodeterioração e Biodegradação (Christine Gaylarde – FA/UFRGS-RS); (Rosana F. Vazoller - Deptº de Hidraúlica/USP/S. Carlos
Microbiologia Médica Veterinária (Silvio A. Vasconcellos – FMVZ/USP-SP); (Antonio F.P. de Castro – IB/UNICAMP-SP)
Infecções Hospitalares (Carlos Emilio Levy – FMRP/USP-SP); (Igor Mimicca –SCM-SP)
Microbiologia de Alimentos (Bernadette de M. G. Franco – FCF/USP-SP); (Sebastião Timo Iaria – ICBII/USP-SP)
Taxonomia e Coleções de Cultura (Wanderley P. Canhos – FTPAT– Campinas – SP); (Christine Gaylarde – FA/UFRGS-RS)
Microbiologia do Solo (Mariângela Hungria da Cunha - EMBRAPA/PR); (SIU MUI TSAI - CENA/ESALQ - Piracicaba)
Micotoxinas (Myrna Sabino – IAL - SP); (Benedito Corrêa – ICBII/USP - SP)
Microbiologia Médica Humana (Sergio E. L. Fracalanza – IM/UFRJ - RJ); (Augusto Cesar Montelli – UNESP/Botucatu – SP)
Microbiologia Ambiental (Elizabeth Marques – CETESB - SP); Regine Helena S. dos F. Vieira - UFCE/Fortaleza/CE)

Objetivos

A Sociedade Brasileira de Microbiologia, fundada em 28 de setembro de 1956, é sociedade civil, sem fins lucrativos, dedicada a agremiar microbiologistas brasileiros; a promover o desenvolvimento da microbiologia através do estímulo à pesquisa científica e suas aplicações, melhorar as qualificações profissionais dos microbiologistas; e manter intercâmbio, a cada dois anos, na última semana do mês de julho, o Congresso Brasileiro de Microbiologia e edita a Revista de Microbiologia, com distribuição trimestral. Para informações sobre a SBM, escrever para qualquer membro da Diretoria.

Endereço

Av. Prof. Lineu Prestes, 1.374
05508 – São Paulo – SP – Brasil
Fone: (011) 813-4392 – Fax: 813-9647

REVISTA DE MICROBIOLOGIA
PUBLICAÇÃO DA SOCIEDADE BRASILEIRA DE MICROBIOLOGIA
VOLUME 25 ABRIL-JUNHO 1994 NÚMERO 2
REV. MICROBIOL. (S. PAULO), 25(2)

CONTEÚDO-CONTENTS PAG

REVISTA DE MICROBIOLOGIA
Depto. de Microbiologia – ICB II – USP
Av. Prof. Lineu Prestes, 1374 – Cid. Universitária
CEP 05508-900 – São Paulo - SP - BRASIL

Rev. Microbiol., São Paulo, 25(2): 77-82, 1994

VIRULENCE FACTORS IN *ESCHERICHIA COLI* ISOLATED FROM BLOOD AND CEREBROSPINAL FLUID

Ângela Maria Girardi Dias
Elena Kano
Leda Kano Nakahara
Sueli Aparecida Fernandes
Maria Aidê Mitico Fukushima Kato
Kinue Irino

SUMMARY

Strains of *Escherichia coli* from the normal intestinal flora of man are responsible for a variety of extraintestinal infections. Numerous investigations have been carried out to establish which factors can be used to define virulence among these microorganisms. In the present paper, the presence of O, H and K1 antigens and the production of hemolysin, aerobactin and colicin were investigated to evaluate virulence among 111 *E.coli* strains isolated from blood (75 strains) and cerebrospinal fluid (36 strains) of inpatients. 75.67% of the isolates were classified into 26 serogroups subdivided into 40 serotypes; 27.02% presented K1 antigen, 39.64% were hemolytic, 74.78% produced aerobactin and 29.46% were colicinogenic, colicin V positive bacteria being the most frequent ones. The majority of both blood and cerebrospinal fluid *E. coli* isolates were classified as serogroup 06; the most frequent serotype detected was 06:H31.

Key words: *Escherichia coli*, serotypes, virulence factors.

INTRODUCTION

Within the group of bacteria associated with extraintestinal infections (EI), *Escherichia coli* is the commonest species. These microorganisms are typically found in the normal intestinal flora and the development of IE depends on both predisposing factors of the host and virulence factors of the bacteria. Certain O and K antigens as well as hemolysin, siderophore and colicin production are considered possible virulence factors.

The prevalence of some O antigens and the frequency of some K antigens have been associated with EI *E.coli* virulence. Among the K antigens, K1 is a particularly relevant one since it has been identified in most isolates from cases of meningitis, septicemia and pyelonephritis in children (11, 26, 30, 33).

The secretion of hemolysin (3, 5, 24) and colicin (14, 24) is more common among EI isolates than among isolates from faeces. Besides the production of a siderophore of the hydroxamate type, aerobactin (4, 8, 21, 25, 27) has also been detected in a significant proportion of EI strains.

In the present study, some of the characteristics associated with EI *E.coli* virulence, namely: the presence of O, H and K surface antigens and the production of hemolysin, aerobactin and coli-

Bacteriology Department; "Adolfo Lutz" Institute, Avenida Dr. Arnaldo, 355 - 9a, CEP - 01246, São Paulo, Brazil.

cin, were investigated among *E.coli* isolates from human blood and cerebrospinal fluid.

MATERIALS AND METHODS

Bacterial strains

111 strains of *E.coli* derived from blood (75 strains) and cerebrospinal fluid (36 strains) of hospitalized patients were studied. Strains were isolated from the collected specimens in the Bacteriology Department of the "Adolfo Lutz" Institute, São Paulo, Brazil, during the January 1985 to December 1989 period.

Identification of surface antigens

All antisera used for screening were prepared in the Bacteriology Department of the "Adolfo Lutz" Institute.

Detection of O antigen - Identification of O antigen was done by the tube agglutination test (16). Initially, all strains were screened with 15 antisera against serogroups 01, 02, 04, 06, 07, 08, 09, 011, 016, 018, 022, 025, 062, 075 and 083, which are the most frequently associated with EI infections (28, 29). Strains that did not present a clear agglutination reaction with these antisera were tested with 51 other antisera.

Detection of H antigen - Strains positive for O antigen were grown in semi-solid agar to promote activation of flagellar antigens and then submitted to the slide agglutination test with 6 polivalent antisera (against antigens H1 to H56), as described by GROSS & ROWE (16). Subsequent to the slide agglutination test with one of these polyvalent antisera, H antigen determination was carried out by tube agglutination test using polyvalent antisera.

Detection of K1 antigen - Isolated strains were cultured in Worfel-Ferguson (6) agar. The presence of K1 antigen was determined by slide agglutination tests using K1 antiserum and confirmed with group B *Neisseria meningitidis* antiserum, since the immunological identity of K1 antigen with the capsular antigen of this bacterial group is now well established (2, 31).

Hemolysin Production

Strains were grown on trypticase soy agar plates containing 5% defibrinated blood (from sheep, horse, guinea pig, rabbit ox, chicken or man). The presence of a clear zone of erythrocyte lysis around the colonies after 24 hours of incubation at 37°C was recorded as a positive result for hemolysin secretion.

Aerobactin Production

Strains were cultured for 10 days in M9 minimum medium supplemented with 2% glucose, 5% casaminoacids and 169 mM a-dipiridil (Sigma). The production of aerobactin was determined in the same medium containing 1% agar using LG 1522 strain as indicator (8).

Colicin Production

The production of colicin was screened by the overlay method (18) using CL-104 and BZB-1011 *E. coli* strains as coligenicity indicators. Identification of the type of colicin secreted was based on the same methodology using 16 reference strains, each being resistant to different colicins of the following types: A, B, E1, E2, E3, E4, E5, E6, E7, Ia, Ib, K, M, N, V (1, 14, 17).

RESULTS

The frequency of production of five possible virulence factors among the isolated *E.coli* strains is shown in Table 1. Strains from both blood and cerebrospinal fluid presented smooth O antigen. K1 antigen was detected in 29.33% of the blood derived bacteria and in 22.22% of the cerebrospinal fluid (CSF) isolates. Out of the 22 K1-positive blood (BL) isolates, 9 (40.80%) were obtained from children of up to 5 years of age and 13 (59.17%) from adults; 5 out of the 8 strains (62.50%) isolated from cerebrospinal fluid were obtained from neonates.

Hemolysin secretion was detected in 42.67% of the BL isolates and 33.33% of the CSF isolates, regardless of the type of blood used in the assay.

Aerobactin production was the second most frequent virulence factor present in 74.67% of the strains isolated from blood and in 75.0% of the CSF derived bacteria.

The percentage distributions of colicin production among BL and CSF isolates were 24.0% and 41.67%, respectively. The most frequent colicin types were K and V. It is interesting to note that out of the 15 colicinogenic CSF strains, 7 (46.67%) secreted V colicin.

TABLE 1 - Frequency of production of virulence factors among *E. coli* strains according to their sources.

Source	Nº	Virulence factors				
		O* antigen	K_1 antigen	Hemolysin	Aerobactin	Colicin
Blood	75	75 (100%)	22 (29,33%)	32 (42,67%)	56 (74,67%)	18 (24,00%)
CSF**	36	36 (100%)	08 (22,22%)	12 (33,33%)	27 (75,00%)	15 (41,67%)
Total	111	111 (100%)	30 (27,03%)	44 (39,64%)	83 (74,77%)	33 (29,73%)

* Smooth 0 antigen
** Cerebrospinal fluid

Table 2 shows the frequency of serotypes (O and H antigens) identified among the 111 strains studied. Twenty six O antigens were identified in 84 (75.67%) strains. Serogroup 06 was detected in a large number of strains isolated from both blood and cerebrospinal fluid (total of 22 strains). Serogroups 021 (6 strains), 015 (5 strains), 01, 04, 07, 018 and 025 (4 strains each) and 02, 020 and 075 (3 strains each) represented 47.61% of all typable strains. A total of 40 serotypes were identified. We would like to point out that 17 (77.25%) out of the 22 strains from serogroup 06 belonged to 06:H31 serotype and that the serogroup 021 strains were positive for H5 antigen.

Considering the presence of K1 antigen and the production of hemolysin, aerobactin and colicin as virulence factors in connection with serotype distribution among the 111 *E.coli* strains analysed, it can be seen that the majority of them were positive for one to three of these factors, which did not exhibit serogroup or serotype specificity. Within the serogroup 06 isolates, that represent the majority of the 111 *E coli* obtained, a

TABLE 2 - *E. coli* serotypes (O and H antigens) identified among isolates from blood and CSF.

Serotypes	Number of strains		
	Total	Blood	CSF *
01:H7	2	2	0
02:H4	3	2	1
04:H-	4	4	0
06:H-	2	1	1
06:H1	3	3	0
06:H31	17	11	6
07:H-	4	3	1
09:H-	2	1	1
015:H-	2	1	1
015:H10	2	1	1
018:H-	3	1	2
020:H-	2	0	2
021:H5	6	5	1
025:H-	3	2	1
075:H-	3	1	2
0111:H-	2	2	0
Others**	24	19	5
Total	84	59	25

* Cerebrospinal fluid
** 24 different serotypes represented by one strain each

TABLE 3 - Frequency of production of hemolysin and/or aerobactin in *E. coli* serogroups isolated from blood or cerebrospinal fluid.

Serogroups	Number of strains			
	Total	Hemolysin+	Aerobactin+	Aerobactin and Hemolysin+
01	2	0	1	1
04	4	0	0	4
06	22	6	2	14
07	4	0	4	0
09	2	0	2	0
015	4	1	3	0
016	2	0	0	2
018	4	1	1	2
020	3	0	3	0
021	6	1	0	5
025	3	0	2	1
075	3	1	2	0
0111	2	0	2	0
0153	2	0	2	0
0158	2	0	2	0
Others*	9	0	9	0
ND	20	1	15	4
Total	94	11	50	33

* 9 serogroups represented by one strain each
** No typable

great variability of virulence factors was observed; most of them (19 out of 22 strains) were positive for at least two of the factors investigated.

The analysis of the frequency of hemolysin and aerobactin production, alone or in association, in relation to serogroups is presented in Table 3. It was observed that 94 out of the 111 strains (84.68%) could be placed in one of the alternatives. It was also observed that, out of the 33 strains of the most frequent serogroups (06, 021 and 015), 32 (96.97%) presented one out of the three alternatives mentioned above.

DISCUSSION

The majority of *E. coli* strains associated with EI have been described as part of a relatively small number of serogroups (28, 29). In the present study, 65.48% of the strains had O antigen identified among 15 serogroups associated with EI.

Serogroup 06 has been identified as the most frequent one among *E. coli* strains isolated from blood both in the U.S.A. (13, 22, 34) and in England (10), and our data are in accordance with this findings. In Denmark, 06 is the third most frequently isolated serogroup. On the other hand, serogroup 06 was the fourth most frequent one according to a study on neonatal meningitis in the U.S.A. yet it was absent in CSF isolates analysed in England.

The most frequent serotype was 06:H31. Studies carried out in several countries also indicated the presence of this serotype, though not at the frequency presently reported.

Among the strains isolated from blood, 055:H-, 055:H6 and 0111:H- are the most commonly associated with gastroenteritis in children. Since these serotypes have been isolated from blood of two years old children, it is possible that the bloodstream invasion followed a gastrointestinal infection. The same possibly occurred with serotypes 028:H- and 0143:H- which may belong to the *E.coli* group called enteroinvasive or "EIEC" (19, 29).

In the present study, it was verified that all strains of serogroups 04, 07, 016 and 018, independently of the material from which they were isolated and of the serotypes to which they belonged, exhibited K1 antigen; additionally, 50.0% of the strains from serogroup 06 presented this antigen. K1 was detected at a frequency compatible with that described in the literature (13), but the use of other K antisera is important to characterize all K antigens.

Among the serogroups most frequently associated with K1 antigen, 01, 06, 07, 016 and 018 are of relevance in America (13, 15, 23, 32). The same O groups are found in Europe with small variations, such as the inclusion of serogroup 04 and the observation that, most of the time, serogroup 06 is found in association with K2 antigen and not with K1 (20). K1 antigen has been detected in strains that belong to all of these serotypes and also to 028, 045 and 0158 serogroups.

The presently reported distribution of hemolysin production is in accordance with that described in the literature (15, 20). Hemolytic strains belong primarily, but not exclusively, to 04, 06, 018 and 075 serogroups (9, 12, 15, 18). Among the samples evaluated in this study, these serogroups accounted for 63.64% of the hemolytic strains. We would like to comment that all strains of serogroup 021 and 20 out of the 22 strains of serogroup 06 were hemolytic.

The majority of the *E.coli* isolates analysed synthetized aerobactin. Similar results have been found in other studies (7, 25, 27). Of the 33 strains that belonged to the most frequent serogroups (06, 021, and 015), 24 (72.72%) were aerobactin producers.

Hemolysin and aerobactin are necessary for *E. coli* to obtain the iron required for survival (27) and the high rate of strains which were hemolytic, aerobactin positive or both probably reflects the important role of these molecules.

Among the colicin positive bacteria investigated, colicin V was produced more frequently. The majority of the colicin V producing strains were isolated from cerebrospinal fluid. No relationship between type of colicin and serotype was found, confirming the results presented by HETTIARATCHY et alii (17).

The data obtained in this study showed that some of the factors investigates are more frequent than others and that this frequency may vary depending on the source of material used for *E. coli* isolation and on the patient's age. Besides the factors analysed, many other possible virulence factors should be evaluated aiming at a more comprehensive elucidation of the pathogenic mechanisms of *E.coli* strains involved in extraintestinal infections.

AKNOWLEDGEMENTS

The authors thank Dr. Mark Achtman from Max Planck Institut fur Molekular Genetik, Ber-

lin, Germany, for the reference strains for colicin production. We also thank Dr. Lilian R. Marques for his suggestions and scientific help.

RESUMO

Fatores de virulência em *Escherichia coli* isolado de sangue e liquor

E. coli da flora normal do intestino humano pode contaminar, colonizar e subsequentemente causar infecções extra-intestinais sendo um dos principais agentes etiológicos de septicemias, meningites e infecções do trato urinário. Numerosas investigações tem sido desenvolvidas a fim de definir os fatores de virulência destes microrganismos. Dentre estes fatores foram pesquisados antígenos de superfície (0, H, K1), hemolisina, acrobactina e colicina em 111 cepas de *E. coli* isoladas de sangue (75 cepas) e líquido cefalorraquidiano (36 cepas) de pacientes hospitalizados. Verificou-se que 75,67% (84 cepas) das cepas foram classificadas sorologicamente em 26 sorogrupos subdivididos em 40 sorotipos; 27,02% (30 cepas) apresentavam o antigeno K1; 39,64% (44 cepas) eram hemolíticas; 74,78% (84 cepas) produziram acrobactina e 29, 46% (33 cepas) eram colicinogênicas, sendo que entre os tipos pesquisados, a colicina V foi a mais frequente. O sorogrupo identificado em um maior número de cepas, isoladas tanto de sangue como de líquido cefalorraquidiano, foi 06 e o sorotipo mais frequente o 06:H31.

Palavras-chave: *Escherichia coli*, sorotipos, fatores de virulência.

REFERENCES

1. Achtman, M.; Mercer, A.; Kusecek, B. Heuzenroeder, M.; Aaronson, W.; Sutton, A.; Silver, R.P. Six widespread bacterial clones among *Escherichia coli* K1 isolates. *Infect Immun.*, 39: 315-35, 1983.
2. Achtman, M. & Pluschke, G. Clonal analysis of descentand virulence among selected *Escherichia coli*. *Ann, Rev. Microbiol.*, 40: 185-210, 1986.
3. Andrade, J.R.C. & Suassuna, I. Atividades citotóxica e hemolítica em *Escherichia coli* uropatogênicas. *Mem. Inst. Oswaldo Cruz*, 83: 193-99, 1988.
4. Aumont, P.; Enard, C.; Expert, D.; Pieddeloup, C.; Tancrede, C.; Andremont, A. Production of haemolysin, acrobactin and enterobactin by strains of *Escherichia coli* causing bacteraemia in cancer patients, and their resistance to human serum. *Res. Microbiol.*, 140: 21-6, 1989.
5. Beutin, L. The different hemolysin of *Escherichia coli*. *Med. Microbiol. Immunol.*, 180: 167-82, 1991.
6. Bier, O. *Bacteriologia e Imunologia em suas aplicações à medicina e à higiene*. 16 ed. São Paulo, Melhoramentos-EDUSP, 1975, p. 1062.
7. Carbonetti, N.H.; Boonchai, S.; Parry, S.H.; Vaisanen-Rhen, V.; Korhonen, T.K. Williams, P.H. Acrobactin mediated iron uptake by *Escherichia coli* isolates from human extraintestinal infections. *Infecti. Immun.*, 51: 966-8, 1986.
8. Carbonetti, N.H. & Williams, P.H. Detection of synthesis, of hydroxamate siderophore acrobactin by pathogenic isolates of *Escherichia coli*. In: The virulence of *Escherichia coli*. Reviews and Methods. M. Sussman (ed.) 1985. Academicpress, INC, Orland, USA. pp. 419-24.
9. Cavaliere, S.J. Bohach, G.A.; Snyder, I.S. *Escherichia coli* alfa haemolysin: characteristics and probable role in pathogenicity. *Microbiol. Rev.*, 48: 326-43, 1984.
10. Cheasty, T.; Gross, R.J. Rowe, B. Incidence of K1 antigen in *Escherichia coli* isolated from blood and cerebrospinal fluid of patients in the United Kingdom. *J. Clin. Pathol.*, 30: 945-7, 1977.
11. Cook, E.M. *Escherichia coli* - an overview. *J. Hyg. Camb.*, 95: 521-30, 1985.
12. Cook, E.M. & Ewins, S.P. Properties of strains of *Escherichia coli* isolated from a variety of sources. *J. Med. Microbiol.*, 8: 107-11, 1975.
13. Cross, A.S., Genski, P.; Sadoff, J.C.; Orskov, F.; Orskov, I. The importance of the K1 capsule in invasive infections caused by *Escherichia coli*. *J. Infect. Dis.*, 149: 184-93, 1984.
14. Davies, D.L.; Falkiner, F.R. Hardy, K.G. Colicin V production by clinical isolates of *Escherichia coli*. *Infect. Immun.*, 31: 574-9, 1981.
15. Evans, Jr., D.J.; Evans, D.J.; Hohne, C.; Noble, M.A.; Haldane, E.V.; Lior, H.; Young, L.S. Hemolysin and K antigens in relation to serotype and hemagglutination type of *Escherichia coli* isolated from extraintestinal infections. *J. Clin. Microbiol.*, 13: 171-8, 1981.
16. Gross, R.J. & Rowe, B. Serotyping of *Escherichia coli*. In: The virulence of *Escherichia coli* reviews and methods. London, Academic Press, 1985, p. 345-63. (Special publications of the Society for General Microbiology).
17. Hettiaratchy, I.G.T.; Cooke, E.M.; Shooter, R.A. Colicin production as an epidemiological marker of *Escherichia coli*. *J. Med. Microbio.*, 6: 1-11, 1973.
18. Hughes, C.; Phillips, R.; Roberts, A.P. Serum resistence among *Escherichia coli* strains causing urinary tract infection in relation to 0 type and the carriage of hemolysin, colicin, and antibiotic resistence determinants. *Infect. Immun.*, 35: 270-5, 1982
19. Kano, E. - *Sorotipos e biotipos de* Escherichia coli *patogênica isolada de coproculturas, no Município de São Paulo*. São Paulo. 1990, 75p. Dissertação de Mestrado - Instituto de Ciências Biomédicas da Universidade de São Paulo.
20. Korhonen, T.K.; Valtonen, M.V.; Parkinen, J.; Vaisanen-Rhen, V.; Finne, J.; Orskov, F.; Orskov, I.; Svenson, S.B., Makela, P.H. Serotypes, hemolysin production, and reptor recognition of *Escherichia coli* strains associated with neonatal sepsis and meningitis. *Infect. Immun.*, 48: 486-91, 1985.
21. Linggodd, M.A.; Roberts, M.; Ford, S.; Parry, S.h.; Williams, P.H. Incidence of the acrobactin iron uptake system among *Escherichia coli* isolates from infections of farm animals. *J. Gen. Microbiol.*, 133: 835-42, 1987.

22. McCabe, W.R.; Kaijser, B.; Olling, S.; Uwaydah, M.; Hanson, L.A.; *Escherichia coli* in bacteremia: K and O antigens and serum sensitivity of strains from adults and neonates. *J. Infect. Dis.*, **138**: 33-41, 1978.
23. McCraken Jr., G.H.; Glode, M.P.; Sarff, L.D. Mize, S.G.; Schiffer, M.S. Robbins, J.B. Gotschlich, E.C.; Orskov, I.; Orskov, F. Relation between *Escherichia coli* K1 capsular polyssacharide antigen and clinical out come in neonatal meningitis. *Lancet*, 246-50, 1974.
24. Minshew, B.H.; Jorgensen, J.; Swanstrum, M.; Grootes-Reuvecamp, G.A.; Falkow, S. Some charactheristics of *Escherichia coli* strains isolated from extraintestinal infections of humans. *J. Infect. Dis.*, **137**: 648-54, 1978.
25. Montgomerie, J.Z.; Bindereif, A.; Neilands, J.B.; Kalmanson, G.M.; Guze, L.B. Association of hidroxamate siderophore (aerobactin) with *Escherichia coli* isolated from patients with bacteremia. *Infect. Immun.*, **46**: 835-8, 1984.
26. Nassif, X. & Sansonetti, P. Facteurs de dissemination septicemique des colibacilles. *Med. Malad. Infect.*, (Nº special): 48-55, 1987.
27. Opal, S.M.; Cross, A.S.; Gemski, P.; Lythe, L.W. Aerobactin and alfa hemolysin as virulence determinantes in *Escherichia coli* isolated from human blood, urine, and stool. *J. Infect.*, **161**: 794-6, 1990.
28. Orskov, F. & Orskov, I. *Escherichia coli* O:H serotypes isolated from human blood. *Acta Path. Microbiol. Scand.* (Sect. B), **83**: 595-600, 1975.
29. Orskov, F. & Orskov, I. Serotyping of *Escherichia coli*. *Methods Microbiol.*, **14**: 43-112, 1984.
30. Orskov, I. & Orskov, F. *Escherichia coli* in extraintestinal infections. *J. Hyg. Camb.*, **95**: 551-75, 1985.
31. Sarff, L.D.; McCracken Jr., G.H.; Schiffer, M.S.; Glode, M.P.; Robbins, J.B.; Orskov, I.; Orskov, F. Epidemiology of *Escherichia coli* K1 in healthy and diseased newborns. *Lancet:* 1099-104, 1975.
32. Silver, R.P.; Aaronson, W.; Sutton, A.; Schneerson, R. Comparative analysis of plasmids and some metabolic characteristics of *Escherichia coli* K1 from diseased and healthy individuals. *Infect. Immun.*, **29**: 200-6, 1980.
33. Silver, R.P.; Aaronson, W.; Vann, W.F. The K1 capsular polysaccharide of *Escherichia coli*. *Rev. Infect. Dis.*, **10** (supl. 2): 282-6, 1988.
34. Vosti, K.L.; Goldberg, L.M.; Monto, A.S.; Rantz, L.A. Host-parasite interaction in patients with infections due to *Escherichia coli*. I-The serogrouping of *E. coli* from intestinal and extraintestinal sources. *J. Clin. Invest.*, **43**: 2377-85, 1964.

Rev. Microbiol., São Paulo, 25(2): 83-5, 1994

INFLUENCE OF LECTINS ON ADHESION OF *STREPTOCOCCUS SALIVARIUS* TO BUCCAL EPITHELIAL CELLS

Maria Regina Lorenzetti Simionato
Marcia Pinto Alves Mayer
Silvana Cai
José Luiz de Lorenzo
Flavio Zelante

SUMMARY

In this study, the influence of pretreating buccal epithelial cells with purified preparations of lectins extracted from food on *S. salivarius* cell adhesion was investigated. All lectins lead to an increase in the number of adhered bacteria, except for the lectin from potato, suggesting that dietary lectins may play an important role on buccal colonization by *S. salivarius*.

Key words: lectins, *S. salivarius*, attachment, buccal epithelial cells.

Streptococcus salivarius is a pioneer colonizer of the mouth, which is present in high proportions on oral epithelial surfaces (11) due to the high affinity binding between adhesins of its superficial fibrils and receptors on epithelial cells (7). This specie plays an important role in oral ecological interactions by producing a variety of bacteriocin-like and other inhibitory substances (4, 10). Several substances, such as lectins, are able to modify the adhesive interactions between bacteria and host either by inhibiting or by promoting adhesion (5). Lectins are a class of proteins which bind to specific carbohydrates (2) present on oral surfaces. Many food contain complex mixtures of lectins and their ingestion may affect host-parasite interactions in the mouth (5, 9), though their ecological role has not been well established yet. The present report describes the influence of lectins on the adhesion of *S. salivarius* to buccal epithelial cells.

The selected lectins were purified preparations from Arachis hypogaea (peanut), Canavalia ensiformis (jack bean - producer of concanavalin A), Glycine max (soybean), Lens culinaris (lentil), Lycopersicon esculentum (tomato), Perseau americana (avocado), Phaseolus vulgaris (kidney bean), Pisum sativum (garden pea), Solanum tuberosum (potato) and Triticum vulgaris (wheat germ) purchased from Sigma Chemical Co. (USA). The strain of *S. salivarius* used in this study was a recent isolate from buccal mucosal surface and identified according to Hardie (8) and Coykendall (3). Stock cultures were made in 50% glycerol and frozen at -20°C until use. For the adhesion experiments, *S. salivarius* was grown in Tryptic Soy Broth (TSB, Difco) at 37°C for 16-20 h. The organism was harvested by centrifugation (5.000 X g, 4°C, 10 min), washed twice in phosphate buffered saline (PBS) pH 7.2, and resuspended in the same buffer at a concentration of $1x10^8$ CFU/ml.

Departamento de Microbiologia, Instituto de Ciências Biomédicas, USP.
Av. Prof. Lineu Prestes, no 1374, Cidade Universitária, São Paulo, SP., Brasil. Caixa Postal 66208. CEP 05508-900.

Buccal epithelial cells (BECs) were obtained by gently scraping the buccal mucosal surface of a volunteer with a sterile wooden spatula. The epithelial cells were dissociated in PBS, harvested by centrifugation (1.000 X g, 4°C, 10 min) washed twice and resuspended in PBS to $1x10^5$ epithelial cells/ml. BECs were pretreated with each one of the lectins at a final concentration of 25 µg/ml for 30 min at 37°C on a rotary device (10 rpm) and then washed twice prior to use (6).

For the assay, equal volumes of each pretreated BECs and bacterial suspensions were mixed and incubated for 60 min at 37°C on a rotary device. Next, the mixtures were washed with 100 ml of PBS through 8 µm membrane filters (Millipore) to remove unattached bacteria and the epithelial cells with adherent bacteria then collected on the membrane surfaces. The filters were gently pressed onto slides to transfer BECs, which were fixed and stained with crystal violet.

All experiments were run in duplicate. Results were expressed as mean bacteria/BEC based on microscopical counts of 25 randomly selected epithelial cells for each of the duplicate set of reaction mixtures (n=50). As control, the numbers of *S. salivarius* present on epithelial cells not pretreated with lectins were also determined following the same procedure. The statistical analysis of the data was done using a paired t-test (significance level at $p<0.05$)

TABLE 1 - Influence of lectins on adhesion of *S. salivarius* to buccal epithelial cells (BECs) pretreated with 25µg of each lectin.

Lectins from:	Average no of adhered bacteria/BEC (n=50) (x±sd)[a]	Percentage in relation to control
Arachis hipogaea (peanut)	131.54 ± 91.63*	208.20
Canavalia ensiformis (jack bean)[b]	119.58 ± 67.30*	189.27
Glycine max (soybean)	137.02 ± 137.52*	216.87
Lens culinaris (lentil)	213.58 ± 176.72*	338.05
Lycopersicon esculentum (tomato)	188.80 ± 128.29*	298.83
Perseau americana (avocado)	189.22 ± 103.94*	299.49
Phaseolus vulgaris (Kidney bean)	244.92 ± 178.48*	387.65
Pisum sativum (garden pea)	98.94 ± 71.76**	156.60
Solanum tuberosum (potato)	39.80 ± 22.92*	62.99
Triticum vulgaris (wheat germ)	232.02 ± 130.92*	367.24
Control	63.18 ± 35.92	100.00

a = mean ± standard deviation
b = producer of concanavalin A
* = significant difference ($p<0.001$)
** = significant difference ($p<0.05$)

In spite of the different sugar specificities of the lectins studied, all of them significantly influenced *S. salivarius* cell adhesion (Table 1).

Lectins are carbohydrate binding proteins that have two critical properties: specificity for particular sugar residues and bivalence or polyvalence (2). Due to the first property, many lectins have the potential to become associated with epithelial surfaces and persist in the oral cavity until 6h after ingestion (6), binding mainly to glycoproteins. Due to their polyvalence, lectins are able to inhibit or promote bacterial attachment to cell surfaces. The results obtained in the present investigation strongly support these views.

Lectins from peanut, soybean, lentil, tomato, avocado, kidney bean, garden pea, wheat germ, and concanavalin A promoted an increase in attachment to cells by this organism at different levels. The greatest influence was associated with Phaseolus vulgaris (kidney bean) lectin. This enhanced adherence could be explained by the polyvalence of these lectins which, by binding to epithelial cells, must offer a high number of receptors to *S. salivarius*.

Solanum tuberosum (potato) lectin was the only one which caused inhibition of *S. salivarius* attachment. Such inhibition may be due to an interaction between the lectins and receptors for the bacteria on mouth surfaces thus masking bacterial binding sites. Other authors have already reported inhibition of adhesion by dietary lectins (5, 12) but, in spite of the frequency of ingestion of dietary lectins and their probable ecological consequences, no studies on *S.salivarius* cell attachment are found in the literature.

Differences on adhesion presumably reflect different numbers of available receptors for the lectins on BECs, or different affinities of the lectins for the receptors present, or both. Other factors such as valency of lectins, their configuration and unlike affinities for the bacteria may also have influenced the results (1).

Gibbons e Dankers (6) have reported significantly higher numbers of *S. sanguis* attached to buccal cells collected from donors who had eaten wheat germ, compared to buccal cells collected before eating. Therefore, sufficient quantities of wheat germ lectin become associated to oral epithelial cells *in vivo* during eating so as to affect bacterial attachment. Whether this occurs with all lectins thereby modifying the coloniza-

tion of *S. salivarius* or other bacteria, remains to be determined.

The authors wish to thank Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) for the financial support (Process 90/0605-1).

RESUMO

Influência de lectinas na adesão de *Streptococcus salivarius* a células epiteliais bucais

Foi verificada a influência do pré-tratamento de células epiteliais bucais com preparações purificadas de lectinas extraídas de alimentos na adesão de *S. salivarius*. Todas as lectinas promoveram um aumento do número de células aderidas, com exceção da lectina de batata, sugerindo que lectinas derivadas da dieta podem desempenhar importante papel na colonização de *S. salivarius*.

Palavras-chave: lectinas, *S. salivarius*, adesão, células epiteliais bucais.

REFERENCES

1. Abaas, S. Binding of lectins to *Streptococcus mitis* cells. Studies of the specificity of ligand mediated aggregation. *Acta Path. Microbiol. Immunol. Scand. Sect. B.*, 93: 15-20, 1985.
2. Barondes, S.H. Soluble lectins: a new class of extracellular proteins. *Science*, 223: 1259-64, 1984.
3. Coykendall, A.L. Classification and identification of the viridans streptococci. *Clin. Microbiol. Rev.*, 2: 315-28, 1989.
4. Fantinato, V. & Zelante, F. *Streptococcus salivarius:* detecção de cepas produtoras de substâncias semelhantes a bacteriocinas contra bactérias bucais. *Rev. Microbiol.*, 22: 49-54, 1991.
5. Gibbons, R.J. & Dankers, I. Lectin-like constituints of foods which react with components of serum, saliva and *Streptococcus mutans*. *Appl. Environm. Microbiol.*, 41: 880-8, 1981.
6. Gibbons, R.J. & Dankers, I. Association of food lectins with human oral epithelial cells in vivo. *Archs. Oral Biol.*, 28: 561-6, 1983.
7. Handley, P.S.; Harty, D.W.S.; Wyatt, J.E.; Brown, C.R.; Doran, J.P.; Gibbs, A.C.C. A comparison of the adhesion, coaggregation and cell-surface hydrophobicity properties of fibrilar and fimbriate strains of *Streptococcus salivarius*. *J. Gen. Microbiol.*, 133: 3207-17, 1987.
8. Hardie, J.M. Oral streptococci. In: Holt, J.G., ed. Bergey's manual of systematic bacteriologic. Baltimore, Williams & Wilkins, v. 2, p. 1054-63, 1986.
9. Nachbar, M.S. & Oppenheim, J.D. Lectins in the United States diet: a survey of lectins in commonly foods and a review of literature. *Am. J. Clin. Nut.*, 33: 2338-45, 1980.
10. Standers, C.C. & Sanders Jr., W.E. Enocin: an antibiotic produced by *Streptococcus salivarius* that may contribute to protection against infections due to group A streptococci. *J. Infect. Dis.*, 146: 683-90, 1982.
11. Smith, D.J.; Anderson, J.M.; King, W.F.; van Houte, J.; Taubman, M.A. Oral streptococcal colonization of infants. *Oral Microbiol. Immunol.*, 8: 1-4, 1993.
12. Staat, R.H.; Doyle, R.J.; Langley, S.D.; Suddick, R.P. Modification "in vitro" adherence of *Streptococcus mutans* by plant lectins. *Adv. Exp. Med. Biol.*, 107: 639-47, 1978.

Rev. Microbiol., São Paulo, 25(2): 86-9, 1994

ANTIGENIC RELATIONSHIPS OF ISOLATES OF *BORDETELLA BRONCHISEPTICA* FROM SWINE WITH ATROPHIC RHINITIS

Paulo R.S. Martins
Carlos Gil-Turnes*

SUMMARY

The antigenic relationships of twenty *Bordetella bronchiseptica* isolates recovered from pigs with atrophic rhinitis were studied using a quantitative seroagglutination test. Sera against each isolate were produced in rabbits and their titers determined using homologous and heterologous antigens. Titers of twelve sera that cross-reacted were also determined after absorption with heterologous isolates. Antigenic relationships were estimated using cross-reactivity indices. Two isolates showed marked cross-reactivity with sixteen of the other isolates whereas one cross-reacted with none. It was concluded that *B. bronchiseptica* recovered from swine is not antigenically homogeneous. The relevance of these observations to epidemiological and immunoprophylactic studies is discussed.

Key words: *Bordetella bronchiseptica*, cross-reactivity indices, atrophic rhinitis.

INTRODUCTION

Bordetella bronchiseptica has been considered a primary cause of porcine atrophic rhinitis (6, 13), a disease that causes severe economic losses due to mortality or poor food conversion (2).

The antigenic heterogencity of *B. bronchiseptica* detected by seroagglutination has already been demonstrated (10,11,12,13). No work has been reported, however, concerning isolates of this bacterium recovered from swine with atrophic rhinitis.

Eldering, Hornbeck and Baker (4) analysed the antigenic relationships of six *B. bronchiseptica* isolates by seroagglutination using absorbed sera and concluded that four were antigenically identical while the other two differed by one or more antigens. Nakase (9) and Pedersen (10) studied the antigenic characteristics of isolates recovered from several species and found that whereas differences could be detected among samples from other species, isolates from the same species were antigenically identical. Cristina Affonso et al. (3) evaluated vaccine-induced cross protection using isolates recovered from rabbits as antigens, and concluded that the observed dissimilar protection indices suggested antigenic variations among the isolates.

In Brazil, where total pig livestocks amount to approximately 35 million animals, porcine atrophic rhinitis was first diagnosed in 1963 (7). Since 1978, work on *Bordetella bronchiseptica* has increased in this country, aiming especially at developing efficient vaccines for disease control (11). The irregular response to vaccines produced with autoctonous isolates as well as to imported

Universidade Federal de Pelotas, Faculdade de Veterinária - UFPEL-Faculdade de Veterinária, Caixa Postal 354, Campus Universitário, 96010-900, Pelotas, RS, Brasil.
* To whom reprints should be requested.

commercial vaccines raised the hypothesis of antigenic variation between isolates recovered from different herds.

This paper reports on the antigenic relationships of twenty isolates of *B. bronchiseptica* observed using a quantitative seroagglutination technique.

MATERIALS AND METHODS

Isolates

Twenty isolates of *B. bronchiseptica* recovered from swine with atrophic rhinitis that belonged to different herds were used. Nineteen samples were recovered from herds in the state of Santa Catarina (SC), Brazil, as follows: 2 and 13 were isolated from herds of the micro region 303 (east of SC) in 1979; 11, 14, 17, 18 and 19 were isolated in 1979 and 3, 4, 16 and 20 in 1981 from herds of the micro region 305 (central region of SC); 1, 5, 6, 7, 8 and 12 were isolated in 1979 and 15 in 1981 from herds of the micro region 306 (west of SC). Isolate 10 was recovered from Casca, Rio Grande do Sul. All samples were kindly provided by the Centro Nacional de Pesquisas de Suinos e Aves (EMBRAPA, Concordia, SC). Isolates were classified biochemically (12) and stored in Dorset medium at 4°C until use. Sample 19 was recovered from lung whereas the remaining ones were recovered from the nasal cavity. Each isolate was multiplied in several tubes at the same passage in order to avoid variation by subculture. All isolates were in phase I.

Sera

Antisera against each isolate were produced in pairs of adult rabbits whose pre-immunization titers against *B. bronchiseptica* were lower than 32. The immunization schedule used has been previously described (5). Twelve sera that cross-reacted with more than one isolate were absorbed. The absorbing isolate was grown on three plates of Bordet Gengou medium at 37°C for 48 h, suspended in PBS and centrifuged at 1500 x g for 30 min. The supernatant was discarded and the bacterial mass suspended in 5 ml of serum diluted 1:100, incubated under continuous agitation at 37°C for the first 4 hours and at 4°C for the next twelve, and then centrifuged at 1500 x g for 30 min. The supernatant was frozen until tested.

Antigens

Cultures stored in Dorset medium were grown on Bordet Gengou at 37°C for 48 h and then suspended in PBS to obtain 60% T at 625 nm in a spectrophotometer.

Seroagglutination

Equal volumes of antigen and doubled dilutions of each serum in PBS were initially incubated at 42°C for 4 h and then at 4°C for the next 48 h. Tubes were left at room temperature for 2 h before taking final readings. Titers were expressed as the reciprocal of the highest dilution at which agglutination was detected.

Cross-reactivity index

The bilateral cross-reactivity index (CRI) (1) was used to estimate antigenic relationships between isolates. CRI represents the geometric mean of the serological relationships derived from the following equation:

$CRI = 100\sqrt{r \times r'}$, where r is obtained by dividing the titer of serum A against antigen B by the titer of serum A against antigen A, and r' is obtained by dividing the titer of serum B against antigen A by that of serum B against antigen B.

RESULTS

Sera

Serum 7 did not cross-react with antigens 6 and 20 nor serum 20 with antigen 7, while the other sera showed some degree of cross-reactivity with the antigens tested.

Absorbed sera

All titers decreased after absorption (Table 1). Some sera were partially absorbed (8 and 12) whereas others were completely absorbed (6 and 15). Several absorbed sera showed differences in the titers against heterologous antigens. Reactivity of serum 6 with antigens 10, 12, 13, 18 and 20 disappeared after absorption with antigen 12, while serum 12 still cross reacted with antigens 1, 2, 5 and 9 after absorption with antigen 20.

TABLE 1 - Titers of sera against *Bordetella bronchiseptica* after absorption.

Serum	Tested with	Titer	Absorbed with	Titer
1	8	2560	2	200
2	8	2560	1	400
6	10	2560	12	<200
6	13	2560	12	<200
6	18	5120	12	<200
6	20	2560	12	<200
8	1	5120	9	800
8	4	2560	9	800
8	5	5120	9	800
12	1	5120	20	400
12	2	5120	20	400
12	5	5120	20	400
12	9	5120	20	400
12	18	5120	20	<200
18	6	5120	12	200
18	10	2560	12	200
18	15	2560	12	200
18	19	2560	12	200
18	20	2560	12	200
19	2	320	14	200
19	6	5120	14	<200
19	12	5120	14	<200

Cross reactivity indices

The cross reactivity indices of non-absorbed sera varied between 0 and 200 (Table 2).

tions, it was not feasible to absorb every serum with each isolate and then test the resulting serum with all the isolates studied. Therefore, only some sera that cross-reacted with more than one isolate were selected to investigate cross-antigenicity in the present work. Serum 2 absorbed with isolate 1 reacted with antigen 8. Similarly, serum 8 absorbed with antigen 9 reacted with three other antigens and serum 12 absorbed with antigen 20 reacted with four other. Isolates 15 and 18, that had a CRI of 141, seemed to be identical by crossed absorption of their sera. Sera that were absorbed with heterologous isolates and then tested against their homologous antigens showed, in every case, a marked reduction in agglutination titers.

Another form of studying antigenic relationships among isolates is by determining the titer of a serum against both its homologous antigen and an heterologous antigen. This procedure, that is extensively used to group subtypes of Foot and Mouth diseases viruses (1) has not been stablished for *B. bronchiseptica*. Therefore, the values of the indices to be employed for grouping isolates, as already conventioned for FMD viruses (13), are not yet available for this bacterium. It was observed that sera that showed the greater number of CRI > 70 also showed the smaller number of CRI < 32, while those that reacted with few or no isolates at

TABLE 2 - Bilateral cross reactivity indices of twenty isolates of *Bordetella bronohiseptica*.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	100	141	100	100	70	50	10	100	100	70	100	70	36	24	36	100	70	100	70	50
2		100	70	50	50	17	0	70	70	50	70	50	17	17	17	70	50	70	50	36
3			100	70	70	50	0	100	100	70	100	70	50	50	36	70	70	70	100	70
4				100	100	100	0	100	141	70	141	141	70	50	70	70	70	100	70	100
5					100	50	14	100	141	70	100	100	36	50	50	100	100	100	100	100
6						100	17	10	10	100	10	141	100	50	70	10	10	141	70	100
7							100	14	14	0	10	14	0	0	0	10	10	10	10	10
8								100	100	50	100	36	36	36	36	70	70	36	70	70
9									100	70	100	70	24	17	36	70	70	50	70	70
10										100	100	70	24	17	36	70	70	50	70	70
11											100	200	70	24	70	70	70	100	100	100
12												100	50	24	36	50	50	70	50	50
13													100	24	36	50	50	70	50	50
14														100	24	50	36	50	141	50
15															100	30	36	141	70	100
16																100	70	50	70	24
17																	100	100	100	100
18																		100	50	50
19																			100	50
20																				100

DISCUSSION

Due to the high number of possible combina-

CRI values > 70 also gave the bigger number of CRI < 32. It was estimated that isolates 4 and 11 are antigenically more complex than the others

tested, since they exhibited strong antigenic relationships with sixteen of the twenty isolates studied. On the other hand, isolate 7 did not show antigenic relationships with any. Between both extremes, a wide range of antigenic relationships were estimated.

Some pairs of sera showed CRI values greater than 100. These values are obtained when the titer of a serum against an heterologous antigen exceeds that against its homologous antigen. Considering that the suspensions of antigens were standardized so as to contain equal concentrations of bacteria, it could be interpreted that the reactivity of some isolates with a heterologous serum is greater than that with the homologous serum. This has also been observed with Foot and Mouth disease viruses, where the use of "dominant" strains in vaccine production is recommended (8).

The complexity of the data obtained makes it difficult to propose a serogrouping of *B. bronchiseptica*. It may be concluded, however, that *B. bronchiseptica* recovered from swine with Atrophic Rhinitis are not antigenically homogeneous and that some isolates are antigenically more complex than others. These observations should be taken into account in epidemiological studies and during the selection of dominant strains for vaccine production.

RESUMO

Relações antigênicas de isolados de *Bordetella bronchiseptica* de suínos com rinite atrófica

Estudaram-se as relações antigênicas de vinte amostras de *Bordetella bronchiseptica* recuperadas de suínos com *Rinite Atrófica* mediante um teste de soroaglutinação quantitativo. Os títulos dos soros produzidos em coelhos contra cada um dos isolamentos foram determinados usando antígenos homólogos e heterólogos. Doze soros que apresentaram reatividade cruzada foram absorvidos e seus títulos determinados com antígenos heterólogos. Estimaram-se as relações antigênicas entre os isolamentos calculando os índices de reatividade cruzada. Dois isolamentos apresentaram alta reatividade cruzada com outros 16, entretanto só um não reagiu com o painel utilizado. Concluiu-se que *B. bronchiseptica* recuperada de suínos é antigenicamente heterogênea.

Palavras-chaves: *Bordetella bronchiseptica*, índice de reatividade cruzada, rinite atrófica.

REFERENCES

1. Arrowsmith, A.E.M. A survey of Foot and Mouth Disease Virus type 0 strains from Far East, *Develop. Biol. Standard*, 35:221-230, 1977.
2. Backstrom, L.; Hoelfling, D.C.; Morkot, A.C. & Cowart, R.P. Effect of atrophic rhinits on growth rate in Illinois swine herds. *J. Am. Vet. Med. Ass.*, 187:712-715, 1985.
3. Cristina Afonso, J.L.F.; Pinto, M.L.M.M. & Silva, J. Imunologia da *Bordetella bronchiseptica*. *Rev. Portug. Cienc. Vet.*, Lisboa, 69:169-186, 1974.
4. Eldering, G.; Hornbeck, C. & Baker, J. Serological study of *Bordetella pertussis* and related species. *J. Bacteriol.*, 74:133-136, 1957.
5. Gil-Turnes, C. & Araújo, F.L. Serological characterization of strains of *Moraxella bovis* using double immunodiffusion. *Can J. Comp. Med.*, 46:165-168, 1982.
6. Goodnow, R.A. Biology of *Bordetella bronchiseptica*. *Microbiol. Rev.*, 44:722-738, 1980.
7. Guerreiro, M. G.; Trein, E.; Warth, W. & Antinolfi, T. Rinite atrófica no Rio Grande do Sul. DIPAN, Porto Alegre, 15:20-23, 1963.
8. Moreau, Y.; Stellmann, C.; Brun, A.; Lombard, M. & Moussa, A. Classification des Souches de Virus Aphteux selon un modele parente dominance pour une meilleure comprehension du concept de sous type serologique et immunologique. *Develop. Biol. Standard.*, 35:195-203, 1977.
9. Nakase, Y. Studies of *Hemophilus bronchisepticus*. IV Serological relation of *H. bronchisepticus* from guinea pigs, dog and human. *Kitasato Arch. Exp. Med.*, 30:85-94, 1957.
10. Pedersen, K.B. The serology of *Bordetella bronchiseptica* isolated from pigs compared with srains from other animal species. *Acta Pathol. Microbiol. Scand.*, 83:590-594, 1975.
11. Piffer, I.A.; Ávila, L.A.F. & Brito, J.R.F. Rinite atrófica dos suínos: isolamento e identificação de *Bordetella bronchiseptica*. *Arq. Esc. Vet.* UFMG, Belo Horizonte, 30:291-296, 1978.
12. Pittman, M. Genus Bordetella. In: Buchanan, R.E. & Gibbons, N.E., eds. Bergey's Manual of Determinative Bacteriology, 8th ed., Baltimore, The Williams and Wilkins Co., p. 967, 1974.
13. Switzer, W.P. Infectious atrophic rhinitis. V concept that several agents may cause turbinate atrophy. *Am. J. Vet. Re.*, 17:478-483, 1956.
14. Symposium Internacional sur la Fievre Aphteuse. Symposia Series in immunological standardization, 8:167-168, 1968.

Rev. Microbiol., São Paulo, 25(2): 90-6, 1994

THE USE OF FLUOROCHROMES IN INSECT AND PLANT MYCOPATHOLOGY

B.P. Magalhães

ABSTRACT

Fluorochromes as tools for the detection and identification of insect and plant pathogenic fungi and as successful reagents for studying fungal physiology, viability and infectivity are discussed to illustrate the versatility of these dyes and suggest further uses in pest biocontrol programs.

Key words: Fluorochromes, entomopathogenic fungi, plant pathogenic fungi, fluorescent microscopy, biological control

INTRODUCTION

Fluorochromes in conjunction with fluorescent microscopy have been used for many years to investigate a wide range of cell components in animals, plants and fungi. The use of fluorochromes for the study of fungal cytology and differentiation processes has been recently reviewed (7). The utilization of these dyes for research on insect and plant pathology has paved the way to the understanding of complex interactions between pathogens and their hosts. This review will focus on some practical aspects of the use of fluorochrome in mycopathology, namely: detection, identification and viability studies and investigations on fungal physiology and infection processes. The possible use of fluorochromes on biocontrol programs is also discussed.

DETECTION OF MYCOPATHOGENS

Soil. Detection of fungal propagules in soil is facilitated by the use of cell wall stains. For example, the fate of *Metarhizium anisopliae* applied against soil pests can be monitored by Tinopal. Propagules of other fungi in soil can also be visualized and quantified using Fluorescent Brightener 28 (39) as well as Calcofluor White (27). Acridine Orange and Europium Chelate are effective for differentiating cells of bacteria and actinomycetes from melanized fungal structures in soil (13).

Ascomycetous hyphae associated with liverwort rhizoids and ericoid mycorrhizal roots can be quickly detected when stained with $DiOC_6$ (3,3' - dihexylocarbocyanine iodide) at low concentrations (1-5 ug/ml). On the other hand, Basidiomycetes forming endophytic associations with liverworts and ectomycorrhizas in seed plants can be stained with $DiOC_6$ only at high concentrations of the dye (50 ug/ml) (10). This fluorochrome is normally used to stain the endoplasmic reticulum.

Detection of fungal hyphae and spores in soil and leaves, where background fluorescence and inert material hamper the quality of results obtained with certain stains, is improved by employing Ethidium Bromide (31), a succesful fluorochrome for revealing dormant structures (e.g. chlamydospores). Ethidium Bromide may also be useful for differential staining when associated with other fluorochromes specific for actively growing cells.

CENARGEN/EMBRAPA - Caixa Postal 02372 - 70.849-970 - Brasília, DF

Insect Surface. A rapid visualization and quantification of insect surface structures is also made feasible by the use of cell wall stains such as Uvitex, Tinopal and Calcofluor White M2R (3, 7, 22). It is possible, for example, to test for the presence of conidia in contact with the insect cuticle after field application of the fungus as mycoinsecticide. One of these fluorochromes, Saturn Yellow, has been employed to monitor the targeting of *M. flavoviride* formulated in oil and applied in field assays to control grasshopers (1). In addition, the use of Uvitex provides a means to follow up pathogen development (germination and differentiation) on the insect cuticle (Fig. 1).

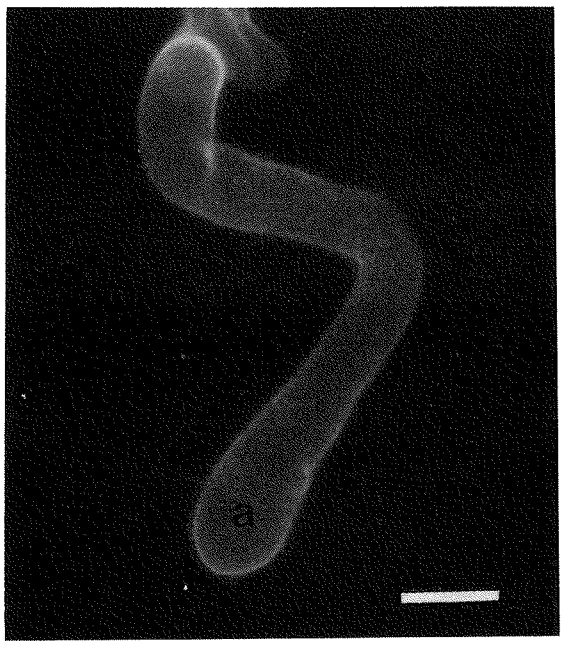


FIGURE 1 - Apressorium (arrow) of *Zoophthora radicans* on the cuticle of *Empoasca fabae* stained with Uvitex (0.1%;w/v). Bar = 30 um.

The microphotographs presented in this review were taken with an Olympus BH-2 microscope coupled to an epifluorescent attachment which comprised a B90-500 excitation filter, a DM455 chromatic beam splitter and a 455 nm barrier filter, using TMAX 400 films. Fig. 6 was taken with a Zeiss Photomicroscope and epifluorescence attachment supplied with a 460 nm chromatic beam splitter together with 390-400 nm excitation and 475 nm barrier filters. A combination of light-field and epifluorescence optics was also used.

Plant surface. The detection of halos in the epidermal cell wall of *Allium cepa* caused by *Colletotrichum dematium* and *Botrytis allii* is done with Acridine Orange and Berberin Sulphate (19). Additionally, spores of both pathogens fluoresce with Brilliant Sulphaflavive, Acridine Orange, Ninhydrin and Dansylchloride. All these stains cause fluorescence of germ tubes, apressoria and primary infection mycelia.

Cells of *Hemileia vastatrix* previously treated with ascorbic acid are detected after staining with Diethanol (38). Rust haustoria of *Puccinia graminis* in wheat leaves are also visualized when stained with Diethanol, Calcofluor and Ethidim Bromide (21). The detection of all infection structures of the yellow rust fungus *P. striiformis* in leaves of contaminated barley plants is made by the use of several optical brighteners, including Wobital BBK (34).

Discrimination between *Pyrenophora tritici-repentis* (wheat tan spot) and its antagonist *Lymonomyces reseipellis* is possible using a double staining technique. *P. tritici-reoentis* is specifically stained by indirect immunofluorescence with fluorescein isothiocyanate (FITC); both fungi are then treated with a nonspecific lectin-conjugate (wheat-germ agglutinin - TRITC); lastly, a counterstain (Toluidine Blue-O) is used to allow visualization of *L. roseipellis* growing in close association with and penetrating hyphae of *P. tritici-repentis* (25).

Specific molecules during development of mycopathogens. Visualization of nuclei by light microscopy is more difficult to achieve with several species of fungi than with other organisms (15). However, there are some dyes such as DAPI and Mithramicin which are highly specific for nucleic acids. DAPI has been used to quantify DNA through image video-analysis (8, 36, 23). Other stains such as Acridine Orange, Propidium Iodide and Ethidium Bromide are used as DNA stains, but they are not considered as efficient as DAPI. Hydroethidine, derived from Ethidium Bromide, has a large spectrum but fades away very quickly compared to DAPI. Ethidium Bromide is higly toxic and nonspecific since it stains other cell components such as vacuoles and lipids (2).

The relative DNA content in nuclei of races of *Phytophthora megasperma* can be estimated by quantitative fluorescence microscopy (cytofluorometry) using DAPI (32). This fluorochrome has also been used to quantify inheritance of DNA contents in sexual progenies of *Phytophthora infestans* (42). Additionally, DAPI staining

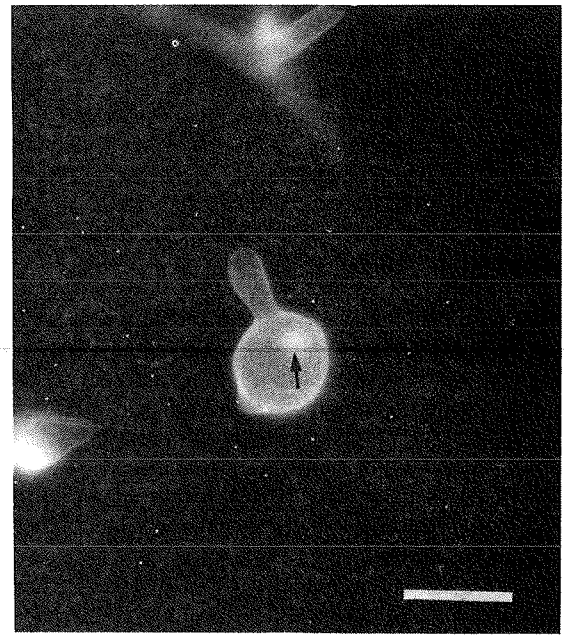


FIGURE 2 - Apressorium (arrow) of *Zoophthora radicans* on the cuticle of *Empoasca fabae* stained with Uvitex (0.1%;w/v). Bar = 30 um.

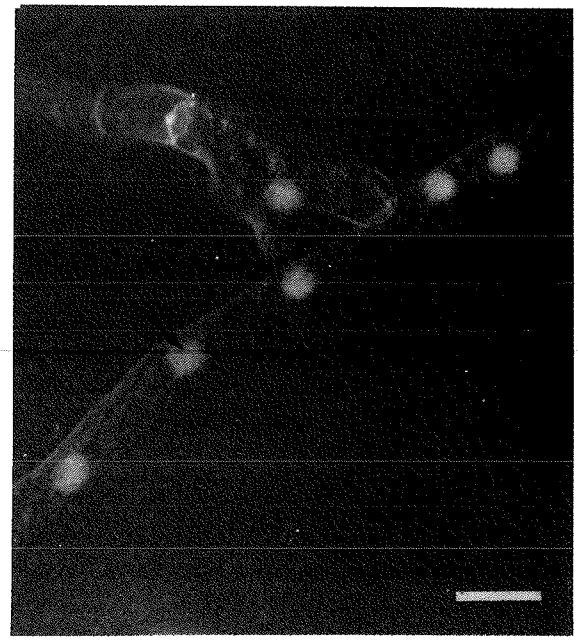


FIGURE 3 - Hyphae of *Zoosphthora radicans* treated with DAPI (1 ug/ml) and observed on a phase-contrast microscope, showing the nucleus (arrow). Bar = 16 um.

has brought about the succesful measurement of DNA contents in zoospores of *P. infestans* (43) and conidia of *Zoophthora radicans* (23) as well as the study of the duplication cycle in nuclei of germinating zoospores from *P. dreschsleri* (17). Finally, it is possible to visualize the nucleus and cell wall in *Z. radicans* by simultaneous utilization of DAPI and Uvitex (Fig. 2), and to detect nuclei and other cell structures by combination of DAPI with differential interference contrast (DIC) (Fig.3).

Immunofluorescence microscopy is another technique also used to study insect fungal mitosis. The mitotic process can be monitored in *Neozygites* sp. when cells of the pathogen are treated with FITC before observation under the fluorescece microscope (6).

Actin filaments are important cytoskeletal elements which participate in mitosis, cytokinesis, cell wall synthesis, cell elongation and formation of apressoria and penetration hyphae. These microfilaments can be indirectly visualized in *Uromyces phaseoli* (16), *Neozygites* sp. (4) and *Z. radicans* (Fig. 4) with the aid of rhodamine-conjugated phalloidin specific for microfilaments.

The importance of the Ca^{2+}/calmodulin system on germination and differentiation of entomopathogenic fungi has been documented (23, 33).

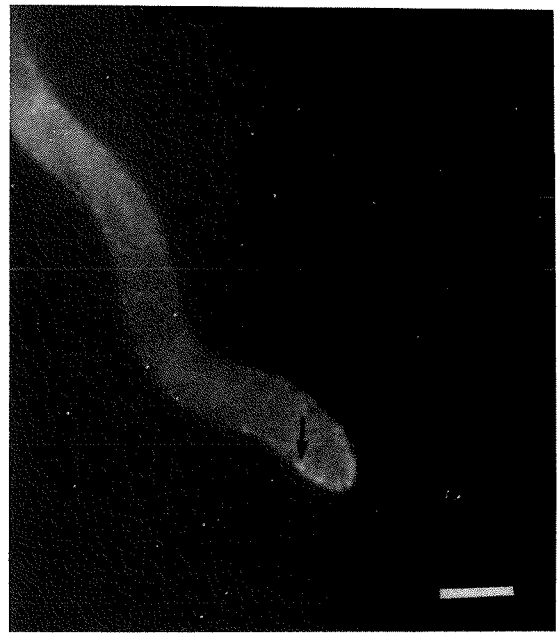


FIGURE 4 - Hyphae of *Zoophthora radicans* treated with rhodamine-conjugated phalloidium (0.5 ug/ml) showing plaques of actin (arrow). Bar = 8 um).

Localization of Ca^{+2} is easily done with the use of CTC (chlorotetracycline = aureomycin) (Fig. 5); this fluorochrome has been used with *Z. radicans*

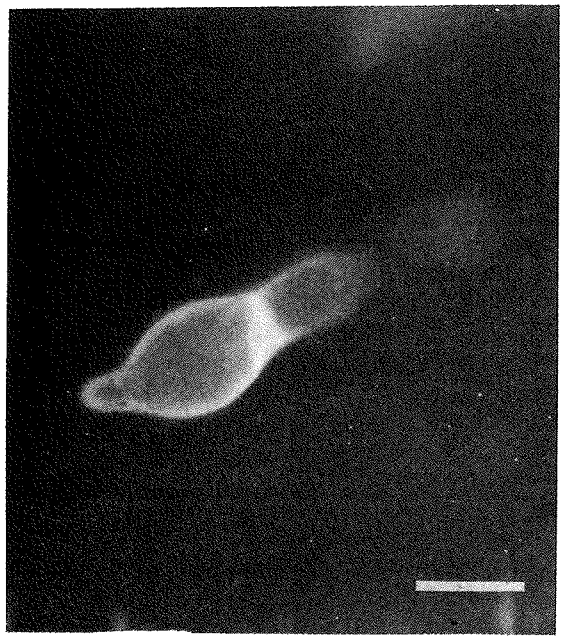


FIGURE 5 - Fluorescence micrograph of an apressorium of *Zoosphthora radicans* (a) treated with CTC (10 uM). Bar = 20 um.

and *M. anisopliae* (33). The calcium-binding protein calmodulin can be visualized by staining the cells with W-7 (N-(6-aninohexyl)-5-chloro-1-naphthalene-sulfonamide).

IDENTIFICATION

Identification of insect and plant pathogenic fungi is another field of biological research that can be facilitated by the use of fluorochromes. For example, it is known that septation can be useful for identifying fungal pathogens: it is complete in Zygomycetes and incomplete in *Basiodiomycetes*. The visualization of septae with the aid of a transmission microscope is, however, not an easy task. This problem is solved using cell wall stains such as Uvitex which, used in combination with an epifluorescence microscope, allows a fast and easy visualization of septae in growing mycelia.

The mitotic pattern of entomophthoralean fungi can also be important for their identification; examples are the central metaphase spindle characteristic of *Neozygites*, and the eccentric metaphase spindle exhibited by *Erynia* (6). Nuclear cytology is yet another significant differential feature: large nuclei are typical of Zygomycetes while small nuclei are typcal of Hyphomycetes.

The number of chromosomes in *Neurospora* can be reliably determined during condensed division stages in ascus using the DNA-specific fluorochrome Acriflavine (28). The fluorochrome Mithramycin has been used to detect nuclei of monokariotic and dikariotic hyphae of the dwarf bunt fungus *Tilletia controversa*, both in wheat plants and in culture (40).

INFECTION PROCESS

Studies on the infection process of insect and plant pathogenic fungi are very important for the establishment of biological control strategies. This type of research has been made easy by the use of fluorochromes. For example, the route of infection of *Z. radicans* (Fig. 1) (44) and *M. anisopliae* (33) can be followed up with the aid of Uvitex, a fluorescent stain that also facilitates studies on development of infection structures *in vitro*.

Formation of apressoria is considered a prerequisite for infection by many parasitic fungi (11) and has been reported to be preceded by DNA synthesis and nuclear division in *M. anisopliae* (33) and in teliomycete and hyphomycete plant pathogens (35). With the combined use of DAPI, video-image analysis and inhibitory drugs, it is

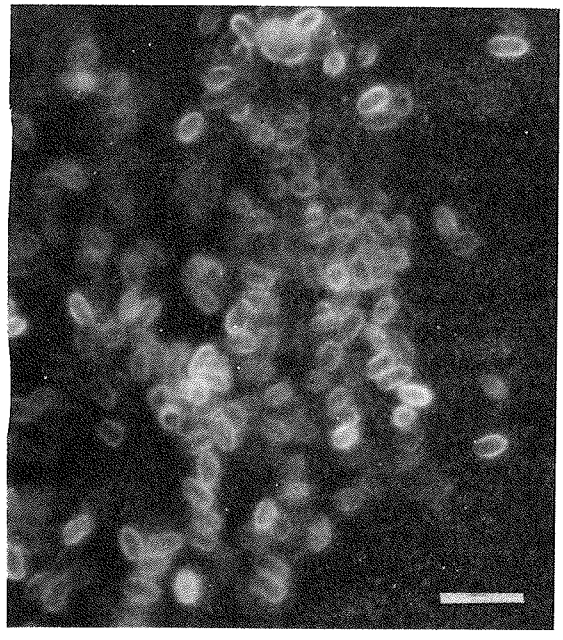


FIGURE 6 - Conidia of *Metarhizium flavoviride* inside the body cavity of *Rhammatocerus schistocercoides* - third instar nymph stained with Uvitex. Bar = 12 um.

possible to demonstrate that Z. *radicans* conidium germination, apressorium formation and secondary sporulation occur independently of DNA replication and nuclear division (23).

The infection process of the plant pathogenic fungus *C. lagenarium* on cucumber leaves (with induced resistance) can be monitored with the fluorochrome Acridine-blue (20).

The development of mycopathogens after host penetration is better undestood with the use of Uvitex. For example, *Erynia neoaphidis* forms protoplasts within its host while Z. *radicans* , another entomophthoralean, does not (Dr. R. A. Humber, USDA/ARS, Ithaca, NY, USA, personal communication), and Uvitex has been used to visualize *E. neoaphidis* protoplasts. Another example is the detection of conidia of *M. flavoviride* inside the body cavity of *Rhammatocerus schistocercoides* (Fig. 6).

FUNGAL PHYSIOLOGY

Fluorochromes have also contributed to a better understanding of fungal physiology. Calcofluor White and Congo Red, for example, can be used to study cell wall morphogenesis in *Geotrichum lactis* (29). Calcofluor White causes cell lysis at hyphal tips, but this can be prevented by addition of an osmotic stabilizer. The rate of chitin synthesis in protoplasts and growing cells is enhanced by addition of Calcofluor White and Congo Red. In contrast, both stains hamper chitin and beta-glucan synthases in cell-free systems and interfere with the growth of *Saccharomyces cerevisiae* through the formation of multicellular aggregates (29). Calcofluor White also affects the synthesis of chitin synthase in *S. cerevisiae.*

Lipids are imortant energy stores for many fungi. Synthesis and breakdown of these reservoirs reflect the physiological status of the fungus. For example, large lipid globules suggest that the fungus is not active. Conversely, globule breakdown into smaller units indicates an active methabolic state.

Cells with large lipid reserves are probably survival stages or propagules. For example, the entomopathogenic fungus *Erynia neophidis* survives as conidium and contains a large central lipid globule. Resting spores also present extensive deposits of lipid. Inactive mycelia of *Hyphomycetes* may also contain large lipid deposits.

Using Congo Red (Eastman Kodak), lipid globules can be detected in several fungal structures such as hyphae, protoplasts, immature resting spores and conidia. This stain is hydrophobic and fluoresces strongly when dissolved in lipid (14). Nile Red is stable and compatible with Primulin (a cell wall stain), and it does not affect fungal growth when incorporated into culture media (1 - 100ul). Luminor 490 PT is another fluorochrome used to detect and measure lipid contents in fungal mycelia (26).

Since mitochondria are structures which generate ATP and are involved in fatty acid metabolism, their spatial distribution may reveal sites of metabolic activity. This occurs during synthesis and secretion of several compounds. Mitochondria are also sites for calcium storage and release. and studies on calcium physiology are facilitated by the use of specific stains such as CTC.

The endoplasmic reticulum of *M. anisopliae* is involved in protein synthesis and glycosylation before their secretion to the exterior of the cell. This organelle may also participate in cell wall synthesis; this becomes evident in *E. neoaphidis* where electron bodies are present in walled cells but not in protoplasts. Studies on fungal cell physiology and detection of mitochondria and endoplastic reticulum can be done with the aid of specific fluorochromes such as Rhodamine 123 and $DiOC_6$ (18, 37).

The vacuoles of fungi are also vital structures in fungal cell physiology. They have a key role as calcium reservoirs and are also essential for the detoxification and displacement of cytoplasm during growth. Specific stains have been utilized in research studies on vacuole formation and function during differentiation and cell growth (41).

CELL VIABILITY

Viability of mycopathogens is traditionally assessed by plating propagules and estimating the number of cells undergoing development (germlings or colony forming units). Fluorescence microscopy can be used as an alternative method to this conventional technique. Esterases are indicators of cell viability and can be detected by using the fluorochrome Fluorescein Diacetate (FDA). When FDA is combined with Propidium Iodide (a stain specific for dead cells), it is possible to distinguish between live (green) and dead (red) cells. This technique has been employed to determine conidial viability of *M. anisopliae* (9) and several species of entomophthoralean fungi (11) and also viability of protoplasts produced by other entomopathogenic fungi.

CONCLUDING REMARKS

The success of mycopesticides on biological control programs depends greatly on a good method of application and monitoring of pathogen development in soil or on insect and plant surfaces. *Is is* always necessary to determine the optimum size of particles containing fungal propagules as a function of fungal virulence and stability under field conditions. It is also necessary to understand the spatial and temporal distribution of fungal propagules (mycelia, protoplasts, or primary and secondary conidia) introduced into the field. Fluorochromes have facilitated these tasks through their use to calibrate particle size and as markers for pathogens or for carriers employed in the formulation of the final product.

The utilization of fluorochromes in insect and plant mycopathology can be optimized by the right choice of the microscope, set of filters and dyes to be employed. The possibility of combining two or more fluorochromes for simultaneous observation is a very attractive feature. The practical use of fluorochromes on field evaluation of fungal propagules will certainly be expanded and highlight the very important role of mycopathogens as biocontrolling agents of insect pests in integrated pest management systems.

ACKNOWLEDGEMENTS

The author is grateful to Dr. Tariq M. Butt (Rothamsted Experimental Station, England, for his encouragement at the very beginning of this project, and to Dr. Eliana M.G. Fontes (CENARGEM/ EMBRAPA) and Boel Sandskaer (Upsalla University, Sweeden) for reviewing the manuscript.

RESUMO

Uso de fluorocromos no estudo da micopatologia de insetos e plantas

O uso de fluorocromos na detecção, identificação, processo de infecção, fisiologia e viabilidade de fungos entomopatogênicos e fitopatogênicos é discutido visando uma indicação de como esses corantes poderiam ser explorados em futuros programas de controle biológico.

Palavras-chave: Fluorocromos, microscopia de fluorescência, fungos entomopatogênicos, fungos fitopatogênicos, controle biológico.

REFERENCES

1. Bateman, R.P.; Godonou, I.; Kpindu, D.; Lomer, C.J. & Paraiso, A. Development of a novel field bioassay technique for assessing mycoinsecticide ULV formulations. In: *Biologica Control of Locusts and Grasshoppers*. Lomer C.J. & Prior, C. eds. CAB International/International Institute of Tropical Agriculture. Redwood Press Ltd, Melksham, UK. pp. 255-262, 1992.
2. Bucana, C.; Saiki, I. & Nayar, R. - Uptake and accumulation of the vitaldye hydroethidine in neoplastic cells. *J. Histochem. Cytochem.*, **34**: 1109-1115, 1986.
3. Butt, T.M. - A fluorescence microscopic method for rapid localization of fungal spores and penetration sites on insect cuticle. *J. Invertebr. Pathol.* **50**: 72-74, 1987.
4. Butt, T.M. & Heath, I.B. - The changing distribution of actin and nuclear behavior during the cell cycle of the mitepathogenic fungus *Neozygites sp. European Journal of Cell Biology*, **46**: 499-505, 1988.
5. Butt, T.M.; Wraight, S.; Galaini-Wraight, S.; Humber R.A.; Roberts, D.W. & Soper, R.S. - Humoral encapsulation of the fungus *Erynia radicans* (Entomophthorales) by the potato leafhopper *Empoasca fabae* (Hemiptera). *J. Invertebr. Pathol.*, **52**: 49-56, 1988.
6. Butt, T.M. & Humber, R.A. - An immunofluorescence study of mitosis in a mite-pathogen, *Neozygites* spp. (Zygomycotina: Entomphthorales). *Protoplasma*, **151**: 115-123, 1989.
7. Butt, T.M.; St Leger, R.J.; Hoch, H.C. & Staples, R.C. - The use of fluorochromes in the study of fungal cytology and differentiation. *Experimental Mycology*, **13**: 303-320, 1989.
8. Coleman, H.A.; Maguire, M.J. & Coleman, J.R. - Mithramicin and 4'-5-diamidina-2-phenyllindole (DAPI)-DNA staining for fluorescence microspectrophotometric of DNA. *Stain Technol.*, **60**: 145-154, 1981.
9. Corrêa, B.; Gambale, W.; Paula, C.R.; Reche, N.O.C. & Purchio, A. - Aplicação do método fluorescente (solução de fluoresceína e brometo de etídio) no estudo da viabilidade de *Metarhizium anisopliae*. *Rev. Microbiol.*, 20: 341-344, 1989.
10. Duckett, J.G. & Read, D.J. - The use of the fluorescent dye, 3,3' - dihexyloxacarbocyanine iodide, for selective staining of ascomycete fungi associated with liverwort rhizoids and ericoid mycorrhizal. *New Phytopathologist*, **118**: 259-272, 1991.
11. Emmet, R.W. & Parbery, D.G. - Appressoria. *Annu. Rev. Phytopathol.*, **13**: 147-167, 1975.
12. Firstencel, H.; Butt, T.M. & Carruthers, R.I. - A fluorescence microscopy method for determining viability of entomophthoralean fungal spores. *J. Invertebr. Pathol.*, **55**: 258-264, 1990.
13. Fradkin, A. & Patrick, Z.A. - Fluorescence microscopy to study colonization of conidia and hyphae of Cochliobolus sativus by soil microorganisms. *Soil, Biology and Biochemistry*, **14**: 543-549, 1982.
14. Greenspan, P.; Mayer, E.P. & Fowler, S.D. - Nile red: A selective fluorescent stain for intracellular lipid droplets. *J. Cell Biol.*, **100**: 965-973, 1985.
15. Heath, I.B. - Fluorescent staining of fungal nuclei. In: *Zoosporic Fungi in Teaching and Research*. Fuller, MS & A Jaworski eds. Southeastern Publishing Co., Athens, GA. pp. 169-171, 1987.
16. Hoch, H.C. & Staples, R.C. - The microtubule cytoskeleton in hyphae of *Uromyces phaseoli* germlings: its relationship to the region of nucleation and to the F-actin cytoskeleton. *Protoplasma*, **124**: 112-122, 1985.

17. Hooley, P.; Fyfe, A.M.; Maltese, C.E. & Shaw, D.S. - Duplication cycle in nuclei of germinating zoospores of *Phytophthora dreschsleri* as revealed by DAPI staining. *Trans. Br. Mycol. Soc.*, **79**: 563-566, 1982.
18. Johnson, G.D. & Nogueira-Araújo, G.M.C. A simple method of reducing the fading of immunofluorescence during microscopy. *J. Immunol. Met.*, **43**: 349-350, 1981.
19. Karabetsos, J.H.; Pappelis, A.J. & Russo, V.M. Visualization of halos in the epidermal cell wall of *Allium cepa* caused by *Colletotrichum dematium f. circinans* and *Botrytis allii* using fluorochromes. *Mycopathologia*, **97**: 137-141, 1987.
20. Koyates, K.; Binder, A. & Hohl, H.R. - Cytology of induced resistance of cucumber to *Colletotrichum lagenarium*. *Mycopathologia*, **183**: 484-490, 1991.
21. Kuck, K.; Tiburzy, R.; Hanssler, G. & Reisener, H. - Visualization of rust haustoria in wheat leaves by using fluorochromes. *Physiol. Plant. Pathol.*, **19**: 439-441, 1981.
22. Magalhães, B.P.; Butt, T.M.; Humber, R.A.; Shields, E.J. & Roberts, D.W. - Formation of appressoria *in vitro* by the entomopathogenic fungus *Zoopthora radicans* (Zygomycetes: Entomophthorales). *J. Invertebr. Pathol.*, **55**: 284-288, 1990.
23. Magalhães, B.P.; St. Leger, R.J.; Humber, R.A.; Allee, L.; Shields, E.J. & Roberts, D.R. - Nuclear events during germination and appressorium formation of the entomopathogenic fungus *Zoophthora radicans* (Zygomycetes: Entomophthorales). *J. Invertebr. Pathol.*, **57**: 43-49, 1991a.
24. Magalhães, B.P.; Wayne, R.; Humber, R.A.; Shields, E.J. & Roberts, D.W. - Calcium-regulated appressorium formation of the entomopathogenic fungus *Zoophthora radicans* Protoplasma, **160**: 77-88, 1991b.
25. Pfender, W.F.; King, L.G. & Rabe, J.R. - Use of dual-stain fluorescence microscopy to observe antagonism of *Pyrenophora tritici-repentis* by *Limonomyces reseipellis* in wheat straw. *Phytopathology*, **81**: 109-112, 1991.
26. Pomoshnikova, N.A.; Korotkov, S.A. & Galanina, L.A. - The application of the fluorecent analysis for studying the synthesis of lipids by mycelial fungi. *Mikrobiologiya*, **52**: 698-701, 1983.
27. Postma, J. & Altemuller, H.J. - Bacteria in thin soil sections stained with the fluorescent brightner calcofluor white M2R. *Soil, Biology and Biochemistry*, **22**: 89-96, 1990.
28. Raju, N.B. - A simple fluorescent staining method for meiotic chromosomes of *Neurospora*. *Mycologia*, **78**: 901-906, 1986.
29. Roncero, C. & Duran, A. - Effect of Calcofluor White and Congo Red on fungal cell wall morphogenesis: In vivo activation of chitin polymerization. *J. Bacteriol.*, **163**: 1180-1185, 1985.
30. Roncero, C.; Valdivieso, M.H.; Ribas, J.C. & Duran, A. - Effect of Calcofluor White on chitin synthases from *Sacharomyces cerevisae*. *J. Bacteriol.*, **170**: 1945-1949, 1988.
31. Roser, D.J.; Keane, P.J. & Pittaway, P.A. - Fluorescent staining on fungi from soil and plant tissues with ethidium bromide. *Trans. Br. Mycol. Soc.*, **79**: 321-329, 1982.
32. Rutherford, F.S. & Ward, E.W.B. - Estimation of relative DNA content in nuclei of races of *Phytophthora megasperma f. sp. glycinea* by quantitative fluorescence microscopy. *Canadian Journal of Genetics and Cytology*, **27**: 614-616, 1985.
33. St. Leger, R.J.; Roberts, D.W. & Staples, R.C. - Calcium and calmodulin-mediated protein synthesis and protein phosphorylation during germination, growth and protease production by *Metarhizium anisopliae*. *J. Gen. Microbiol.*, **135**: 2141-2154, 1989.
34. Schlegel, H.; Noll, B. & Opel, B. - Zentralbl. Bakteriol., Parasitenkd., Infektionskr. *Hyg.* **137**: 36-41. 1982.
35. Staples, R.C.; Laccetti, L. & Yaniv, Z. - Appressorium formation and nuclear division in *Colletotrichum truncatum*. *Arch. Microbiol.*, **109**: 75-84, 1976.
36. Staples, R.C.; Gross, D.; Tiburzy, R.; Hoch, H.C.; Hassouna, S. & Webb, W.W. - Changes in DNA content of nuclei in rust uredospore germlings during the start of differentiation. *Exp. Mycol.*, **8**: 245-255, 1984.
37. Teraski, M.; Song, J.; Wong, J.R.; Weiss, M. & Chen, L.B. - Localization of endoplasmic reticulum in living and glutaraldehyde-fixed with fluorescent dyes. *Cell*, **38**: 101-108, 1984.
38. Tiburzy, R.; Martins, E.M.F. & Moraes, W.B.C. - Visualization of *Hemileia vastatrix* structures in coffee leaves by fluorescence microscopy. *Fitopatologia Brasileira*, **8**: 461-466, 1983.
39. Tippkotter, R. - Staining of soil microorganisms and related materials with fluorochromes. Developments in Soil Science, 19: 605-611. In: *Soil micromorphology: a basic and applied science* (# 19), 1988.
40. Trione, E.H.; Hess. W.M. & Stockwell, V.O. - Growth and sporulation of the dwarf bunt fungus in wheat plants and in culture. *Canadian Journal of Botany*, **67**: 1671-1680, 1989.
41. Weisman, L.S.; Bacallao, R. & Wicker, W. - Multiple methods of visualizing the yeast vacuole permit evaluation of its morphology and inheritance during cell cycle. *J. Cell Biol.*, **105**: 1539-1547, 1987.
42. Whittaker, S.L.; Shattock, R.C. & Shaw, D.S. - Inheritance of DNA contents in sexual progenies of *Phytophthora infestans*. *Mycol. Res.* **95**: 1094-1100, 1991a.
43. Whittaker, S.L.; Shattock, R.C. & Shaw, D.S. - Variation in DNA content of nuclei of *Phytophthora infestans* as measured by a microfluorimetric method using the fluorochrome DAPI. *Mycol. Res.* **95**: 602-610, 1991b.
44. Wraight, S.P.; Butt, T.M.; Galaini-Wraight, S.; Allee, L.; Soper, R.S. & Roberts, D.W. - Investigation of the germination and infection process of *Erynia radicans on Empoasca fabae*. *J. of Invertebr. Pathol.*, **56**: 157-174, 1990.

Rev. Microbiol., São Paulo, 25(2): 97-100, 1994

POST HARVEST CONTROL OF AFLATOXIN PRODUCTION ON IN-SHELL MOIST PEANUTS BY SODIUM ORTHO-PHENYLPHENATE. II. HARVESTER TESTS

H. Fonseca*
M.A. Calori-Domingues*
P.J. Approbatto*
I.V.Zambello*
E.L. Fonseca*

SUMMARY

This work was carried out to optimize a spraying method for the application of sodium ortho-phenylphenate (SOP) solution on in-shell moist peanuts during crop gathering with a combine harvester, aiming at control of aflatoxin production. In a previous work, it was verified that the SOP spraying operation under field conditions did not attain total pod coverage, indicating the need for its optimization.

In the present work, SOP spraying was carried out during mechanical harvesting of the 1988 rainy season peanut crop. The spraying attachment was adapted to a combine harvester and a 0.5% SOP solution used. Despite the improvements made for SOP application, technical problems were still encoutered, such as the accumulation of peanuts inside the bag filler pipe which hampered spraying efficiency. Both treated and control lots showed an increase in initial aflatoxin levels during storage. High aflatoxin contamination of treated samples may have occurred due to the still technically inadequate coupling of the spraying attachment to the harvester and/or to a suboptimal SOP concentration used for the chemical control of aflatoxin.

Key words: aflatoxin, peanut, chemical control, postharvest, fungi, sodium orthophenylphenate.

INTRODUCTION

Aflatoxins were the first fungal products identified as toxic agents for man and animals. These mycotoxins are produced by *Aspergillus flavus* Link and *A. parasiticus* Speare, which are found worldwide in soil and the atmosphere and may contaminate peanuts, corn, cottonseed and other crops (4).

The presence of aflatoxin on Brazilian peanuts has contributed to limit the country's exports of peanuts and peanut meal. Aflatoxin production results not only in economic loss for agricultural producers but also in serious health hazards to consumers exposed to contaminated goods.

Prevention of contamination may be accomplished by using adequate techniques and procedures from harvest to storage of the agricultural produce. However, adverse weather conditions may difficult considerably preventive strategies, and the use of chemical agents on crops during such periods may be a good alternative for the control of aflatoxigenic fungi.

* Depto. de Ciência e Tecnologia Agroindustrial — Escola superior de Agricultura " Luiz de Queiroz" da Universidade de São Paulo. Av. Pádua Dias, 11 — 13418-900 — Piracicaba — SP.

Field application of chemicals has produced good results in the control of aflatoxin contamination (2, 3, 8). The effect of eighteen fungicides on thirteen species of fungi obtained from peanut samples was evaluated *in vitro*; some of the chemicals were found to be efficient at restrainig growth of many of the isolated microorganisms, including *A. flavus* (7).

Sodium ortho-phenylphenate (SOP), together with other fungicides, was tested *in vitro* for anti-fungal activity by Fonseca *et al* (5) and was shown to totally inhibit the growth of *A. flavus*. However, when testing SOP efficiency during field trials, Fonseca *et al* (6) did not obtain the same successful results; total coverage of pods by SOP during application under field conditions was not attained leading to inefficient control of the aflatoxigenic mould and thus of aflatoxin production.

Therefore, to improve pod coverage with the SOP solution, a spraying attachment was adapted to a combine harvester and the chemical applied to pods inside the bag filler system of the machine. The purpose of this work was to test the efficacy of these technical modifications on optimization of fungicide application, which may then provide an alternative method for control of peanut aflatoxin contamination whenever unfavourable weather conditions do not allow a rapid and efficient drying of produce before storage.

MATERIALS AND METHODS

This experiment was conducted in the peanut producing area of Marilia region, São Paulo, Brazil, during harvesting of the rainy season peanut crop of 1988.

Four lots of 120 bags of in-shell non-dried peanuts (14-18% moisture content) were used. Two lots were employed for spray application of SOP solution (5%) and the remaining two taken as controls. Lots were stored for two month before final evaluation.

Crop spraying was carried out with a combine harvester which had a spray attachment adapted at the bag filler point. Bagged peanut was subsequently moved to a grain and seed station and submitted to a pre-cleaning operation in a ventilation machine; stacks of 3X4X10 bags high were then piled up. Fifteen samples from each lot were drawn to determine moisture content and aflatoxin contamination and thus establish initial produce conditions. Ten external and ten internal 2 Kg samples were taken from the stacks after one month and two month of storage, respectively, to evaluate final levels of aflatoxin and moisture contents.

Analytical Methodology

Moisture content: Field determinations were carried out using a portable equipment of the resistant type (ELOTEST). Laboratory determinations were done according to the oven method (1).

Aflatoxin contents: A combined methodology based on Pons Jr. *et al* (9) and Velasco & Morris (12) was used. The modifications were: a) the peanut kernel: water ratio in slurry was 1: 1.5; a total of 50g of the slurry was transferred to a 250 ml Erlenmeyer flask and 100 ml acetone added for extraction; b) the clean-up procedure was done with lead acetate solution without boiling (10); the amount of chloroform for partitioning was 2 X 25 ml (11).

RESULTS AND DISCUSSION

The results on peanut moisture and aflatoxin content of treated and control lots are summarized in Tables 1 and 2.

TABLE 1 - Initial aflatoxin (μg/kg of B_1 + G_1) and moisture contents in peanut samples of treated and control lots.

Sample	SOP	SOP(r)	Control	Control (r)
1	n.d.	9	n.d.	37
2	61	n.d.	37	9
3	n.d.	73	74	73
4	n.d.	37	161	73
5	9	37	37	9
6	n.d.	37	n.d.	9
7	n.d.	n.d.	32	16
8	n.d.	73	n.d.	9
9	n.d.	n.d.	322	9
10	n.d.	18	37	37
11	9	184	n.d.	184
12	n.d.	92	n.d.	184
13	n.d.	37	n.d.	9
14	9	37	n.d.	9
15	n.d.	37	9	37
Mean*	22	56	89	47
Moisture content (%)	13.4	17.5	13.3	15.0

SOP = sodium orthophenylhenate
(r) = repetition
* = mean of contaminated samples
n.d. = not detected

TABLE 2 - Aflatoxin (μg/kg of B_1 + H_1) and moisture contents in treated and control peanut lots, after one and two months of storage.

Sample	After one month storage				After two month storage			
	SOP	SOP(r)	Control	Control (r)	SOP	SOP(r)	Control	Control (r)
1	1104	1860	237	149	74	930	71	51
2	319	379	239	744	322	930	1999	639
3	75	9	12	372	9	368	253	88
4	88	538	2209	162	232	814	74	61
5	990	1860	505	1767	2556	184	37	37
6	242	186	12	465	2208	92	1627	88
7	9	372	3952	148	149	232	31	88
8	511	387	319	74	149	465	322	74
9	970	379	256	828	767	465	65	80
10	464	149	406	9	534	930	80	644
Mean*	477	612	815	472	700	541	456	185
Moisture content (%)	10.0	9.5	9.3	9.6	8.9	9.7	9.5	8.8

SOP = sodium orthophenylhenate
(r) = repetition
* = mean of contaminated samples

Initial moisture contents were above 11% and could thus enable fungal growths and aflatoxin production to take place.

The data show that, starting at initial aflatoxin levels of 22 and 56 mg/Kg for the two treated lots (which were slightly lower than those observed for control lots, namely: 89 and 47 mg/Kg), mean values for sprayed samples after one and two months of storage (477 and 612 mg/Kg; 827 and 208 mg/Kg, respectively) were high and did not present a significant reduction compared to control values recorded after the same storage periods (815 and 472 mg/Kg; 456 and 185 mg/Kg, respectively).

Crop spraying with the SOP did not succeed in controlling aflatoxin production under the conditions used. The persistent contamination of treated lots may be still due to technical deficiencies of the spray system, despite improvements achieved with respect to previous trials (6). The spraying attachment was adapted to the two bag filler pipe of a model of combine harvester typically used in Brazil: once a bag is filled, pod flow has to be interrupted to replace it with an empty one. Thus, homogeneous application of the fungicide becomes difficult because, when the pipe door is opened again to fill the next bag, an initially fast flow occurs that makes it impossible to spray all pods adequately. A suboptimal concentration of the SOP solution may also have contributed to the unsuccesful outcome of the experiment.

The authors intend to continue the present investigation testing higher concentrations of SOD and the effect of transferring the site for produce spraying to the pre-cleaning machine in the warehouse, where it is believed that continuous pod flow will allow for a homogeneous distribution of the fungicide.

RESUMO

Controle da produção de aflatoxinas no amendoim em casca úmido com ortofenilfenato de sódio, no pós-colheita. II. Testes na colhedora

O objetivo deste trabalho foi o de otimizar o sistema de pulverização, em campo, da solução de ortofenilfenato de sódio (OFS) sobre amendoim em casca, para verificar a eficiência desta substância no controle da produção de aflatoxinas. Em trabalho realizado anteriormente por Fonseca et al. (6) verificou-se que a pulverização sob condições de campo foi deficiente uma vez que a cobertura completa da vagem, com a solução de OFS não foi conseguida, indicando assim a necessidade de otimização desta operação.

Deste modo, na safra das águas de 1988, a pulverização foi realizada na própria colhedora mecânica onde o sistema de pulverização foi adaptado. A concentração da solução de OFS utilizada foi de 0,5%.

Nesta safra, a despeito da melhor cobertura das vagens com a solução ocorreu o acúmulo de vagens na bica de saída da colhedora, por ocasião da troca da sacaria já cheia pela vazia, o que prejudicou a pulverização, dificultando a cobertura de todas as vagens com a solução. Observou-se que o teor inicial de aflatoxinas aumentou durante o período de armazenamento, tanto nos lotes tratados como nos lotes controle. A alta contaminação com aflatoxinas pode ter ocorrido por não se ter obtido ainda uma pulverização perfeita de todas as vagens e/ou devido à concentração insuficiente da solução de OFS para o controle da produção da toxina.

Palavras-chave: aflatoxinas, amendoim, controle, ortofenilfenato de sódio, pós-colheita, fungo.

ACKNOWLEDGMENTS

The authors wish to thank the "Empresa Brasileira de Pesquisa Agropecuária" for partially financing the experiment, the "Máquinas Agricolas Jacto S.A." company for the mechanical adaptation of the spraying system and technical support, the unvaluable cooperation of directors and staff of "Cerealistas IHARA Ltda" which included the supply of peanut samples, and the Grain and Seed Station of the city of Marilia from the Department of Agriculture of the State of São Paulo, without which the present research work would not have taken place.

REFERENCES

1. BRASIL. Ministério da Agricultura. *Regras para análise de sementes*. Depto. Nacional de Produção Vegetal, DISEN. 188p. 1976.
2. BELL, D.K. & DOUPNIK JUNIOR, B. Chemical treatment of peanuts in windrow to control *Aspergillus flavus* and aflatoxins 1. *Journal American Peanut Research and Education Association*, 3(1):31-32, 1971.
3. BELL, D.K. & DOUPNIK JUNIOR, B. Chemicals in the windrow for controlling aflatoxins in peanuts. *Journal of American Peanut Research and Education Association*, 4:18-20, Aug. 1972.
4. DIENNER, U.L.; COLE, R.J.; SANDERS, T.H.; PAYNE ,G.A.; LEE, L.S.; KLICH, M.A. Epidemiology of aflatoxin formation by *Aspergillus flavus*. *Annual Review of Phytopathology*, Palo Alto, 25: 249-270, 1987.
5. FONSECA, H.; SAVY, A.; SOAVE, J.; CANECCHIO, V. Estudo do controle químico do *Aspergillus flavus* e produção de aflatoxina no amendoim *(Arachis hypogaea)* no campo. *Anais da ESALQ*, Piracicaba, 33:337-347, 1976.
6. FONSECA, H.; NOGUEIRA, J.N.; CALORI-DOMINGUES, M.A.; ZAMBELLO, I.V.; FONSECA, E.L. Post-harvest chemical treatment of in-shell moist peanuts with sodium ortho-phenylphenate to control aflatoxigenic fungi. I. Field tests. *Revista de Microbiologia*, São Paulo, 23(4): 279-283, 1992.
7. JACKSON, C.R. Evaluation of fungicides for control of some fungi found on peanuts. *Plant Disease Reporter*, Washington, 49(11): 928-31, Nov., 1965.
8. MADAAN, S.L. & CHOHANN, J.S. Efficacy of antimicrobial chemicals to control postharvest occurrence of *Aspergillus flavus* in groundnut kernels. *Indian Phytopathology*, New Deli, 31(1):57-59, 1978.
9. PONS JUNIOR, W.A.; CUCULLU, A.F.; LEE, L.S.; ROBERTSON, J.A.; FRANZ JUNIOR, A.O.; GOLDBLATT, L.A. Determination of aflatoxins in agricultural products: use of aqueous acetone for extraction. *Journal of the AOAC*, Chicago, 49(3): 554-562, 1966.
10. PONS JUNIOR, W.A.; CUCULLU, A.F.; LEE, L.S.; ROBERTSON, J.A.; FRANZ JUNIOR, A.O.; Rapid quantitative TLC method for determining aflatoxins in cottonseed products. *Journal of the AOAC*, Chicago, 55 (4):768-774, 1972.
11. STOLOFF, L. & SCOTT, P.M., eds. 14 ed. *Natural poisons*. Arlington, AOAC, 1984. cap 26. p. 477-500.
12. VELASCO, J.; MORRIS, S.L. Use of water slurries in aflatoxins analysis. *J. Agric. Food Chem.*, Easton, 24 (1):86-88, 1976.

Rev. Microbiol., São Paulo, 25(2): 101-6, 1994

AFLATOXIN REMOVAL FROM PEANUT MEALS WITH COMMERCIAL AQUEOUS ETHYL ALCOHOL

H. Fonseca[1]

SUMMARY

Removal of aflatoxin B1 (AF) from contaminated peanut meals with commercial aqueous ethyl alcohol (fuel alcohol) in an actual scale vessel was tried in two experiments (TESTS 1 and 2) run in an oil mill in the State of São Paulo, Brazil. In TEST 1, 90°GL alcohol (downgraded from commercial 96°GL) heated to 75°C was utilized to make three one hour-period extractions (each one using fresh alcohol), with samples taken for AF analyses after each extraction. In TEST 2, straight commercial 96°GL alcohol was used to make four extractions under the same conditions as in TEST 1 but without stopping the process to take samples and also introducing a 30 min soaking period in the middle of each extraction. In this experiment, pieces (about 2 cm thick) and course ground meal were used to check the influence of size of material on the efficiency of AF extraction by the solvent. Twenty samples (10 of each type) were taken at the end of the process for AF evaluation. The results showed that extraction of AF with commercial aqueous ethyl alcohol in actual scale is time-dependent and technically feasible. 90°GL alcohol removed 87.4% (average) of total AF content after three one-hour extraction periods. 96°GL alcohol removed an average of 87.3% from the pieces and 95.3% from the course ground meal after four one-hour extraction periods. Samples from the lower part of the vessel showed better AF removal than those from the upper part. It was also observed that coarse ground material allows a more efficient removal of AF than pieces. Protein content was evaluated before and during TEST 1 and showed a slight increase in mean values (from 60.19 to 63.79%).

Key words: aflatoxin, removal, peanut meal, commercial aqueous ethyl alcohol, detoxification.

INTRODUCTION

The contamination of peanuts with aflatoxins (AF) renders them improper for human consumption. In Brazil, most of the contaminated peanuts are used for oil extraction. The meal, a residue of high protein content, holds practically all the toxin present in the raw material. Depending on the final concentration of AF, the meal cannot be used as animal feed. The detoxification of contaminated meals by inactivation through chemical reactions, high pressure, etc., or by extraction using an organic solvent or a combination of them has been tried since the AF problem was detected in 1960.

Several solvents or solvent combinations have succeeded at, under laboratory conditions, eliminating the AF retained in peanut meals, once the

1. Departamento de Ciência e Tecnologia Agroindustrial, Escola Superior de Agricultura "Luiz de Queiroz", USP.

toxin is not removed by the hexane used for extraction of residual oil from meals.

Mixtures of hexane-methyl alcohol, hexane-ethanol, hexane-ethanol-water and hexane-acetone-water were evaluated for AF extraction by Vorster (1966). The greatest reduction on a laboratory scale was obtained with hexane-acetone-water and hexane-methyl alcohol.

Gardner *et al.* (1968) reported the effectiveness of a binary system of 90% acetone and 10% water (by weight) for reducing AF cont102)fof contaminated peanut and cottonseed meals.

Removal of AF by aqueous alcohols has also been studied. Rayner and Dollear (1968) reported good extraction using aqueous isopropyl alcohol at 60°C. Rayner *et al.* (1970) found that good results could be obtained by extraction with 95% ethanol.

In 1985, Fonseca and Regitano-d'Arce (1992), using anhydrous, 96, 93 and 90°GL ethanol found, by Soxhlet extractions, that 90°GL alcohol was able to remove 100% AFB1 in less than 3 hours, while the 96°GL in less than 4 hours, from peanut meal containing 400 mg/Kg. Anhydrous alcohol was the least efficient.

However, these solvents or solvent mixtures were not evaluated neither with commercial products or on a industrial scale.

MATERIALS AND METHODS

Two experiments were conducted in the largest peanut crusher mill, located at Pirapozinho, State of São Paulo, Brazil, where one actual-size unit for batch solvent extraction was especially allocated to run these experiments, with all the characteristics and dimensions of the industrial hexane semi-continuous batch extractors which can hold about 4 tons of peanut meal. The equipment was composed of a steam jacketed extraction vessel, complete equipment for steam, vacuum system, circulating pumps, fresh and used alcohol tanks, etc. The direction of solvent circulation was up-flow.

Oil-extracted peanut meal from the industrial solvent extraction unit was used.

TEST 1

The first experiment was made with a contaminated meal whose previous analyses of 10 samples showed a mean AF content of about 1000 mg/kg of meal. The choice of 90°GL alcohol was based on our previous laboratory experiment (Fonseca and Regitano-d'Arce, 1992). 90°GL was obtained by downgrading commercial 96°GL with water. The procedure of TEST 1 is shown in Figure 1.

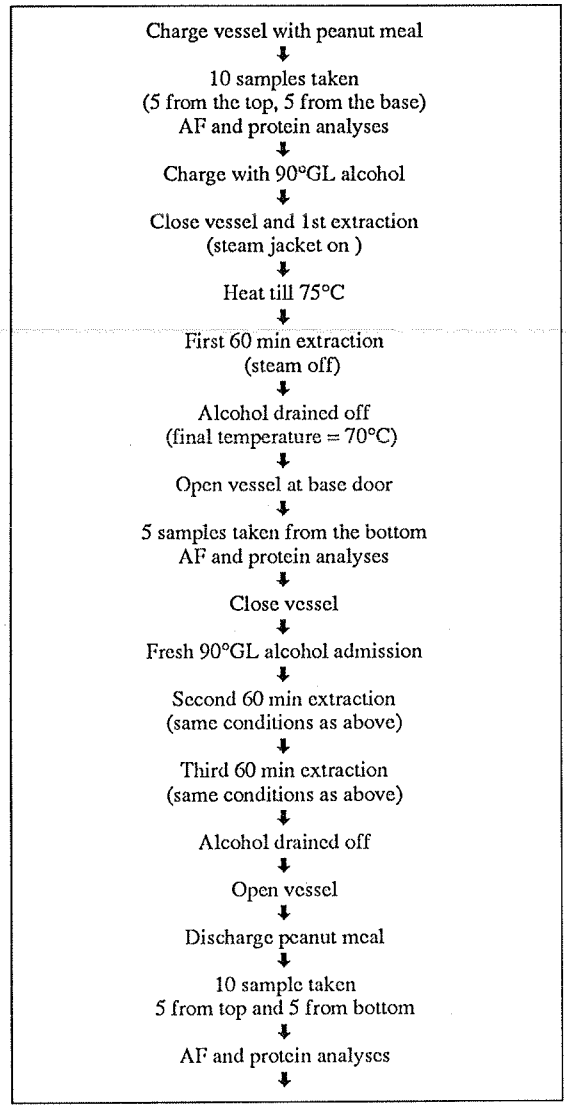


FIGURE 1 - Flow scheme of TEST 1.

TEST 2

In the second experiment, the procedure was modified as illustrated in Figure 2. Previous analyses of 10 samples of the peanut meal employed in this experiment showed an AF contamination content of about 1200 mg/kg. Unchanged commercial 96°GL fuel alcohol was chosen due to the difficulty to downgrade 96°GL to 90°GL. So, no alcohol strength correction was needed since the distilleries produce it at 96°GL.

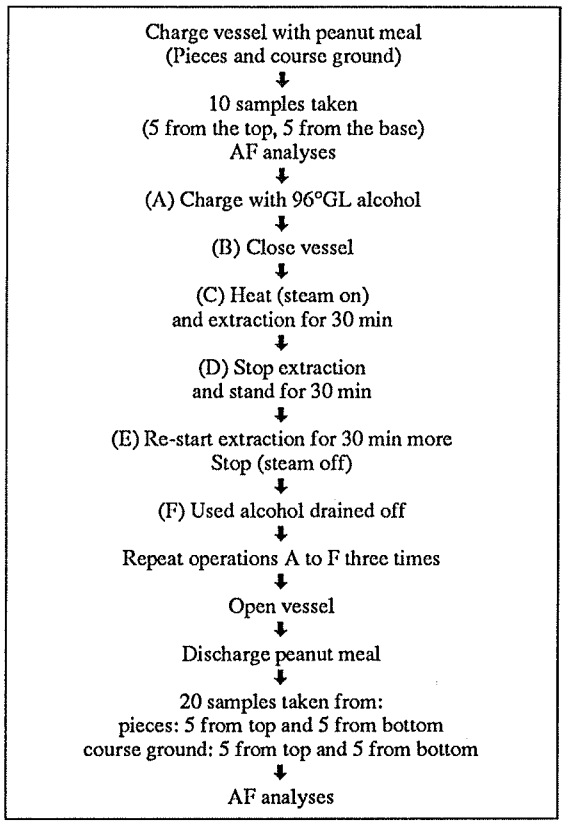


FIGURE 2 - Flow scheme of TEST 2.

At the end of the operation, separate samples of pieces and ground meal were taken for AF analysis, being 5 from top and 5 from the base of the vessel for each type, making up a total of 20.

Three thousand litres of alcohol were used for each extraction process in both experiments.

The AF analyses were performed according to the Pons *et al.* (1966) method combined with that of Velasco and Morris (1976).

TABLE 1 - AFB_1 content (µg/kg) of peanut meal before extraction in TEST 1.

Site	Site
Top 1 = 800	Base 1 = 800
Top 2 = 530	Base 2 = 800
Top 3 = 1200	Base 3 = 800
Top 4 = 1200	Base 4 = 800
Top 5 = 1200	Base 5 = 1600
Mean = 986	Mean = 960
Average = 973	

Percentage protein content had been previously analyzed by the industry and was also evaluated on homogenized samples after each extraction in TEST 1, by the macro Kjeldahl method, as an additional information.

RESULTS AND DISCUSSION

AF content of peanut meals before and af-

TABLE 2 - AFB_1 content (µg/kg) of peanut meal after the first and second extractions of 90°GL ethyl alcohol at 75°C for 60 min in TEST 1.

First	Second
Base 1 = 400	Base 1 = 240
Base 2 = 400	Base 2 = 400
Base 3 = 533	Base 3 = 160
Base 4 = 800	Base 4 = 320
Base 5 = 533	Base 5 = 400
Mean = 533	Mean = 304
Mean AFB_1 removal = 45.9%	Mean AFB_1 removal = 68.3%

TABLE 3 - AFB_1 content (µg/kg) of peanut meal after a third extraction of 90°GL ethyl alcohol at 75°C for 60 min in TEST 1.

Site	Site
Top 1 = 160	Base 1 = 160
Top 2 = 160	Base 2 = 80
Top 3 = 160	Base 3 = 80
Top 4 = 106	Base 4 = 80
Top 5 = 80	Base 5 = 160
Mean = 133	Mean = 60
Mean AFB_1 removal = 86.5%	Mean AFB_1 removal = 88.3%
Average AFB_1 removal = 87.3%	

TABLE 4 - AFB_1 content (µg/kg) of peanut meal before extraction in TEST 2.

Site	Site
Top 1 = 1600	Base 1 = 1600
Top 2 = 1066	Base 2 = 1600
Top 3 = 800	Base 3 = 2000
Top 4 = 800	Base 4 = 1066
Top 5 = 800	Base 5 = 600
Mean = 1013	Mean = 1373
Average = 1193	

TABLE 5 - AFB_1 content (µg/kg) of peanut meal after four extractions of 90°GL ethyl alcohol at 75°C for 60 min each in TEST 2.

Course ground		2 cm pieces	
Top 1 = 65		Top 1 = 160	
Top 2 = 57		Top 2 = 80	
Top 3 = 54		Top 3 = 80	
Top 4 = 57		Top 4 = 200	
Top 5 = 57		Top 5 = 266	
Base 5 = 50		Base 5 = 133	
Base 4 = 50		Base 4 = 200	
Base 3 = 50		Base 3 = 200	
Base 2 = 50		Base 2 = 100	
Base 1 = 57		Base 1 = 80	
Mean AFB_1	Mean Removal	Mean AFB_1	Mean Removal
Top = 58.0	94.3%	Top = 157.2	84.5%
Base = 51.4	94.3%	Base = 89.6	89.6%
Average AFB_1 Removal = 95.3%		Average AFB_1 Removal = 87.3%	

TABLE 6 - Protein content (%, dry basis) of peanut meal before and after each extraction in TEST 1.

Before	Top = 60.37 Base = 60.01 Mean = 60.19
After 1 hour of extraction	Top = 61.20 Base = 61.88 Mean = 61.54
After 2 hours	Top = 61.49 Base = 62.55 Mean = 62.05
After 3 hours	Top = 63.05 Base = 64.52 Mean = 63.79

ter extractions are presented in Tables 1 to 5. Protein contents before and after extractions, as determined by the industry, are shown in Table 6.

TEST 1

As can be inferred from the results of TEST 1, 45.9%, 68.3% and also 86.5% (top) and 88.3% (base) - averaging 87.4% - of the contaminating AF was removed after the first, the second and the third one-hour extraction period, respectively, using fresh 90°GL alcohol. The curve tends to be very close to zero between 4 and 5 one-hour extraction periods, which can be considered a good result (Figure 3).

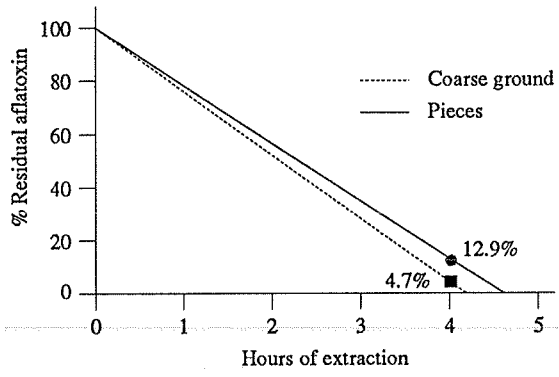


FIGURE 3 - Removal of AF from peanut meal with 96°GL ethanol: TEST 2

The problem that emerged during TEST 1 was that it took a long time to get rid of the last portion of water+dissolved alcohol from the meal, which is usually the last operation done with steam jacket heating under vacuum.

Considering the information obtained from the intermediate analyses of TEST 1, it was thought unnecessary to stop the process for sampling, since that would make the processing time longer and turn TEST 1 more complicated. However, the experiment provided useful data such as the trend of residual AF versus time of extraction shown in Figure 3.

TEST 2

This test showed an average removal of 87.3% for 2 cm thick pieces and of 95.3% for course ground meal after four one hour extraction periods with 96°GL alcohol, a result that can be considered very good.

The lower extraction efficiency of 96°GL alcohol is due to its reduced water content compared to 90°GL since water is needed to unbind AF from the protein, and this data corroborates the results previously obtained in our laboratory (Fonseca and Regitano-d'Arce 1992).

In both TEST 1 and 2, it is possible to see that the removal of AF is slightly greater at the base of the vessel than that at the top. As in TEST 1, there was some difficulty in evaporating residual moisture-dissolved alcohol from the meal in TEST 2 also, but this time to a lesser extent.

The experiments illustrate the technical feasibility of removing AF from contaminated peanut meal on an industrial scale. We think that, concerning semi-continuous processes, the time for

complete removal of AF can be reduced. Research aimed at overcoming the problem of residual moisture+alcohol in the meal will be carried out next.

Protein content increased slightly after each extraction increasing from 60.19% before the process to 63.79% (mean values) after the third extraction,on a dry basis (Table 6). This may be due to the probable extraction of alcohol soluble substances tha increases the relative concentration of those remaining. This is a relevant observation because it shows that protein, which is the most important component as feed and the basis for meal pricing, is not negatively affected.

CONCLUSIONS

From the results obtained under the experimental conditions used, the following main conclusions can be drawn:

1. It is technically feasible to remove AF from peanut meals utilizing commercial 96°GL carburant alcohol, in an actual scale;
2. AF removal is time-dependent and an efficiency of about 95% after 4 to 5 hours of batch extraction with 96°GL alcohol can be obtained;
3. Removal of AF is greater in the coarse ground material than in pieces, as would be expected;
4. Removal is more efficient in the material located at the bottom of the vessel;
5. The protein content is not negativelly affected, actually showing a slight increase in content at the end of the process.
6. Further research is needed to optimize conditions of extraction (e.g. a comparison of semicontinuous with continuous processes) and to attain removal of the residual moisture+alcohol from the decontaminated peanut meal.

RESUMO

Remoção de aflatoxinas de farelo de amendoim com álcool etílico aquoso comercial

A remoção de aflatoxinas (AF), com álcool etílico aquoso comercial (carburante), de farelo de amendoim contaminado em vaso extrator de escala real foi testada em dois experimentos (TESTES 1 e 2) realizados numa indústria de extração de óleo no Estado de São Paulo, Brasil. No TESTE 1, álcool 90°GL (diluído a partir do 96°GL) aquecido a 75°C, foi utilizado para fazer três extrações de uma hora cada (sempre com álcool novo), tendo sido retiradas amostras após cada extração, para análise de AF e de proteína. No TESTE 2, álcool 96°GL foi utilizado em quatro extrações de uma hora, nas mesmas condições porém, sem parar o processo para retirada de amostras intermediárias mas, introduzindo um período de 30 minutos de maceração entre as extrações. Neste experimento, pedaços de cerca de 2 cm de espessura e farelo grosseiramente moído, foram utilizados para testar a influência do tamanho da partícula na eficiência da extração de AF pelo solvente. Vinte amostras (10 de cada tipo) foram retiradas no fim do processo para análises de AF. Os resultados mostraram que a extração de AF com álcool etílico aquoso carburante, em escala real, é dependente do tempo e tecnicamente possível. Álcool 90°GL removeu, em média, 87,4% da AF presente depois de três extrações de uma hora. Álcool 96°GL removeu, em média 87,3% do farelo em pedaços e 95,3% do moído, após quatro extrações de uma hora. Houve uma melhor extração na parte inferior do que na parte superior do vaso. O conteúdo de proteína foi avaliado antes e durante o TESTE 1 e mostrou um pequeno aumento de 60,19% para 63, 79%.

Palavras-chave: aflatoxina, remoção, farelo de amendoim, álcool etílico aquoso, destoxificação.

ACKNOWLEDGEMENT

The author wishes to thank BRASWEY S.A. INDUSTRIA E COMERCIO, de São Paulo, SP, Brazil, for providing equipments, personnel and for financially supporting the whole set of experiments run in the company's plant at Pirapozinho.

REFERENCES

1. FONSECA, H., and REGITANDO-d'ARCE, M.A.B., 1992. Aflatoxin removal removal from peanut meals with aqueous ethanol. *Research note. Sci agric.* (Piracicaba), 50(1): 154-156, 1993.
2. GARDNER Jr. , H.K., KOLTUN, S.P. and VIX, H.L.E. Solvent extraction of aflatoxins from oilseed meals. *J. Agric. and Food Chem.*, 16:990-993, 1968.
3. PONS Jr., W.A., CUCULLU, A.F., LEE.L.S., ROBERTSON J.r., J.A., FRANZ, A. O., and GOLDBLATT, L.A. Determination of aflatoxins in agricultural products: use of aqueuos acetone for extration. *J.A.O.A.C.*, 49: 554-562, 1966.

4. RAYNER, E.T., and DOLLEAR, F.G. Removal of aflatoxins from oilseed meals by extraction with aqueous propanol. *J.AM. Oil Chem. Soc.*, **45**:662-664, 1968.
5. RAYNER, E.T., DOLLEAR, F.G., and CODIFER Jr., L.P. Extraction of aflatoxin from cottonseed and peanut meals with ethanol. *J.AM. Oil Chem. Soc.*,**47**: 26, 1970.
6. VELASCO, J., and MORRIS, S.L. Use of water slurries in aflatoxin analysis. *Journal of Agriculture and Food Chemistry*, **24**:86-88, 1976.
7. VORSTER, L.J. Studies on the detoxification of peanuts contaminated by aflatoxins. *Revue Fracaise des Corps Gras.*, **13**:7-13, 1966.

Rev. Microbiol., São Paulo, 25(2): 107-11, 1994

DETERMINATION OF OCHRATOXIN A IN BLOOD SERUM OF PIGS BY USING THIN LAYER CHROMATOGRAPHY

Mallmann, C.A.[1]
Santurio, J.M.[2]
Baldissera, M.A.[3]
Mickwitz, G. von[1]

SUMMARY

The determination of Ochratoxin A (a secondary metabolite of *Aspergillus* and *Penicillium*) in the blood serum of pigs by using thin layer chromatography (TLC) and liquid-liquid extraction is a sensitive and exact method, which leads to a recovery rate of 92.5%. This method allows to confirm a clinically suspected Ochratoxicosis by determination of Ochratoxin A in blood serum. Ochratoxin A has a high binding affinity for serum albumin. This characteristic allows the utilization of this method to screen a previous ingestion of this mycotoxin without examination of feed samples. A contamination of 32.21% (out of 444 investigated blood samples) has been detected in sera of pigs from Schleswig-Holstein and Niedersachsen (Northern Germany) with an average of Ochratoxin A concentration of about 0.6 ng/ml. Due to its sensitivity and the low input of time and material, this method is highly recommendable for routine use by any mycotoxicology laboratory.

Key Words: Ochratoxin A, survey, serum, pigs, TLC.

INTRODUCTION

Ochratoxin A (OA) is a powerful metabolite (9) produced by fungi of the genus *Aspergillus* (6) and *Penicillium* (5) which are ubiquitous in the enviroment. Ochratoxin A, classified as a pentacetic within the policetic group (10), is a crystalline, colourless compound with a fusion temperature of about 168-173°C (5).

Ochratoxin A is frequently found in a variety of cereal products such as maize, wheat, barley, malt and their by-products. It is also found in products of animal origin such as meat and its by-products (5). Because of its widespread distribution among these products, this toxin represents a serious hazard for both human and animal health (1). Pharmacokinetic studies show that Ochratoxin A displays a high affinity for serum albumin, especially that from humans, bovines and swine (5).

The detection of mycotoxins in products of animal origin by chemical analysis is a relatively complex and time consuming process. The chemical analysis requires previous trituration, homogenization, organic solvent extraction, filtration, elution and evaporation of the extract. The result-

1. Freie Universität Berlin - Tierärtzliche Ambulanz Schwarzenbek - Röntgenstraße, 12 - 21493 - Schwarzenbek - Alemanha.
2. Departamento de Medicina Veterinária Preventiva - Universidade Federal de Santa Maria - 97119-900 - Santa Maria, RS - Brasil.
3. Departamento de Análises Clínicas e Toxicológicas - Universidade Federal de Santa Maria - 97119-900 - Santa Maria, RS - Brasil.

ing extract is then submitted to the thin layer chromatography technique (TLC) for qualitative and semi-quantitative analyses (8). This technique involves the use of large amounts of highly toxic organic solvents and is relatively expensive. Likewise, these alimentary materials contain a series of substances that might interfer with the determination of Ochratoxin A under ultraviolet light (1). Sometimes it is necessary to run several different chromatographies using different solvents, nebulization by developing substances or even a chemical derivation to confirm the presence of the toxin.

On the other hand, the serum is a less complex material and yields a relatively clear extract with smaller amounts of potentially interfering substances. Therefore, the use of serum for the detection of Ochratoxin A by thin layer chromatography represents a rapid, sensitive and low cost diagnostic method. This technique involves minimal handling of toxic substances and allows a clear and definitive reading and interpretation of the results.

Hasert (1988) reported that concentrations of 1 µg/l of Ochratoxin A can be detected in swine serum up to 28 days after the toxin has been given to the animals with a diet containing 0.25 mg of Ochratoxin A daily during a period of three weeks.

The objectives of the study reported here were to investigate the efficiency of the Ochratoxin A determination in blood serum of pigs using TLC and the use of this technique for serological surveys on herds.

MATERIALS AND METHODS

A) Biologic material

The sera were obtained from 444 swine blood samples collected at the slaughterhouse from 14.01.91 to 13.05.91. The sampled pigs originated from 236 herds in the areas of Schleswig-Holstein and Niedersachsen (North Germany).

B) Extraction

The method used for Ochratoxin A extraction has been previously described by MORTENSEN et al (1983) and modified by Hasert (1988). The identification and quantification were performed according to techniques described by Nesheim et al (1973).

FIGURE 1 - Scheme of the analysis for detection of Ochratoxin A in swine blood serum:

Extraction:	- mix 5 ml of serum with 5 ml of HCl 1 mol. - shake two times with 10 ml of $CHCL_3$ - centrifuge at 4000 rpm for 10 min.
Extract clearification:	- remove chloroform phase; - filter through sodium sulphate; - evaporate (rotavapor) - dilute the extract in 100 V 1 of $CHCL_3$
Chromatography:	- apply 40 µl of the extract to the chromotographic plate - to elute in: a) benzene: methanol: acetic acid (90:5.5) b) toluene: methanol: acetic acid (90:5:5)

For the extraction (figure 1) 5 ml of serum were filled in a centrifuge tube (Mod. Kranich, (100x44mm high x Ø)) (Erich Wiegand GmbH) to which 5 ml of cloridric acid 1 mol (E. Merck) were added, gently mixed and allowed to rest for 3 minutes. After that, 10 ml of chloroform (E. Merck) were added followed by stirring for 1 minute with a stirrer (Vibrofix VF2) until a milky emulsion was obtained. By using this procedure it is possible to obtain both protein precipitation and Ochratoxin A chloroform solubility. Then the samples were centrifuged at 4000 rpm for 10 minutes in order to let the separation of the three phases occur. After that, the chloroform phase (bottom) was aspirated by using a needle attached to a 10 ml disposable syringe. The needle had to be carefully introduced strictly close to the tube wall to avoid aspiration of the protein and aqueous phase. By using the needle, the protein layer was fractionated, added to 10 ml of chloroform and mixed until a milky emulsion was obtained, whereon the extract was finally centrifuged.

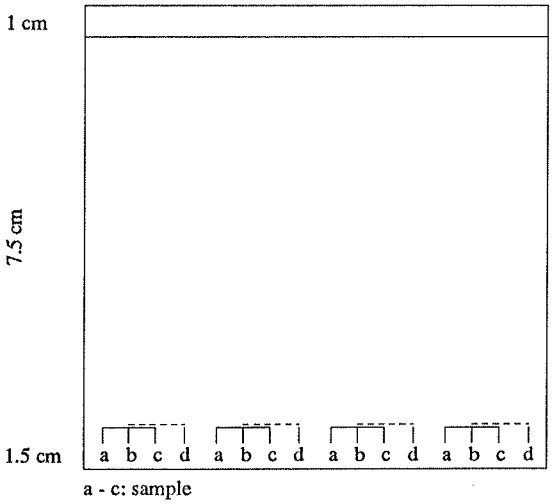
The chloroform phases were filtered through anidre sodium sulphate (Na_2SO_4) (approximately 1 g) (E. Merck) and placed on an extrelut 20 tube (E. Merck). A piece of cotton served as a lid to close the tube base and then 2 ml of chloroform were added. After that, the filtrate was collected in a 25 ml volumetric bottle (Erich Wiegand GmbH). Finally, 2 ml of chloroform were added to the Na_2SO_4 and allowed to stand until the dripping has ceased. Under pressure, the rest of the extrelut tube contents was expelled. After that, the contents of the volumetric bottle was evaporated in a Rotavapor.

C) Identification by the thin layer chromatography

The extract obtained was dissolved in 100 µl of chloroform, 40 µl being linearly applied over the G 60 silica gel chromatoplates (E. Merck) at A to C segments by using a microsyringe (Hamilton Bonaduz AG) and then the application line was dried with a stream of hot air. Ten microliters of Ochratoxin A standard (2 ng Ochratoxin A/µl) were applied from point B to D, in order to obtain both internal and external standards (figure 2).

The plates were developed in a saturation chamber (Erich Wiegand GmbH) containing benzene:methanol:acetic acid (90:5:5 v/v/v) or toluene:methanol:acetic acid (90:5:5 v/v/v) (E. Merck). After the solvent had reached the front line (20-25 min), the plates were removed and allowed to dry at room temperature for 30 min.

The chromatographic plates were examined under long-wave (360nm) ultraviolet light (Desaga GmbH) where a blue-green fluorescence derived from the Ochratoxin A. The tests were regarded as positive when the fluorescence was observed through all the A-D application segment (figure 2).



a - c: sample
b - d: standard
b - c: Sample with internal standard
c - d: External standard

FIGURE 2 - Graphic representation of the thin layer chromatographic plate.

The semi-quantitative analysis of the Ochratoxin A concentration was made by comparing the fluorescence by different standard concentrations.

The following formula was used to calculate the concentration of the toxin/ml serum:

$$OA\ ng/ml = \frac{1 \times 2 \times 3}{4 \times 5 \times 6} \times CF \text{ being:}$$

1 = OA visualized on the plate (ng)
2 = vol. of utilized CHC13 (20 ml)
3 = vol. of CHC13 in the redilution (100 µl)
4 = vol. of recovered eluate
5 = serum vol. (5 ml)
6 = vol. applied on the plate (40 µl)
FC = correction factor

The correction factor (CF) was calculated based on the following formulae:

$$RR = \frac{dt}{jd} \times 100 \text{ being:}$$

RR = recovery rate
dt = detected toxin (ng/ml of serum)
at = added toxin (ng/ml of serum)

$$CF = \frac{100}{RR} \text{ being:}$$

CF = correction factor
RR = recovery rate

RESULTS AND DISCUSSION

In this study we used OA-free serum samples to which increasing concentrations of the toxin were added. By analyzing these samples we were able to obtain a recovery rate of 92.5% (Standard deviation = 12.16).

The recovery rate obtained leads to a correction factor of 1.1.

TABLE 1 - Ochratoxin A recovery rates (RR) in swine blood serum.

Added Ochratoxin A (ng/ml)	Recovery of Ochratoxin A (ng/ml) in the sera					Mean	% of recovery
	1	2	3	4	5		
1	1	1	1	0.6	1	0.9	90
5	6	4	4	5	5	4.8	96
10	10	10	8	8	10	9.2	92
50	50	50	32	45	45	44.4	88.8
100	100	100	90	90	100	96.0	96
200	180	180	180	200	160	180.0	90
RR Mean							92.5%

In those tests in which the serum concentration of Ochratoxin A was above 30 ng/ml (thus leading to a too high fluorescence in the plate) the quantification was quite difficult to obtain, because almost no difference was evident between bands with close concentrations such as 31 or 32 ng/ml. In those cases, a new extraction was performed, either using a smaller amount of serum or adding less extract (10-20 µl), in order to have less toxin reacting and consequently a fluorescence intensity which allowed more exact quantification.

On the chromatoqraphic plates it is possible, to distinguish bands of fluorescence down to 1 ng. The use of this value in the formula mentioned above for the Ochratoxin A concentration in the sample leads to a detection level of 0.6 ng/ml.

One hundred and forty five (37.21%) out of the 444 samples collected from 14.01.91 to 13.05.91 were found to be contaminated with concentrations of Ochratoxin A above 0.6 ng/ml. Eighty two (39.81%) out of the 206 sampled herds showed at least one positive sample.

The levels of contamination of OA and the relation of contaminated samples detected per herd are shown in table 2.

TABLE 2 - Ochratoxin A levels in swine sera from Schleswing - Holstein and Niedersachsen (North Germany) from 14.01.91 to 13.05.1991.

Group ng/ml	Nº of samples	%
< 0.6	301	67.79
0.6 - 5.0	95	21.40
5.1 - 10	30	6.76
> 10	18	4.05
Total	444	100.00

The average Ochratoxin A concentration determined in the positive samples was 4.69 ng/ml, with values ranging from 0.6 ng/ml to 37 ng/ml. In a previous experiment, studying 786 serum samples, Hasert (1988) found 76 being (26.6%) positive, of which 7 samples out of 10 (2.45%) were found to be contaminated with Ochratoxin A concentrations above of 10 µg/l. Haupt(1989) found that 54.09% (of 1200 samples) were contaminated with doses above 0.6 ng/ml and 4.79% were contaminated with Ochratoxin A concentrations above 10 ng/ml.

In order to substantiate the statistical accuracy of testing with only one sample per herd, as described by Hult (1980), in this study we tested sera from 1 to 10 (average of 2.15) per herd (table 3). The sum of the cases in which no sample was found contaminated in the herd plus the total of cases in which all samples were positive resulted in a rate of 87.5%. The results already known are quite promising regarding the use of this technique for serological survey, since it allows a highly precise estimation (nearly 88%) of the contamination rate by testing only one sample per herd. Furthermore, this study continues to be carried on by testing a larger number of samples in order to achieve a higher statistical accuracy.

TABLE 3 - Distribution by percentage of positive sera related to the total samples collected per herd.

% positive sera	Herds n	Sera Positive/total	% tested samples
0	124	0/256	60.19[a]
1 - 20	1	1/5	0.49
21 - 40	9	10/31	4.37
41 - 60	12	16/33	5.83
61 - 80	2	6/8	0.97
81 - 99	1	8/9	0.49
100	57	102/102	27.66[b]
Total	206	143/444	100.00

a + b = 87,85%

Since Ochratoxin A displays a long residual activity in swine serum, contamination can be detected even a long time after consumption of contaminated feed (3). Likewise, because sampling of feed for analysis often involves a risk of taking non-representative samples thus leading to less reliable results, the serum is easy to obtain and constitutes an unequivocally representative sample.

Based on our results we conclude that the detection of Ochratoxin A in serum represents an accurate, useful and suitable method to be used in epidemiological surveys of this mycotoxicosis in swine.

RESUMO

Determinação de ocratoxina A no soro de suínos através de cromatografia em camada delgada

A determinação de Ocratoxina A (metabólito secundário de fungos do gênero *Aspergillus* e *Penicillium*) no soro de suínos por cromatografia em camada delgada (CCD) e a extração líquido-líquido representa um método eficiente e preciso com uma

taxa de recuperação de 92.5%. O método permite a confirmação da suspeita de Ocratoxicose pela determinação da Ocratoxina A no soro sangüíneo. A grande afinidade da Ocratoxina A pelas albuminas séricas tornam este método recomendável para levantamentos epidemiológicos permitindo o estudo de contaminações por Ocratoxina A em rebanhos, sem que seja necessário o exame das rações consumidas. Neste trabalho pode-se verificar uma contaminação de 32.21% de um total de 444 soros coletados na região de Scheleswig-Holstein, norte da Alemanha, em concentrações acima de 0.6 ng/ml. Além disto os custos relativamente baixos e a rapidez com que se obtém o diagnóstico permitem o emprego rotineiro da técnica em qualquer laboratório de micotoxicologia.

Palavras-chaves: Ocratoxina A, Diagnóstico, Soro, Suíno, CCD.

ACKNOWLEDGEMENTS

Ms. U. Braun and Prof. E. Furtado Flores for the English translation.

REFERENCES

1. BAUER, J.; GAREIS, M.; GEDEK, B. Zum Nachweis und Vorkommen von Ochratoxin A bei Schlachtschweinen. *Berl. Münch. Tierärztl. Wschr.* 97(8):283-297, 1984.
2. HAUPT, C. Untersuchungen zum Vorkommen von Ochratoxin A im Blut von Schlachtschweinen im Raum Schleswig-Holstein mit Berücksichtigung regionaler und wetterbedingter Einflüsse. *Thesis*. Freie Universität Berlin. 211p. 1989.
3. HASERT, W. Untersuchungen zum Nachweis des Mykotoxins Ochratoxin A im Schweineblut nach Aufnahme toxinkontaminierten Futters und zum Vor-kommen von Ochratoxin A im Blut von Schlachtschweinen. *Thesis*. Freie Universität Berlin. 115p. 1988.
4. HULT, K.; HORKY, E.; GATEMBECK, S.; RUTQUIST, L. Ochratoxin A in blood from slaughter pigs in Sweden: Use in evaluation of toxic content of consumed feed. *Appl. Environ. Microbiol.*, **49**:1014-1015.
5. Enviromental Health Criteria 105, Selected mycotoxins: Ochratoxin, Tricho-thecenes, Ergot. *World Healt Organization*. 263p. 1990.
6. MERWE, K.J. van der; STEYN, P.S.; FOURI, L. Mycotoxins. Part II. The constitution of ochratoxins A, B, and C, metabolites of *Aspergillus ochraceus* Wilh. *J. Chem. Soc.*, :7083-7088, 1965.
7. MORTENSEN, H.P.; HALD, B.; MADSEN, A. Feeding experiments with ochratoxin A contaminated barley for bacon pigs. 5. Ochratoxin in pig blood. *Acta Agric. Scand.*, **33**:235-239, 1983.
8. NESHEIM, S.; HARDIN, N.F.; FRANCIS, O.J.; LANGHAM, W.S. Analysis of ochratoxin A and B and their esters in barley, using partition and thin layer chromatography. I. Development of the method. *J. Assoc. Offic Anal. Chem.*, 56:817-821, 1973.
9. PECKHAM, J.C.; DOUPNIK, Jr. B; JONES, Jr. O.H. Acute toxicity of ochratoxins A and B in chicks. *Appl. Environ. Microbiol.*, **21**:492-4, 1971.
10. SCOTT, P.M. *Penicillium* mycotoxins. In: Wyllie, T.D.; Marehouse, L.G., Mycotoxic fungi, mycotoxins, mycotoxicoses. New York, Marcel Dekker, pp. 383-356, 1977.

Rev. Microbiol., São Paulo, 25(2): 112-8, 1994

PURIFICATION AND CHARACTERIZATION OF THERMOSTABLE XYLANASES FROM THERMOPHILIC *HUMICOLA* SP. AND THEIR APPLICATION IN PULP IMPROVEMENT

Roberto da Silva
Dong K. Yim
Yong K. Park

ABSTRACT

A thermophilic *Humicola* sp. isolated from decayed wood produced thermostable extracellular xylanase at 50°C. Enzyme purification was done by DEAE-Sephadex and CM-Sephadex C-50 gel column chromatography and three protein fractions with xylanase activity were found. Optimal pH and temperature values for the three xylanases were pH 5.0 and 5.6 and 75°C. Other characteristics of the enzymes were investigated and it was found that xylanase I was an endoxylanase while xylanase II was a xylosidase (exoxylanase). Xylanase III was an endoxylanase and also showed arabinosidase and CMCase activities. Treatment of bleached Kraft eucalyptus pulp with purified and crude enzymes enhanced pulp brightness compared to untreated pulp. Xylanase I and crude xylanase increased pulp viscosity whereas xylanase II and III decreased it due to the presence of CMCase activity.

Key words: Thermophilic *Humicola* sp.; thermostable xylanases; endoxylanase; xylosidase; Bleached Kraft eucalyptus pulp

INTRODUCTION

Lignocellulose consists of three major components which are cellulose, hemicellulose and lignin. Xylans compose most of the hemicellulose fraction. Agricultural residues contain 20-40% of hemicellulose which thus represents an important resource that could be employed for conversion of biomass to xylose or xylooligosaccharides by chemical processes or by the use of xylanases.

In recent years, there has been an increasing interest in utilizing xylanases for conversion of xylans to xylose or in pulping processes. These enzymes have been particularly used to facilitate the bleaching of Kraft pulp and to improve fiber properties (2, 3, 7, 8, 13). During the early stages of alkaline-based pulping processes, xylans are largely dissolved but can be reprecipitated onto the fiber surface, so that 10-30% (W/W) remain in the finished alkali of Kraft pulp (10). Since removal of xylans from pulp is a desirable step that can be achieved using xylanases, xylose can be obtained as a by-product of pulp treatment.

The lack of thermostability exhibited by xylanases from microorganisms results in low hydrolysis efficiencies. Therefore, the use of thermostable xylanases to carry out xylan hydrolysis at high temperatures over prolongued periods of time might enhance both the technical and economic feasibility of the hydrolysis process (16).

Universidade Estadual de Campinas, Faculdade de Engenharia de Alimentos (UNICAMP), Campinas, SP, Brasil.
Endereço para correspondência: Yong K. Park - FEA, UNICAMP - Caixa Postal 6121 - Campinas, SP, Brasil.

The aim of the present work was to screen thermophilic strains of microorganisms for thermostable xylanase production and to purify and characterize the enzymes involved. Purified xylanases were also added to bleached Kraft eucalyptus pulp in an attempt to improve pulp quality.

MATERIALS AND METHODS

Isolation of xylanase-producing thermophilic microorganisms. Aproximately 1g of soil or decayed wood was inoculated into test-tubes containing 10ml of the enrichment medium and incubated at 50°C for 1 week. After incubation, cultures were dilutes with sterilized water and inoculated onto potato dextrose agar plates. Plates were then kept at 50°C and the colonies subsequently transferred to slant cultures. The composition of the enrichment medium was as described by Mandels and Sternberg (6), with 1% of cellulose being substituted by xylan (oat spelt xylan, Sigma). Isolated thermophilic microorganisms from the slant cultures were inoculated into 100ml of culture medium inside 500ml Erlenmeyer flasks and incubated at 50°C with shaking (200rpm) for 5 days. At the end of the incubation period, flask contents were filtered and the xylanolytic acitivities of the filtrates determined.

Production of enzyme by solid state fermentation. The production of enzyme by solid state fermentation was carried out using isolated strains of microorganisms which produced the highest xylanase activity but lowest levels of cellulolitic enzymes. Solid culture medium was prepared by mixing equal weights of wheat bran and water and then placing 20g of this mixture in 500ml Erlenmeyer flasks, which were then sterilized in an autoclave. Spores of a selected strain from a slant culture were inoculated into the flasks and kept at 50°C for 4 days. After incubation, 100ml of water were added to each flask for enzyme extraction; the obtained extracts were filtered through filter paper.

Assays for enzyme activities. The activities of endo-1,4-β-D-xylanase (1,4-β-D-xylan xylanohydrolase, EC 3.2.1.8) and carboxy-methylcellulase or CMCase (endo-1,4-β-D-glucan glucanohydrolase, EC 3.2.1.4) were determined by incubating a mixture of 0.9ml of a 1% substrate solution in 0.1M acetate buffer pH 5.0 (larchwood xylan and carboxymethylcellulose, Sigma Co.,) and 0.1ml of adequately diluted enzyme at 60°C for 10 min. Reducing substances were then quantified using a dinitrosalicylic acid (DNS) solution. Avicelase activity (exo-1,4-β-D-cellobiohydrolase, EC 3.2.1.91) was also determined as described above, substituting substrate with avicel (Sigma Co.). Activities of exo-1,4-β-D-xylosidase (β-D-xylan xylohydrolase, EC 3.2.1.37), β-glucosidase or cellobiase (β-D-glucoside glucohydrolase, EC 3.2.1.21) and α-glucosidase were determined by incubating a mixture of 0.9ml of their respective substrates 1 mM in 0.1M acetate buffer, pH 5.0 (namely: p-nitrophenyl β-D-xylopyranoside, pNPX (Sigma); p-nitro-phenyl β-D-glucopyranoside, pNPG (Sigma)); p-nitrophenyl α-D-glucopyranoside (Koch-Light Lab.)) and 0.1ml of the adequately diluted enzyme, at 60°C for 10 min. The activity of α-L-arabinofuranosidase, EC 3.2.1.55 was determined using p-nitrophenyl-α-L-arabinofuranoside, pNPA (Sigma) as substrate. The reaction mixture, consisting of 0.5ml of 1 mM pNPA in 0.1M acetate buffer pH 5.0, 0.3ml of 0.1M acetate buffer pH 5.0 and 0.2ml of the adequately diluted enzyme, was incubated at 60°C for 10 min. All the reactions were terminated by addition of 6ml of 0.1N NaOH and the amount of p-nitrophenol released determined at 420 nm. One unit of the respective enzyme activity was defined as the amount of enzyme needed to liberate 1 umole of p-nitrophenol, xylose and glucose per minute under the assay conditions.

Purification of xylanases. The enzyme extract (1.500ml pooled from 20 Erlenmeyer flasks when obtained by solid state fermentation) was precipitated by addition of absolute ethanol to 70%vol., centrifuged and the precipitate dried. Two grams of dried precipitate (crude enzyme) were dissolved in 20ml of 0.05M acetate buffer pH 5.0 and centrifuged to discard insoluble solids. The supernatant was applied to the top of a column charged with DEAE-Sephadex A-50 which equilibrated with the same acetate buffer, and 6 ml/30 min eluted with the same acetate buffer until complete elution of the non-adsorbed protein. A saline concentration gradient, 0.1-1.0 N, was then applied to the column. Enzyme fractions obtained by DEAE-Sephadex A-50 column chromatography were dialysed against 0.05M acetate buffer pH 5.0 and then applied to a CM-Sephadex C-50 column equilibrated with the same acetate buffer. Elution was carried out as described for DEAE-Sephadex A-50 column chromatography. All samples from CM-Sephadex C-50 column chromatography were dialysed against deionized water and freeze dried.

Analysis of enzymatic action pattern. Mixtures of 9ml of 1% solutions of both purified larchwood xylan and oat spelt xylan (Sigma) in 0.1M acetate buffer pH 5.0 and 0.1ml of xylanase (0.5 unit) were incubated at 60°C for 1 hour, followed by inactivation of enzyme activity by heating. The hydrolysates were examined by descending paper chromatography using a Butanol-Pyridine-Water (6:4:3) solvent system and silver nitrate staining (12). Authentic standard samples of D-xylose, xylobiose, L-arabinose and glucose were purchased from Sigma, and xylotriose and xylotetraose were prepared as described by Lee et al (4). Two substrates (Larchwood and oat spelt xylans from Sigma Co.) were used in this study. Larchwood xylan was purified to obtain pure β-1,4-linked xylan as described by Taiz and Honingman (11), and oat spelt xylan was not purified.

SDS-Polyacrylamide gel electrrophoresis. SDS-Page was performed by the method of Weber and Osborn (14), using an electrophoresis calibration kit for molecular weight determination (Pharmacia Co.). Protein bands were stained with Comassie brilliant blue dye.

Quantitative determination of proteins. Proteins were measured by the method of Lowry et al (5), using bovine serum albumin as standard.

Xylanase treatment of pulps. Bleached Kraft eucalyptus pulps which had been prepared for making printing paper by Champion Paper & Cellulose Ltd. Mogi Guaçu, SP, Brazil, were suspended in enzyme solutions (non-purified crude enzyme, xylanase I, II and III, respectively). Each suspension, which consisted of 1.5% bleached Kraft pulp (dry base) and enzyme solution containing 1 unit of xylanase activity per ml, pH 5.0, was incubated at 50°C for 5 hr and then washed with distilled water by filtration. The viscosities of enzyme-treated pulps were measured by determining the viscosity of the 0.5% pulp solutions using 0.5M cupriethylenediamine as a solvent and the capillary viscometer described in TAPPI (Technical Association of Pulp and Pulp Industry) T-230 OM-82. Pulp brightness was measured by directional reflectance at 457 nm as described in TAPPI T-452 OM-87.

RESULTS AND DISCUSSION

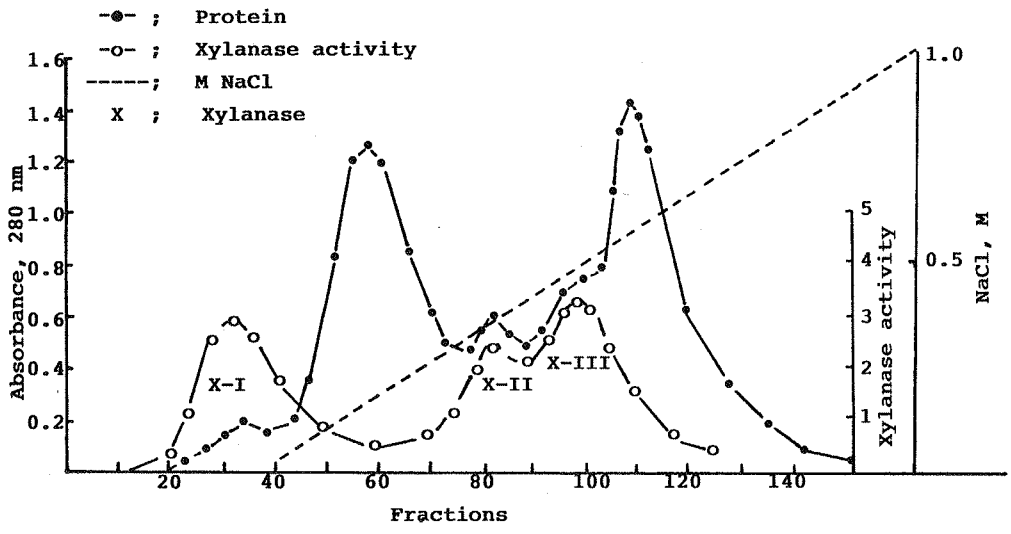
Screening and identification of the microorganisms. It was found that one strain of thermophilic fungus isolated from decayed wood of the Amazon (Manaus, Brazil) produced the highest xylanase activity when compared to other isolated thermophilic fungi. Furthermore, maximum xylanase production from this strain was obtained by solid state fermentation at 50°C for 4 days. The strain was identifiifed as a species of the genus *Humicola* based on the Manual of Thermophilic Fungi (1).

Purification of xylanase. Ethanolic precipitates from extract of wheat bran medium fermented by *Humicola* sp. demonstrated extracellular xylanase, xylosidase and arabinosidase activities, as shown in Table 1. The fungus also produced endoglucanase, β-glucosidase and α-glucosidase at levels which were low compared to those reported for other cellulose-producing microorganisms; no aviceclulase activity was detected. The ethanolic precipitates were chromatographed on DEAE-Sephadex A-50 gel as described in Materials and Methods, and three protein fractions with xylanase activity (Xylanase I, II and III) were obtained (Figure 1). Each fraction was further purified using a CM-Sephadex C50 gel (a cation exchange resin). The yields and purity of the three xylanases at each purification step were calculated and are presented in Table 2. The specific activities of Xylanase I, II and III after final purification were 585 units mg^{-1} (14.8% recovery), 430 units mg^{-1} (2.5% recovery) and 109 units mg^{-1} (2.3% recovery), respectively. Other enzyme activities were also examined and the data are shown in Table 1. It was found that xylanase I hydrolysed both oat spelt xylan and purified larchwood xylan to xylose, xylobiose and xylotriose, resembling endoxylanase (Fig. 2). Xylanase II hydrolysed p-nitrophenyl β-D-xylopyranoside, indicating xylosidase activity (exoxylanase). This result was confirmed by paper chromatography, as shown in Fig. 2; the majority of the xylooligosaccharides were converted to xylose (Fig. 2B), indicating that the enzyme had an

TABLE 1 - The activity of the purified xylanases against various substrates.

Enzyme	Ethanol precipitates	Xylanase I	Xylanase II	Xylanase III
Xylanase	23	585	430	109
Xylosidase	0.1	<0.01	2.7	<0.01
Arabinosidase	0.21	<0.01	2.0	5.5
CMCase	9.7	<0.01	14.0	270.0
Aviceclase	0.02	<0.01	<0.01	<0.01
β-Glucosidase	0.7	<0.01	5.9	2.0
α-Glucosidase	0.05	<0.01	1.6	3.0

Number represent enzyme units mg^{-1}



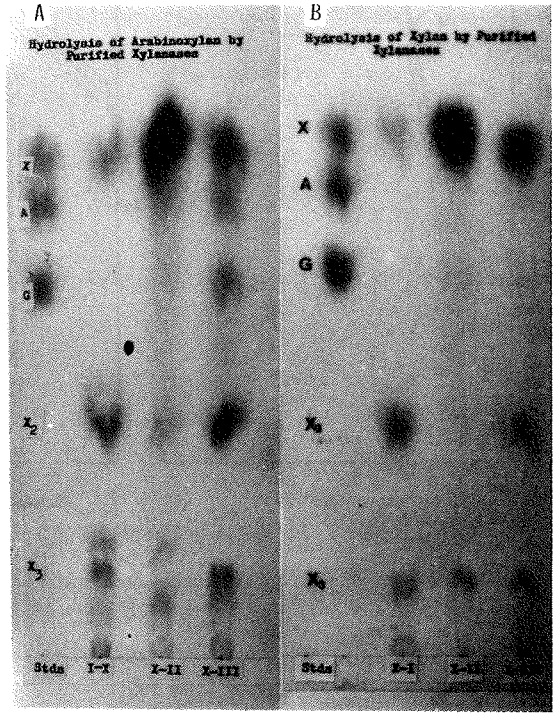
All xylanase activities were determined by using xylan

FIGURE 1 - DEAE-Sephadex A-50 column chromatograohy.

TABLE 2 - Summary of purification of xylanases from ethanol precipitates.

Purification steps	Total protein (mg)	Total activity (units)	Specific activity (units mg^{-1})	Recovery (%)
Ethanol precipitates	510	11,850	23	100
Xylanase I				
DEAE-Sephadex A-50	18	5,760	320	48.6
CM-Sephadex C-50	3	1,755	585	14.8
Xylanase II				
DEAE-Sephadex A-50	7	1,230	176	10.4
CM-Sephadex C-50	0.7	301	430	2.5
Xylanase III				
DEAE-Sephadex A-50	6.2	443	71	3.7
CM-Sephadex C-50	2.5	273	109	2.3

exo-type of function (hydrolysis of the terminal xylose of xylan) hydrolysing the terminal chains of xylooligosaccharides. The action of xylanase II is therefore similar to that of a xylosidase. On the other hand, xylanase III hydrolysed oat spelt xylan to

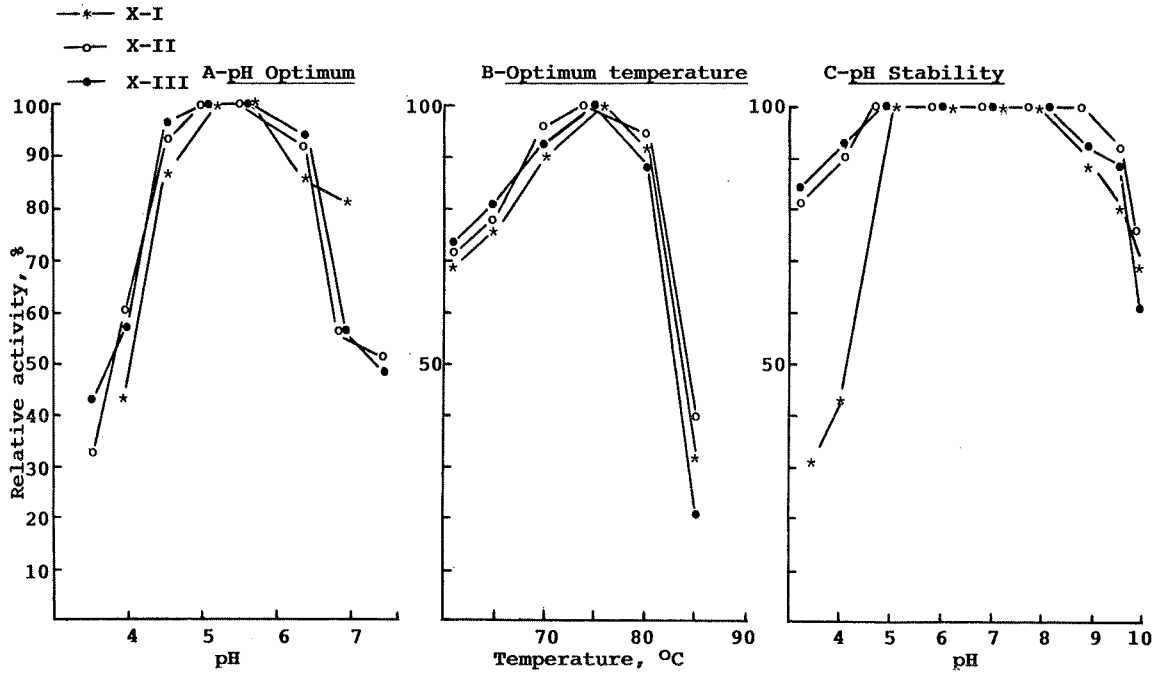


A; Oat spelt xylan
X; Xylose
G; Glucose
X_3; Xylotriose
B; Purified larchwood xylan
A; Arabinose
X_2; Xylobiose

FIGURE 2 - Paper chromatographgy of xylan hydrolysates by the purified xylanases.

remarkable amounts of xylose, xylobiose, xylotriose, arabinose and glucose (Fig. 2A), but degraded purified larchwood xylan mainly to xylose, xylobiose and xylotriose, thus resembling an endoxylanase. Formation of arabinose and glucose from oat spelt xylan is due to activities of arabinosidase and other enzymes described in Table 1. It is known that xylan consists of a β-D-1,4-D-xylopyranose backbone which commonly contains side branches of α-1,3-linked L-arabinofuranose and α-1,4-linked D-glucopyranose or its 4-O-methyl ether (15). Oat spelt xylan contains approximately 10% arabinose and 15% glucose residues according to Sigma Co., information. Xylanase III was also found to contain CMCase activity, indicating that the enzyme was not completely pure.

Enzyme treatment of bleached Kraft eucalyptus pulps. It is known that the pulp obtained by combined xylanase pretreatment and bleaching shows increased brightness and viscosity compared to conventionally bleached pulp. Xylanase pretreatment of Kraft pulp also has the advantage of allowing brightness and viscosity to be reached with reduced chlorine charge during the bleaching stage (3, 13). In the present investigation, we analysed the effect of enzyme treatment after bleaching of Kraft pulp. The bleached Kraft eucalyptus pulp, which is used for print paper making, was obtained by a five-stage process (C, Ep,H,D and H) that involves treatment with free chlorine (C), NaOH and peroxide (Ep), Calcium hypochlorite (H) and chlorine dioxide (D). The



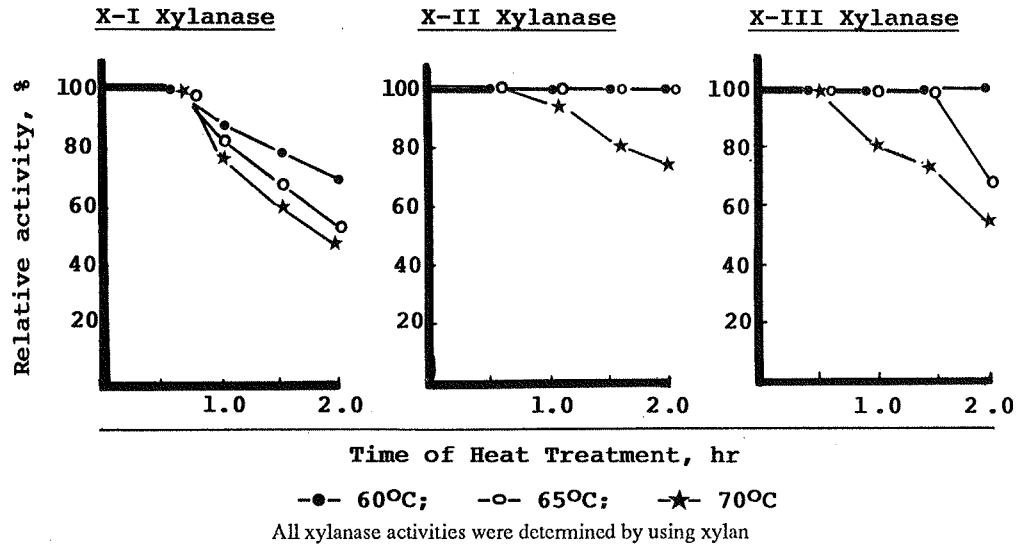
pII 3.0 - 5.6 Acetate buffer; pH 5.7 - 8.0 phosphate buffer; pH 8.6 - 10 Glycine-NaOH buffer

Determination of enzyme activity is described in the text. For pII stability, enzyme in each pH solution was incubated for 24 hrs at room temperature, and then measured enzyme activity.

FIGURE 3 - Effects of pH and temperature on xylanase activity.

Determination of molecular weight. Molecular weights as determined from the relative mobility of six standard proteins on SDS-PAGE were 30.000 for xylanase I and 43.000 for xylanase II. SDS-PAGE of xylanase III did not show a single band of protein.

bleached Kraft pulp was treated with purified xylanase I, II and III, and with ethanol precipitate (crude xylanase). The results are shown in Table 4. Enzyme-treated pulps were brighter than untreated control pulp. This was probably caused by a loss of xylan during exposure to the enzymes.



All xylanase activities were determined by using xylan

FIGURE 4 - Thermostability on xylanase activity.

TABLE 3 - Effect of metal ions and chemicals on xylanase activity.

1x10^{-3} M	Relative xylanase activity		
	X-I	X-II	X-III
Control	100	100	100
$CaCl_2$	106	123	128
$ZnCl_2$	90	93	96
KCl	100	102	100
$BaCl_2$	100	100	102
$MgSO_4.7H_2O$	66	71	72
$HgCl_2$	0	0	0
$AgNO_3$	93	35	24
$CuSO_4.5H_2O$	18	15	24
$CoCl_2.6H_2O$	85	77	84
$FeCl_3.6H_2O$	81	58	66
EDTA	55	51	58

TABLE 4 - Enzyme-treated bleached kraft Eucalyptus pulps.

	Brightness of pulp (%)	Viscosity of pulp mPás (cp)
Xylanase I	87.1	20.0
Xylanase II	87.3	16.0
Xylanase III	86.1	13.0
Ethanol precipitates	87.1	19.5
Control	85.8	19.0

Control is same as described in the method without enzyme.

Pulp viscosity was slightly higher after xylanase I and crude enzyme treatmet; xylanases II and III, on the other hand, decreased considerably the viscosity of the pulp, indicating degradation of cellulose chains. As shown in Table 1, xylanase III contained a remarkable level of CMCase activity which was only slight for xylanase II. Crude enzyme increased pulp viscosity very slightly, indicating that trace amounts of CMCase do not affect viscosity. Considering the results obtained, it is concluded that a sinple enzyme extract from culture medium (crude xylanase) can be succesfully employed for pulp treatment and improvement of paper cellulose.

RESUMO

Purificação e caracterização das xilanases termoestáveis e *Humicola* sp. e suas aplicações no melhoria da polpa

A linhagem termófila *Humicola* sp., que foi isolada de madeira em decomposição, produz xilanases extracelulares termoestáveis a 50°C. As xilanases foram purificadas e foram encontradas três frações de proteínas com atividade de xilanase. As características das três xilanases foram estudadas. Verificou-se que a xilanase I é uma endoxilanase; a xilanase II é uma xilosidase (exoxilanase) enquanto que a xilanase III é uma endoxilanase que também apresenta atividade de arabinosidase e CMCase. O tratamento da polpa "Kraft" branqueada obtida de eucalipto com enzima purificadas e bruta aumentou o brilho da polpa quando compa-

rada com a polpa sem este tratamento. Xilanase I e xilanase bruta aumentaram a viscosidade da polpa enquanto xilanase II e III diminuíram a viscosidade devido à presença de atividade CMCase.

Palavras-chave: *Humicola* sp. termófila, Xilanases termoestáveis, endoxilanases, xilosidases, Kraft polpa branqueada.

ACKNOWLEDGEMENT

The authors thank Dr. Guido N.P. Cespedes of Champion Paper Cellulose Ltd., Mogi Guaçú, SP, Brazil, for his unvaluable technical assistance and advice during this research work.

REFERENCES

1. Cooney, D.G. and Emerson, R. In Thermophilic Fungi. edited by Freeman, W.H. Publ. Co., San Francisco, pp. 72, 1964.
2. Jurasek, L. and Paice, M.C. Biological bleaching of pulp. In International Pulp Bleaching Coference, Orlando, Fla. Edited by Salvo, T.J. pp. 11-13. TAPPI Proceeding. Technical Association of the Pulp and Paper Industy, Atlanta. 1988.
3. Kantelinen, A.; Ratto, M.; Sundquist, J.; Ranua, M. Viikari, L. and Linko, M. Hemicellulases and their potential role in bleaching. In International Pulp Bleaching Conference, Orlando, Fla. Edited by Salvo, T.J. pp. 1-4, TAPPI Proceeding. Technical Association of the Pulp and Paper Industy, Atlanta. 1988.
4. Lee, S.F.; Forsberg, C.W. and Rattray, J.B. Purification and characterization of two endoxylanases from Clostridium acetobutylicum ATCC 824. *Appl. Environ. Microbiol.* **53**: 644-650, 1987.
5. Lowry, O.H.; Rosebrough, N.J.; Farr, A.L. and Randall, R.J. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193**: 265-275, 1951.
6. Mandels, M. and Sternberg, D. Recent advances in cellulose technology. *J. Ferment. Technol.* **54**: 267-286, 1976.
7. Mora, F.; Comtat, J.; Barnoud, F.; Pla, F. and Noe, P. Action of xylanases on chemical pulp fibers. Part 1; Investigation on cell-wall modifications. *J. Wood Chem. Technol.* **6**: 147-165, 1986.
8. Noe, P.; Chevalier, F.; Mora, F. and Comtat, J. Action of xylanases on chemical pulp fibers. Part II; Enzymatic beating. *J. Wood Chem. Technol.* **6**: 167-184, 1986.
9. Paice, M.C.; Bernier, R. Jr. and Jurasek, L. Viscosity-enhancing bleaching of hardwood Kraft pulp with xylanase from a cloned gene. *Biotechnol and Bioeng.* **32**: 235-239, 1988.
10. Roberts, J.C.; McCarthy, A.J.; Flynn, N.J. and Broda, P. Modification of paper properties by the pretreatment of pulp with Saccharomonospora viridis xylanases. *Enzyme Microb. Technol.* **12**: 210-213, 1990.
11. Taiz, L. and Honigman, W.A. Production of cell wall hydrolyzing enzymes by barley aleurone layers in response to gibberellic acid. *Plant Physiol.* **58**: 380-386, 1976.
12. Trevelyan, W.E.; Procter, D.P. and Harrison, J.G. Detection of sugars on papers chromatograms. *Nature*, **166**: 444-445, 1950.
13. Viikari, L.; Kantelinen, K.; Poutanen, K and Rauna, M. Characterization of pulps treated with hemicellulolytic enzymes prior to bleaching. In *Biotechnology in pulp and paper manufacture*. Butterworth-Heinemann, Stoncham, Mass. Edited by Kirk, K and Chang, H.M. pp. 145-151, 1990.
14. Weber, K. and Osborn, M. The reliability of molecular weight determination by dodecyl sulfate-polyacrylamide gel electrophoresis. *J. Biol. Chem.* **244**: 4406-4412, 1969.
15. Whistler, R.L.A. and Richards, E.L. In the Carbohydrates. Edited by Pigman, W. and Horton, D. Academic Press, New York, pp. 447-469, 1970.
16. Yu, E.K.C.; Tan, L.U.L; Chan, M.K.H.; Deschatelets, L. and Saddler, J.N. Production of thermostable xylanase by a thermophilic fungus, Thermoascus aurantiacus. *Enzyme Microb. Technol.* **9**: 16-24, 1987.

Rev. Microbiol., São Paulo, 25(2): 119-25, 1994

EFFECT OF pH AND TEMPERATURE ON *BACILLUS SUBTILIS* ATCC 601 α-AMYLASE PRODUCTION. SOME PROPERTIES OF THE CRUDE ENZYME

Terezinha de Jesus Garcia Salva[1]
Iracema O. Moraes[2]

SUMMARY

The influence of initial culture medium pH and growth temperature on *Bacillus subtilis* ATCC 601 alfa-amylase production as well as some properties of the crude enzyme were investigated. Enzyme production was highest at 37°C. The overall specific enzyme activity (E_{max}/X_{max}) showed that maximum enzyme synthesis was not due to greater microbial growth but to an increase in enzyme synthesis per cell mass unit. The study of the effect of culture medium pH on enzyme production revealed that the rise observed at pH 7 was also due to increased synthesis per cell mass unit. Optimum temperature for enzyme activity was around 50°C and optimum pH between 5.4 and 6.4. The enzyme was not very stable at 60°C and was completely inactivated after 10 minutes at 80°C. 10mM Ca^{2+} stabilized the enzyme, whereas 10mM EDTA destabilized it. EDTA action was such that the residual activity of the EDTA-treated enzyme incubated at 50°C was similar to that of the crude enzyme incubated at 80°C. The action of the enzyme on starch did not result in glucose generation. The sugars formed varied from maltose to maltoheptaose.

Key words: α-amylase, Bacillus subtilis

INTRODUCTION

The α-amylase enzymes (α-1,4 glucan 4 glucanohydrolase, EC 3.2.1.1.) are synthetized by almost all microorganisms of the *Bacillus* genus. Amongst the producers are *Bacillus subtilis, Bacillus macerans, Bacillus licheniformis, Bacillus amyloliquefaciens, Bacillus coagulans and Bacillus stearothermophilus.*

Experiments on microbial enzyme production have shown that optimal conditions for cell growth are not always adequate for enzyme production. *Bacillus licheniformis* CUM 305 did not produce α-amylase at 30°C altough it grew very well at this temperature. The strain did not grow at pH 4.0 but was able to do so and also secrete the enzyme between pH 5.0 and 10.0, with maximal production at pH 6.5 (3). Bajpai & Bajpai (1) isolated a *Bacillus licheniformis* that produced greater enzyme quantities when incubated at 35°C and pH values that ranged from 6.0 to 9.0.

Although Saito & Yamamoto (18) did not carry out a systematic investigation on the effect of the fermentation temperature on α-amylase synthesis, they studied a *Bacillus licheniformis* which produced α-amylase at temperatures around 50°C and never produced the enzyme at temperatures lower than 45°C.

With respect to enzyme properties, the thermosensibility of the α-amylase produced by the

1. Instituto de Tecnologia de Alimentos -ITAL - Av Brasil, 2880, Caixa Postal, 139 -CEP 13.073-001 - Campinas - SP - Brazil. To whom correspondence should be adressed.
2. Departamento de Engenharia de Alimentos -DETA - UNESP - SJRP, Caixa Postal, 136 - São José do Rio Preto - SP - Brazil.

Bacillus genus varies not only as a function of strain characteristics but also as a function of the reaction medium composition. Enzyme concentration (2), substrate concentration (2, 6, 8) and the concentrations of metal ions (7, 10), urea and EDTA (9, 10) may affect enzyme activity and enzyme stability at different pH values (10). The calcium ion is particularly important for α-amylase activity. All α-amylases have calcium bound to their molecules and the strength of the bond varies with the enzyme source. In the presence of calcium ions, this enzyme is more resistant to extreme pH and temperature values, to urea treatment and to the action of some proteases (16, 19).

Bacillus α-amylases have maximum activity between 50°C and 90°C (8, 11, 14, 17). There are strains belonging to this genus that produce enzymes with optimum activities at pH values as low as 3.5 or as high as 10.6 (7, 8).

The purpose of this research was to study the effect ot the initial pH of the culture medium and growth temperature on *Bacillus subtilis* ATCC 601 α-amylase synthesis, as well as some properties of the crude enzyme.

MATERIALS AND METHODS

Effect of pH of the Culture Medium and Fermentation Temperature on α-amylase Production

Microorganisms - The microorganism used throughout this study was *Bacillus subtilis* ATCC 601 maintained on nutrient agar slants at 30°C for 3 days. Stock cultures were maintained on nutrient agar slants immersed in mineral oil at 5°C.

Medium - The culture medium composition was (g/l): $(NH_4)_2SO_4$-2.5; $MgSO_4.7H_2O$-0.5; KCl-0.5; K_2HPO_4-1.0; yeast extract (Difco)-5.0; dextrin (Merck)-4.0; bacto peptone (Difco)-10.0; $CaCl_2$-0.148. $CaCl_2$ was added to the sterilized medium as a concentrated sterilized solution. The pH was adjusted to 7.0 with KOH 5N before sterilization at 121°C for 15 minutes, except when the effect of pH on enzyme production was studied.

Inoculum - A loopful of microorganisms was transferred to 100ml of growth medium contained in a 500ml Erlenmeyer flask. The culture was incubated for 18hr at 37°C in an incubator shaker, New Brunswick Sci. Co, at 200rpm.

Fermentation - This was carried out in 1000ml Erlenmeyer flasks containing 400ml of culture medium to which 20ml of inoculum adjusted to 24mg of dry cell mass with sterilized water were added. The inoculated medium was incubated for 72 hours at 37°C, 200 rpm, except for the study of the effect of temperature on α-amylase production. All the samples for enzyme assaying were collected from the same flask, the results presented to the mean of two experiments.

The effect of pH on enzyme production - In this study, the pH of the medium was adjusted to cover the 4.0 to 10.0 range using KOH 5N or HCl 5N before sterilization.

The effect of temperature on enzyme production - This study was carried out in the inoculated culture medium incubated for 72hr at 30°C, 33°C, 37°C, and 40°C at 200rpm.

α-Amylase assay - Enzyme activity was determined in the clear solution obtained by centrifuging the fermented liquid at 13.000g (10 minutes, 5°C). The dextrinizing activity was assayed by the method of Medda & Chandra (14) at 50°C and pH 6.0 using 0.1M citric acid-sodium phosphate buffer. The reaction mixture contained 0.5ml of 1% starch solution, 0.3ml of distilled water, 0.1ml of buffer and 0.1ml of enzyme solution suitably diluted. After incubation for 5 minutes, the reaction was stopped by adding 0.5ml of 1N HCl. In determining dextrinizing activity, colour was developed by adding 0.1ml of iodine solution (0.3% I_2 in 3.0% KI solution) and diluting to 15ml. The optical density of the blue-coloured solution was measured at 620nm. One unit of enzyme activity (DU) was defined as the amount of enzyme which brings about the hydrolysis of 1mg of starch per minute in the presence of 5.0mg of substrate.

Bacterial dry weight - Measurements were made in triplicates and averaged. Samples were centrifuged and the precipitates placed in preweighed pans.The precipitates were dried at 105°C to constant weight. One unit of optical density at 660nm corresponded to a bacterial dry weight of 0.6051mg/ml. During the experiments, optical density was measured and converted to dry weight.

Properties of the crude enzyme

Enzyme extract - The crude enzyme was obtained from a fermentation flask where the initial pH of the medium had been adjusted to 7.0. At the end of the incubation time, the broth was centrifuged at 13.000g for 10 minutes at 5°C. The supernatant at pH 8.4 to 8.8, dialysed or otherwise, was used in the experiments. When the experiment re-

quired a dialysed solution, this was carried out using distilled water at 5°C for 64 hr, with periodic changes of water.

Thermal stability - After incubation at appropriate temperatures, the samples were immediately immersed in an ice bath and assayed for dextrinizing activity. The effect of Ca^{2+} on the thermal stability of the enzyme was investigated by adding sufficient amounts of $CaCl_2$ to the non-dialysed enzyme solution to obtain levels of 10mM Ca^{2+} and 20mM Ca^{2+} . The effect of substrate was studied by adding 2 ml of a 2% soluble starch solution prepared in 0.1M citric acid-sodium phosphate buffer at pH 6.0 to 3ml of a non-dialysed enzyme solution.The effect of EDTA (disodium ethylenediaminetetracetic acid) was studied in a non-dialysed solution containing 10mM of the salt.

Effect of temperature on enzyme activity - The enzyme's activity profile was obtained by measuring enzymatic activities in 0.1M citric acid-sodium phosphate buffer, pH 6.0, between 30°C and 90°C.

Effect of pH on enzyme activity - The effect of pH and buffer composition on α-amylase activity was investigated by measuring enzyme activity at 50°C in different buffer solutions.

Action pattern of the enzyme on soluble starch - Five millilitres of a 2% soluble starch solution prepared in 0.1M citric acid-sodium phosphate buffer at pH 6.0 were incubated at 37°C with 2ml of dialysed enzyme solution containing 37DU/ml. The mixture was incubated at 37°C and the hydrolysis stopped using 0.2ml of 6N HCl after 24hr or 48hr. The sugars in the digestion mixture were identified by descending chromatography. Fifteen microlitres of solution were applied to Whatman nº1 paper. Column development of the chromatograms was achieved by spraying the paper strips with a solution of 22.75ml aniline, 4.15g phthalic acid, 120ml n-butanol, 120ml ethyl ether and 10ml distilled water (4).

RESULTS AND DISCUSSION

Effect of pH and Temperature on Enzyme Production

Effect of temperature - The microorganism grew well at all the temperatures assayed. Cell mass increased for up to 24hr, the maximum cell concentrations being 7.0g/l, 5.0g/l, 5.8g/l and 5.8g/l at 30°C, 33°C, 37°C and 40°C, respectively. The maximum enzyme concentration in the broth was obtained at 30°C and 33°C at time 48hr and at 37°C and 40°C at time 72hr. Enzyme inactivation was not observed under any of these conditions. The greatest enzyme production occurred at 37°C. At this temperature, the enzyme concentration in the culture broth after 72hr was 20DU/ml. The lowest enzyme production was observed at 40°C with a maximum of 8DU/ml (Fig. 1). The overall specific enzyme activity, E_{max}/X_{max}, was highest at 37°C (32DU/mg) with the lowest value also occurring at 40°C (11DU/mg) (Fig. 1), thus showing that the increased enzyme production at 37°C was not due to greater microbial growth but to higher enzyme synthesis per cell mass unit. Figure 1 also shows that there is a smaller difference in the overall productivity ($E_{max}/X_{max}/t$) at 33°C and 37°C than there is in the overall specific enzyme activity at these temperatures. This result was a consequence of the shorter fermentation time required to reach maximum enzyme activity at 33°C. Both overall specific enzyme activity and overall productivity indicated that an appropriate temperature may stimulate the microbial cell to produce the enzyme.

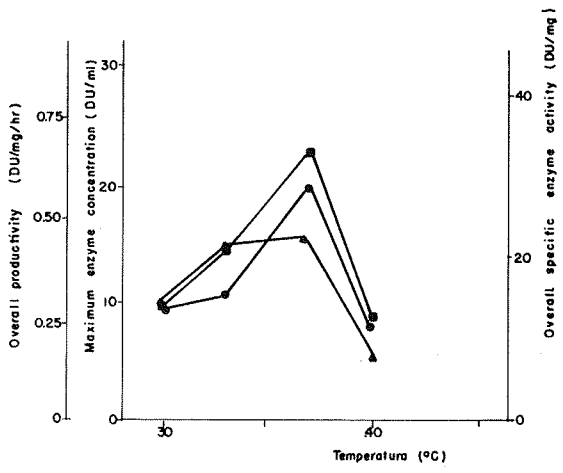


FIGURE 1 - Effect of growth temperature on α-amylase production —●— maximum enzyme concentration; —■— overall specific enzyme activity; —▲— overall productivity.

Effect of initial pH - *Bacillus subtilis* ATCC 601 strain did not grow in the culture media adjusted to pH 4.0, 5.0 and 10.0. Autoclaving caused a pH reduction of 0.3 pH units in all culture media. In those media where bacterial growth occurred, pH increased after 10 hours of fermentation, never reaching values greater than 9.0. The enzyme activity of the broth increased during the 72 hours of

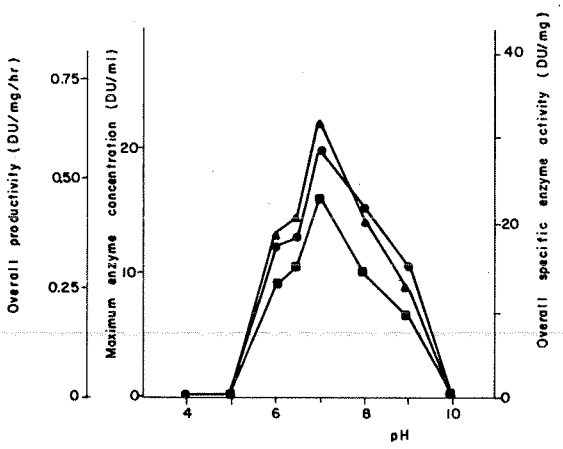


FIGURE 2 - Effect of the initial pH of the culture medium on α-amylase production —●— maximum enzyme concentration; —▲— overall specific enzyme activity; —■— overall productivity.

incubation at all pH values. The greatest enzyme production occurred at pH 7.0 (20DU/ml - Fig. 2) and was associated with the greatest overall specific enzyme activity (32DU/mg - Fig. 2) and overall productivity (0.45DU/mg/hr - Fig. 2). The same was observed with the lowest enzyme production (10DU/ml - Fig. 2) which was associated with the lowest overall specific enzyme activity (13DU/mg - Fig. 2) and lowest overall productivity (0.17DU/mg/hr - Fig. 2). These results show that there is a stimulation of enzyme synthesis at pH 7.0 and that the higher enzyme production at this pH was not a result of increased cell growth.

Properties of the crude enzyme

Thermal stability - The enzyme extract retained 100% activity when incubated for 24 hours at 37°C (Fig. 3). Poor stability was observed at 60°C and the activity was completely lost after 10 minutes at 80°C (Fig. 4). At 37°C, the non-dialysed enzyme extract was more stable than the dialysed extract, probably due to the removal of the Ca^{2+} cofactor and Mg^{2+} ions during dialysis. Starch stabilized the enzyme after one hour of heating, which could be important in the hydrolysis of starch by this enzyme. The addition of 10mM and 20 mM Ca^{2+} also stabilized the enzyme, as shown in Figure 3. After 24 hours at 50°C, the enzyme retained 64% and 100% of its initial activity in the presence of 10mM and 20mM Ca^{2+}, respectively, whereas it exhibited only 25% residual activity in the absence of the ion. As shown in Figure 4, the enzyme was less thermostable in the presence of 10mM EDTA, so that the stability of the EDTA-treated enzyme incubated at 50°C was similar to that recorded at 80°C. In the absence of EDTA, the enzyme retained, for example, 98% of its activity after 40 minutes at 50°C; however, in the presence of the chelant, the enzyme preserved only 9% of its activity after 20

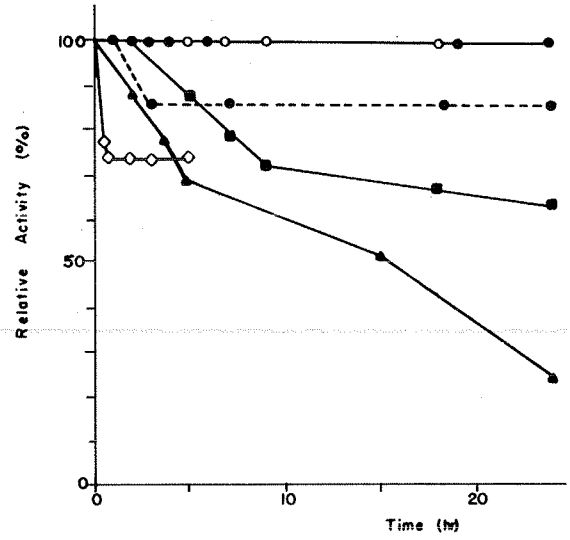


FIGURE 3 - Thermal stability of the *Bacillus subtilis* ATCC 601 α-amylase at pH 6,0 —●— 37°C; —▲— 50°C; —■— 50°C with 10 mM Ca^{+2}; —O— 50°C with 20 mM Ca^{+2}; —◊— 50°C with 1,3% soluble starch; ---●--- dialysed solution at 37°C.

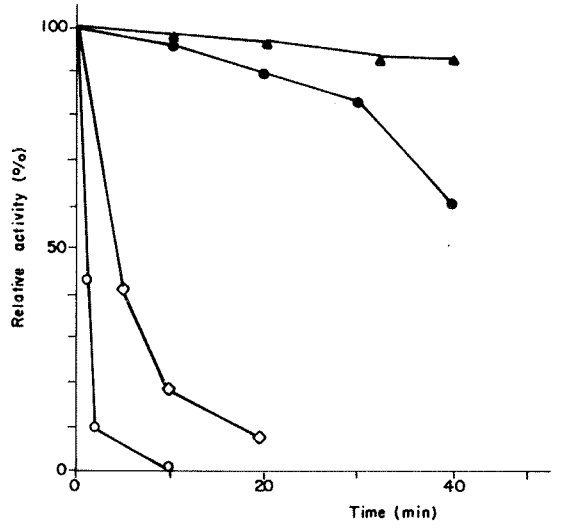


FIGURE 4 - Thermal stability of the *Bacillus subtilis* ATCC 601 α-amylase at pH 6,0 —●— 60°C; —▲— 50°C; —◊— 50°C with 10 mM EDTA; —O— 80°C.

minutes at the same temperature. Both the Ca^{2+} and the EDTA results reflect the action of the calcium ion as an α-amylase stabilizer, since the deleterious effect of EDTA is due to its ability to bind the Ca^{2+} of the enzyme molecule. Although the intensity of the effect of both Ca^{2+} and EDTA may vary with enzyme source, one may say that the observed behaviour is very commom among *Bacillus* enzymes. The calcium ion normally increases α-amylase thermal stability whereas EDTA reduces it (3, 10, 14, 15).

Effect of temperature on enzyme activity - Enzyme activity increased with temperature within the range of 30°C to 50°C. A reduction in enzyme activity was observed at values above 50°C, at a rate of 35% for each increase of 10°C in the hydrolysis temperature (Fig. 5). The optimum temperature of this α-amylase was around 50°C, which is lower than that described for other crude (10, 15) or purified (5, 20) α-amylases produced by *Bacillus subtilis*. Those other crude enzymes mentioned in the literature showed optimum temperature values between 60°C and 70°C at pH 5.8 to 6.0.

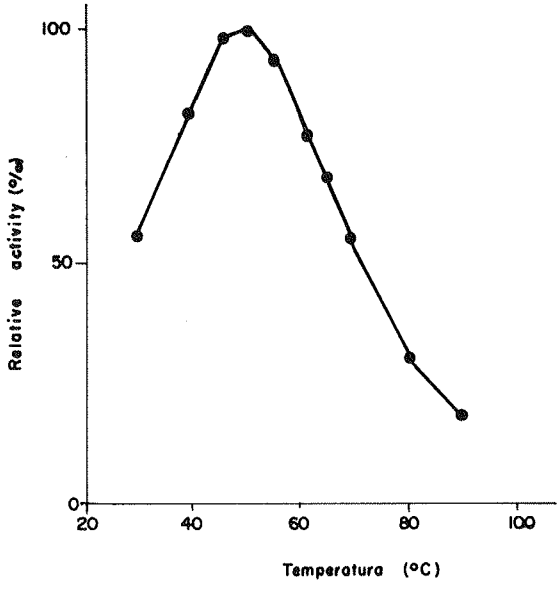


FIGURE 5 - Effect of temperature on *Bacillus subtilis* ATCC 601 α-amylase activity at pH 6,0.

Effect of pH on enzyme activity - The optimum pH for enzyme activity was between 5.4 and 6.4. There was a nearly 15% reduction in maximum activity at pH 5.0 or 7.0 (Fig. 6). The enzyme activity of the extract varied not only with buffer pH but also with buffer composition. At pH 6.0, 96% of the maximum activity was observed in citric acid-sodium phosphate buffer as opposed to 86% in citric acid-sodium citrate buffer. Since the ionic strength of the latter buffer at pH 6.0 was 0.535 whereas that of the citric acid-sodium phosphate buffer was 0.278, it is possible that such difference affects enzyme activity, but more detailed studies will have to be carried out to confirm this hypothesis.

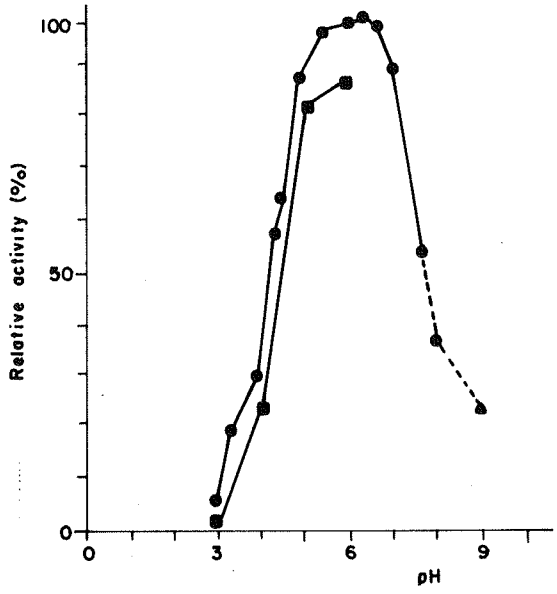


FIGURE 6 - Effect of pH on the *Bacillus subtilis* ATCC 601 α-amylase at 50°C; —■— citric acid - trisodium citrate buffer; —●— citric acid-sodium phosphate buffer; ---●--- phosphate buffer; --▲--- borate buffer.

Products of starch hydrolysis - Figure 7 shows that the action of the enzyme on starch resulted in the production of maltose to maltoheptaose oligosaccharides after either 24hr or 48hr. The action of *Bacillus subtilis* ATCC 601 α-amylase on soluble starch did not result in glucose generation in amounts detectable by paper chromatography, resembling the α-amylase of *Bacteroides amylophilus* studied by *McWethy* & Hartman (13).

RESUMO

Efeito do pH e temperatura na produção de α–amilase por *Bacillus subtilis* ATCC 601. Algumas propriedades da enzima bruta

Neste trabalho foram estudadas a influência do pH inicial do meio de cultura e da temperatura

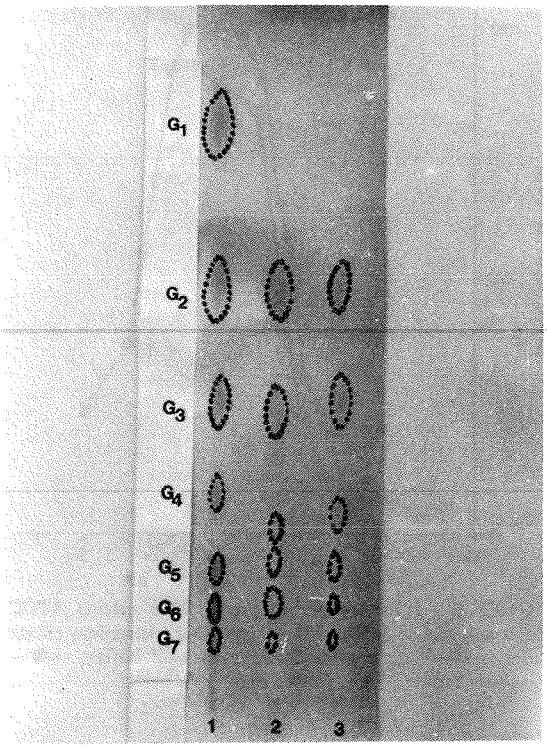


FIGURE 7 - Chromatografic analysis of *Bacillus subtilis* ATCC 601 α-amylase action on soluble starch. G_1, G_2, G_3, G_4, G_5, G_6, and G_7 are glucose, maltose, maltotriose, maltotetrose, maltopentaose, maltohexaose and maltoheptaose respectively. Digestion was performed with crude amylase for 24hr (2) and 48hr (3).

de crescimento sobre a produção de a-amilase por Bacillus subtilis ATCC 601, bem como algumas propriedades da enzima bruta. Houve maior produção de enzima quando a fermentação foi realizada a 37°C. A atividade enzimática específica global, E_{max}/X_{max}, mostrou que a maior concentração enzimática no caldo de cultura fermentado a 37°C não foi devida a um maior crescimento celular e sim a uma maior síntese de enzima por unidade de massa celular. O estudo do efeito do pH inicial do meio de cultura sobre a produção da enzima mostrou que ela ocorreu preferencialmente a pH 7,0, também devido a um estímulo da síntese por unidade de massa celular. A temperatura ótima da enzima está ao redor de 50°C e o seu pH ótimo está entre 5,4 e 6,4. A α-amilase de *Bacillus subtilis* ATCC 601 mostrou-se pouco estável a 60°C foi completamente inativada quando mantida durante 10 minutos a 80°C. Íons Ca^{+2}, nas concentrações de 10mM e 20mM, estabilizaram sensivelmente a enzima, enquanto que a adição de EDTA 10mM a desestabilizou de tal modo que a enzima apresentou a 50°C um comportamento semelhante ao observado a 80°C. Não houve formação de glicose quando o amido foi hidrolisado por esta α-amilase. Os açúcares formados variaram de maltose à maltoheptaose.

Palavras-chave: α-amilase, *Bacillus subtilis*.

REFERENCES

1. BAJPAI, P. & BAJPAI, P.K. High temperature alkaline α-amylase from *Bacillus licheniformis* TCRDC-B13. *Biotechnol. Bioeng.* **33**: 72-78, 1989.
2. BUONOCORE, V.; CAPORALE, C.; ROSA, M. & GAMBACORTA, S. Stable, inducible thermoacidophilic α-amylase from *Bacillus acidocaldarius*. *J. Bacteriol.*, **128**(2): 515-521, 1976.
3. CHANDRA, A.K.; MEDDA, S. & BHADRA, A.K. Production of extracellular thermostable α-amylase by *Bacillus licheniformis*. *J. Ferment. Technol.*, **58**(1): 1-10, 1980.
4. FRANCIS, F.J.; DRAETTA, I.S.; BALDINI, V.L.S. & IADEROZA, M. New anthocyanins from *Tibouclina granulosa*. *J. Am. Soc. Hortic. Sci.* **107**(5): 789-791, 1983.
5. GANDHI, A.P. & KJAERGAARD, L. Effect of carbon dioxide on the formation of α-amylase by *Bacillus subtilis* growing in continuous and batch cultures. *Biotechnol. Bioeng.*, **17**: 1109-1118, 1975.
6. GRUENINGER, H.; SONNL, B. & FIECHTER, A. Bacterial diversity in thermophilic aerobic sewage sludge. III. A source of organisms producting heat stable industrial useful enzymes, e.g. α-amylase. *Appl. Microbiol. Biotechnol.*, **19**(6): 414-422, 1984.
7. HAYASHI, T.; ABIBA, T.; HORIKOSHI, K. Properties of new alkaline maltohexaose forming amylases. *Appl. Microbiol. Biotechnol.*, **28**(3): 281-285, 1988
8. HORIKOSHI, K. Production of alkaline enzymes by alkalophilic microorganisms. Part II. Alkaline amylase produced by *Bacillus* nº A-40-2. *Agric. Biol. Chem.* **35**(11): 1783-1791, 1971.
9. JUNGE, J.M.; STEIN, E.A.; NEURATH, H. & FISHER, E.H. The amino acid composition of α-amylase from *Bacillus subtilis*. *J. Biol. Chem.*, **234**: 556-561, 1989.
10. KENNEDY, F. & WHITE, C.A. Characteristics of α-amylase K, a novel amylase from a strain of *Bacillus subtilis*. *Starch*, **31**(3): 93-99, 1979.
11. KRISHNAN, T. & CHANDRA, A.K. Purification and characterization of α-amylase from *Bacillus licheniformis* CUM305. *Appl. Env. Microbiol.*, **46**: 430-437, 1983.
12. MANNING, G.B.; CAMPBELL, L.L. & FOSTER, T.J. Thermostable α-amylase of *Bacillus stearothermophilus*. III. Physical properties and molecular weight. *J. Biol. Chem.*, **236**(11): 2958-2961, 1961.
13. MCWETHY, S.J. & HARTMAN, P.A. Purification and some properties of an extracellular α-amylase from *Bacteroides amylophilus*. *J. Bacteriol.*, **129**(3): 1537-1544, 1977.
14. MEDDA, S. & CHANDRA, K. New strains of *Bacillus licheniformis* and Bacillus coagulans producing thermostable α-amylase active at alkaline pH. *J. Appl. Bacteriol.*, **48**: 47-58, 1980.

15. MOSELEY, M. & KEAY, L. Purification and characterization of the amylase of *Bacillus subtilis* NRRL3411. *Biotechnol. Bioeng.*, **12**: 251-271, 1970.
16. OGASAHARA, K.; IMANISHI, A. & ISEMURA, T. Studies on thermophilic α-amylase from *Bacillus stearothermophilus*. II. Thermal stability of thermophilic α-amylase. *J. Biochem.*, **67**(1) 77-82, 1970.
17. SAITO, N. A thermophilic extracellular α-amylase from *Bacillus licheniformis*. *Arch. Biochem. Biophy.*, **155**: 290-298, 1973.
18. SAITO, N. & YAMAMOTO, K. Regulatory factors affecting α-amylase production in *Bacillus licheniformis*. *J. Bacteriol.*, **121**(3): 848-856, 1975.
19. STEIN, E.A. & FISCHER, E.H. The resistence of α-amylase towards proteolitic attack. *J. Biol. Chem.* **232**: 867-879, 1958.
20. WELKER, N. E. & CAMPBELL, L.L. Comparation of the α-amylase of *Bacillus subtilis* and *Bacillus amyloliquefaciens*. *J. Bacteriol.*, **94**(4): 1131-1135, 1967.

Rev. Microbiol., São Paulo, 25(2): 126-8, 1994

RHEOLOGICAL BEHAVIOR STUDIES ON *ASPERGILLUS* SP. SUBMERGED CULTURE

Willibaldo Schmidell[1]
Estevão Rosim Fachini[2]
Maria Cândida Reginato Facciotti[1]
Beatriz Vahan Kilikian[1]

SHORT COMMUNICATION

SUMMARY

The authors report preliminary results concerning the rheological behavior of culture medium during submerged culture of *Aspergillus* sp. The broth was pseudoplastic and, according to the "power law", the flow behavior index (n) was almost constant above a cell concentration of 5g/l, while the consistency index (K) exhibited an exponential behavior.

Key words: rheological behavior; glucoamylase production; *Aspergillus* sp. culture.

Rheological studies involving non-Newtonian fermentation broth, particularly those related to the growth of microorganisms in the mycelial form, are rather scarce in the specialized literature (1, 5). Nevertheless, this kind of data is of great importance for an adequate estimation of power inputs in a typical stirred reactor. In fact, some recent publications have proposed the control of fermentation processes through on-line rheological measurements (4).

The present study was undertaken to determine rheological characteristics during submerged culture of *Aspergillus awamori* NRRL 3112 in a culture medium containing cassava flour as main carbon source.

Fungal growth was carried out in a 10-litre fermenter at a controlled temperature of 35°C and pH 4.0. Aeration flow rate and agitation frequency were 10 $l.min^{-1}$ and 700 min^{-1}, respectively, maintaining an internal pressure of 1.2 atm.

The methodology employed for culture medium preparation, inoculum growth and analytical treatment of samples are described elsewhere (3).

The rheological properties of the culture broth were determined for all collected samples, employing a Brookfield Viscometer (Brookfield Engineering Lab., Inc., USA, Model LVT).

As it is normal practice in the pertinent literature (1), generally, for a pseudoplastic fluid, the "power law" permits a very good fit to experimental data, being it formerly stated as:

$$\tau = K\,(dv/dx)^n \qquad \text{Equation 1}$$

where: τ = shear stress (dyne.cm^{-2} or g.cm^{-1} . sec^{-2})
(dv/dx) = rate of shear (sec^{-1})
K = consistency index (g.cm^{-1}. sec^{n-2})
n = flow behavior index

1. Escola Politécnica da Universidade de São Paulo - Depto. de Engenharia Química, Caixa Postal 61548 - CEP 05424-970 - São Paulo - SP - Brasil.
2. Bolsista da Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP).

Defining apparent viscosity, ηa as the relashionship bewtween a certain measured shear stress (1) corresponding to an imposed rate of shear (dv/dx), it is possible to state that:

$$\eta_a = K\,(dv/dx)^{n-1} \qquad \text{Equation 2}$$

$$\text{or: } \log\eta_a = \log K + (n-1)\log(dv/dx) \qquad \text{Equation 3}$$

In a typical rotational viscometer, it is possible to obtain different values for ηa by submitting fluid samples to different values of (dv/dx), which are, in turn, directly correlated with the rotational speed (N).

The experimental results were fitted quite well by Equation 3, thus permitting the determination of parameters n and K.

The obtained values of n and K were plotted against cell mass concentration (X), as shown in Figure 1.

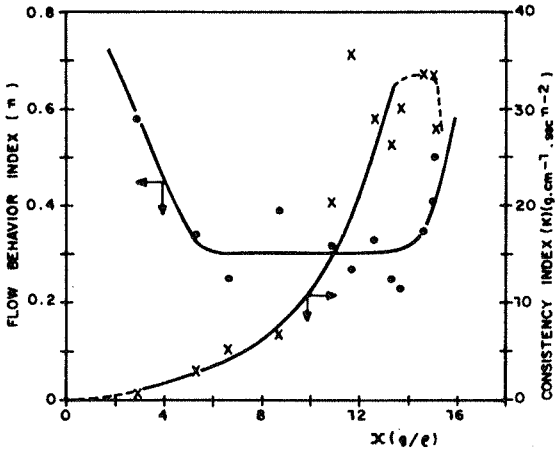


FIGURE 1 - Flow behavior index n (●), and consistency index K (x) as a function of the cell mass concentration X (solid line for K following equation 4).

It can be observed that there is a sharp decrease in the values of n at the beginning of the process, until X is close to 5g/l. Subsequent to this initial behavior, n becomes nearly constant at around 0.3 until exhaustion of the carbon source, exhibiting a tendency to increase after this period.

On the other hand, K values are very low at the beginning of the culture but, for values of X above 5 g/l, start to increase sharply, leading thus to a highly viscous broth.

The observed behavior for both n and K is quite similar to the data presented by Taguchi (5) on culture of *Endomyces* sp.

The experimental values of K as a function of X suggest an exponential behavior, as can be seen in Figure 1. Based on this observation, the following equation was fitted to the experimental values:

$$K = 0.51\, e^{0.31\,X} \qquad \text{Equation 4}$$

As proposed by Calderbank & Moo-Young (2), it is possible to define a modified Reynolds number (N'_{Re}) for pseudoplastic fluids, such as:

$$N'_{Re} = \frac{Di^2\, n^{2-n}\, \rho \left(\frac{n}{6n+2}\right)^n}{0.1\,K} \qquad \text{Equation 5}$$

where: Di = impeller diameter (cm)
N = impeller rotational speed (sec^{-1})
ρ = fuid density ($g.cm^{-3}$)

As discussed before, the flow behavior index (n) can be considered nearly constant (n = 0.3) during most part of the culture process, while the consistency index (K) can be estimated through Equation 4. In this way, assuming that p = 1.0 g. cm^{-3}, the modified Reynolds number for the *Aspergillus* sp. culture broth can be expressed as follows:

$$N'_{Re} = \frac{4.67\, Di^2\, n^{1.7}}{0.51\, e^{0.31X}} \qquad \text{Equation 6}$$

The above equation can be very useful, since it makes possible the estimation of N'_{Re} as a function of cell concentration (X) for a certain set of values of Di and N.

Through the estimates of N'_{Re} it is then possible to predict the power input in a non-aerated system employing for this purpose the empirical correlation proposed by the previously mentioned Calderbank & Moo-Young (2).

Further rheological studies on *Aspergillus* sp. submerged culture are presently being carried out in our laboratory, employing several different conditions of both air flow rate and agitation frequency.

RESUMO

Estudo do comportamento reológico no cultivo de *Aspergillus* em cultura submersa

Apresentam-se resultados preliminares relativos ao comportamento reológico observado durante o cultivo submerso de *Aspergillus*. O caldo comportou-se como pseudoplástico, e de acordo com a lei da potência, o índice de comportamento do fluxo (n) manteve-se aproximadamente constante para concentrações celulares acima de 5 g/l, enquanto o índice de consistência (K) apresentou um comportamento exponencial.

Palavras-chave: comportamento reológico, produção de glicoamilase, cultivo de *Aspergillus*.

REFERENCES

1. Aiba, S.; Humphrey, A.E. & Millis, N.F. - Biochemical Engineering. New York, Academic Press Inc., 1973.
2. Calderbank, P.H. & Moo-Young, M. - The prediction of power consumption in the agitation of non-Newtonian fluids. *Trans. Inst. Chem. Eng., Lond.*, **37**: 26-33, 1959.
3. Facciotti, M.C.R.; Kilikian, B.V.; Schmidell, W. & Fachini, E.R. - Processo descontínuo de síntese de amiloglicosidase por *Aspergillus awamori*: influência do pH e da concentração inicial de polissacarídeo. *Rev. Microbiol.*, São Paulo, **20**(1): 108-114, 1989.
4. Olsvik, E.S. & Kristiansen, B. - On-line rheological measurements and control in fungal fermentations. *Biotechnol. Bioeng.*, **40**: 375-387, 1992.
5. Taguchi, H. & Miyamoto, S. - Power requirement in non-Newtonian fermentation broth. *Biotechnol. Bioeng.*, **8**: 43-54, 1966.

Rev. Microbiol., São Paulo, 25(2): 129-35, 1994

OPTIMAL CONDITIONS FOR SCP PRODUCTION BY MIXED CULTURES OF *A. NIGER* AND *CR. LAURENTII* GROWN IN SUGAR CANE VINASSE MEDIUM

Sandra Regina Ceccato-Antonini[1]
Sâmia Maria Tauk-Tornisielo[2]

SUMMARY

Mixed cultures of *Aspergillus niger* and *Cryptococcus laurenti* grown in sugar cane vinasse medium under continuous agitation were studied in order to establish optimal conditions (carbon:nitrogen:phosphorus ratios; carbohydrate concentration; initial pH; incubation temperature; incubation period) for biomass production and biological depuration of vinasse, a waste product from the ethanol industry. Pure (single-species) cultures of each microorganism were also analysed. Addition of nitrogen and phosphorus to vinasse medium altered significantly biomass production, carbohydrate consumption and BOD reduction. Protein content of biomass was greatly increased and BOD reduction diminished at carbohydrate concentrations higher than 3 gl^{-1}. Both initial pH and incubation temperature had little influence on BOD reduction (most levels at around 50%). Vinasse pH was always around 7.0 after 48h of incubation. Mixed cultures of *A. niger* and *Cr. laurentii* in sugar cane vinasse medium modified for optimal growth (20: 3.0: 0.1 C:N:P ratio; 8 gl^{-1} carbohydrate content; pH 4.6; 30°C) reached maximal biomass concentrations after 24 hours of incubation; best results for BOD reduction (around 80%) and protein yields (40%) were recorded after 72 h of culture. Compared to pure cultures, the optimized mixed cultures of *A. niger* and *Cr. laurentii* produced lower concentrations of biomass.

Key Words: vinasse, fungi, SCP.

INTRODUCTION

Vinasse (or stillage) is a waste product generated during ethanol production, and its current disposal poses environmental problems. The product is a dark, viscous, concentrated liquid with low dissolved oxygen content, high turbidity and low pH. However, due to its rich organic and mineral contents and absence of toxic substances (20), vinasse can be viewed as potential raw material and many procedures have been suggested to convert it into useful products such as microbial protein.

Microalgae (9), bacteria (13), filamentous fungi (12), mushrooms (3) and yeasts (20) have been grown in vinasse either for biomass generation or to decrease biochemical oxygen demand (BOD). Attempts to reach high yields have been made by supplementing culture media with nitrogen, using mixed cultures and a two-stage process.

The use of mixed cultures for protein convertion is a particularly attractive prospect and good

1. Departamento de Tecnologia Agroindustrial e Socio-Economia Rural, Centro de Ciências Agrárias, Universidade Federal de São Carlos-Campus de Araras, Caixa Postal 153, CEP 13600-970, Araras, Brazil.
2. Centro de Estudos Ambientais (CEA) e Departamento de Ecologia, Instituto de Biociências, UNESP-Campus de Rio Claro, Caixa Postal 199, CEP 13506-900, Rio Claro, SP, Brazil.

results have been obtained with a two-stage process involving the growth of filamentous fungi and yeasts. A 93% reduction in chemical oxygen demand (COD) and a cell concentration equivalent to 24 $g.l^{-1}$ of dried biomass have been attained (22).

In a previous screening study, the combination of *Aspergillus niger* isolated from vinasse and *Cryptococcus laurentii* isolated from soil produced significantly more biomass in fermentation experiments than pure cultures (4). In this paper, we report the effects of carbon, nitrogen and phosphorus ratios, carbohydrate concentration, initial pH, temperature and time of incubation on the growth of mixed cultures of *A. niger* and *Cr. laurentii* under continuous agitation to evaluate their possible use as source of single-cell protein (SCP). Estimates on vinasse depuration by the microorganisms are also analysed.

MATERIALS AND METHODS

Media. The physical and chemical characteristics of sugar cane vinasse from Usina São João, Araras, São Paulo, were: pH = 4.2; BOD 16,000 mg O_2/l; nitrogen 0.059%; Ca^{+2} 0.054%; Mg^{+2} 0.011%; K^{+} 0.09%; PO_4^{-3} 0.0006%; total carbohydrate 0.6%; organic matter 1.25%. The composition of culture media used are shown in Table 1. Vinasse was supplemented with ammonium sulphate, phosphoric acid and molasses to reach the desired C:N:P ratios. After pH adjustmment with 1N NaOH or 1N HCl, final media were pasteurized at 85°C for 20 minutes and cooled to room temperature.

TABLE 1 - Composition of vinasse media and levels of treatment for each parameter tested.

	Parameters		
Medium	C:N:P ratio	Carbohydrate concentration	Initial pH
I	a	3 gl^{-1}	4.6
II	20:3.0:0.1	b	4.6
III	20:3.0:0.1	8 gl^{-1}	c

a = 20:3.0:0.1; 24:3.0:0.1; 27:3.0:0.1; 30:3.0:0.1
b = 3, 8, 20 and 32 gl^{-1}
c = 3.5; 4.0; 4.3; 4.6; 4.9; 5.2; 5.5

Microorganisms, inocula and culture conditions. *A. niger* was originally isolated from vinasse and *Cr laurentii* from soil. 0.5 cm^2 of *A. niger* mycelia from four-day stock slants on Sabouraud-dextrose agar were transferred to 250 ml Erlenmeyer flasks containing 7.5 or 15 ml of vinasse medium for mixed and pure cultures, respectively. Loops of *Cr. laurentii* cells from three-day stock slants on Sabouraud-dextrose agar were transferred to 250 ml Erlenmeyer flasks containing 50 ml of vinasse medium. All flasks were incubated on a rotary shaker (250 rpm) at 30°C for 24-48 hours, or at 25°C, 30°C or 35°C in experiments where the influence of temperature was being investigated. The suspensions of inocula were handled separately and each sown to give final mixed-culture concentrations and pure-culture concentrations of 7.5% and 15% (vol/vol), respectively.

Culture conditions for the fermentation experiments were as follows: for optimization of C:N:P ratios, carbohydrate concentrations and initial pH, 500-ml Erlenmeyer flasks containing 100 ml of vinasse media (I, II or III, see Table 1) were incubated on a rotary shaker (250 rpm) for 48h at 30°C. For the incubation temperature tests, flasks containing 100 ml of vinasse medium III (pH 4.6) were kept at 25°C, 30°C and 35°C. Growth curves for pure and mixed cultures were established in 100 ml of vinasse medium III (pH 4.6) at 30°C for a period of 72 hours.

Analytical procedures. Samples (i.e. flask contents) were vacuum-filtered through Whatman paper (no 1) and the cells centrifuged at 10.000 g for 20 minutes at 5°C. The pellet was then resuspended in distilled water and centrifuged once more under the same conditions. Both sediment and filtrate were dried overnight at 105°C. Protein content was quantitated by the Kjeldahl method for total nitrogen multiplied by 6.25 in the dry biomass (1). Total carbohydrate content was determined by the anthrone method (23). Final culture pH was measured with a digital pH-meter. Soluble biochemical oxygen demand (BOD) was determined by the incubation method at 20°C for 5 days (2).

Statistical Analysis. The Bartlett's test of homogeneity of variance (16) was applied, followed by the Kruskal-Wallis test or the one-way analysis of variance for the heterogeneous and homogeneous variances, respectively (17). When these tests rejected the multisample hypothesis of equal means, the non-parametric multiple comparison (NMC) or the Student-Newman-Keuls (SNK) test were then applied to the means with heterogeneous or homogeneous variances, respectively (24). All tests were analysed at the 1% significance level.

RESULTS AND DISCUSSION

As shown in Table 2, highest biomass production and BOD reduction were obtained in vinasse medium with a C:N:P ratio of 20: 3.0: 0.1. Final culture pH, carbohydrate consumption and protein content were not significantly affected by C:N:P ratios (except for a lower carbohydrate consumption in medium with a 30: 3.0: 0.1 ratio). Previous studies have demonstrated that nitrogen and phosphorus supplementation of medium by salts and mineral acids can increase biomass and/or protein content in cultures of yeasts and filamentous fungi grown in vinasse (14, 19, 21).

progressively as carbohydrate levels increased in the medium. A similar finding has been described for dilution of rum distillery slops, which led to an increase in BOD reduction from 56% (non-diluted media) to 75% (diluted media) (10).

Attempts to establish ideal concentrations of solids (solube or total) in vinasse are scarce. Maximun vinasse carbohydrate content for *Candida* sp. was set at 1% to avoid alchool production (15). Almost all the parameters analysed herein pointed to 8 gl^{-1} as the optimal carbohydrate concentration in vinasse medium for the species studied.

Biomass production was maximal when the initial pH ranged between 3.5 and 4.0 (Table 4).

TABLE 2 - Effect of variation in C:N:P ratios on growth of mixed cultures of *A. niger* and *Cr. laurentii* in vinasse medium[1].

C:N:P ratios	Biomass (gl^{-1})	Protein (%)	Final pH	Carbohydrate consumption (%)	BOD reduction (%)
20:3.0:0.1	6.71 b ± 0.93	22.9 a ± 1.1	5.5 a ± 2.1	77.6 b ± 6.3	78.1 b ± 3.6
24:3.0:0.1	4.70 c ± 0.44	19.9 a ± 2.6	4.5 a ± 1.0	78.5 bc ± 5.2	62.5 a ± 11.4
27:3.0:0.1	4.24 ac ± 0.09	21.7 a ± 0.9	5.6 a ± 1.0	81.2 b ± 8.3	60.9 a ± 3.1
30:3.0:0.1	3.10 a ± 0.45	22.4 a ± 2.6	4.9 a ± 0.7	62.3 ac ± 6.6	53.1 a ± 3.6

1 Culture conditions: carbohydrate concentrations of vinasse medium: 3 gl^{-1}; T = 30°C; N = 250 rpm; pH = 4.6; time of incubation = 48 hours.
Means values (n=4) followed by the same letters in the columns did not differ statistically at the 1% significance level according to the SNK or NMC tests.

According to the present data, protein content of the biomass was not affected by C:N:P ratios but varied as a function of carbohydrate availability, reaching about 40% at carbohydrate concentrations of 8 gl^{-1} or higher (Table 3). Carbohydrate consumption peaked but biomass production showed a significant decrease at a carbohydrate concentration of 8 gl^{-1}. The differences found between mean carbohydrate consumption values may indicate the existence of low efficiency mechanisms for carbon uptake and metabolism when this element is present in excess, as described elsewhere (8). Likewise, BOD reduction diminished

Given that spores of *A. niger* are known to be more sensitive to low pH than vegetative cells (7), the ability of the mixed cultures to grow at lower pH values may be explained by the fact that the authors used the latter as inoculum. Protein content of the biomass, on the other hand, built up significantly with greater initial pH values, reaching 46% at pH 4.9. Alcalinization of the medium was observed from initial pH values of 4.6 upwards. The increase in pH observed during fungal growth in a substrate can be due to anion absorption (e.g. organic acids) or ammonium production from nitrogen compounds (7). Both possibilities may ex-

TABLE 3 - Effect of initial carbohydrate concentrations on growth of mixed cultures of *A. niger* and *Cr. Laurentii* in vinasse medium [1].

Carbohydrate conc.	Biomass (gl^{-1})	Protein (%)	Final pH	Carbohydrate consumption (%)	BOD reduction (%)
3 gl^{-1}	6.71 a ± 0.93	22.9 a ± 1.1	5.5 a ± 2.1	77.6 a ± 6.3	78.1 a ± 3.6
8 gl^{-1}	4.02 b ± 0.32	40.0 b ± 2.1	5.8 a ± 0.7	93.1 b ± 1.3	50.0 ab ± 14.4
20 gl^{-1}	6.27 a ± 1.31	40.0 b ± 0.9	3.9 a ± 0.1	77.6 a ± 5.6	33.3 b ± 15.7
32 gl^{-1}	6.68 a ± 0.50	39.6 b ± 1.0	4.1 a ± 0.1	73.7 a ± 2.1	32.7 b ± 14.5

1 Initial culture conditions: C:N:P: ratio = 20: 3.0: 0,1; T = 30°C; N = 250 rpm; pH = 4.6; time of incubation = 48 hours.
Means values (n=4) followed by the same letters in the columns did not differ statistically at the 1% significance level according to the SNK or NMC tests.

TABLE 4 - Effect of initial pH on growth of mixed cultures of *A. niger* and *Cr. laurentii* in vinasse medium[1].

Initial pH	Biomass (gl^{-1})	Protein (%)	Final pH	Carbohydrate consumption (%)	BOD reduction (%)
3.5	10.22 a ± 1.95	35.3 a ± 1.2	3.1 a ± 0.3	86.0 ac ± 0.8	47.7 a ± 23.9
4.0	9.19 a ± 1.11	33.6 a ± 0.9	3.8 ab ± 0.2	85.0 ac ± 2.6	18.0 a ± 19.6
4.3	5.66 b ± 0.37	36.8 ab ± 1.3	4.2 b ± 0.1	83.9 a ± 0.3	38.2 a ± 9.5
4.6	4.02 c ± 0.32	40.0 b ± 2.1	5.8 c ± 0.7	93.1 b ± 1.3	50.0 a ± 14.4
4.9	4.85 b ± 0.27	46.0 c ± 1.8	5.0 c ± 0.4	86.4 ac ± 2.0	57.2 a ± 9.5
5.2	5.22 b ± 0.22	39.3 b ± 2.3	5.2 c ± 2.2	88.7 c ± 2.2	43.0 a ± 15.5
5.5	5.78 b ± 1.00	36.9 ab ± 0.9	5.1 bc ± 0.4	88.0 ac ± 1.5	43.0 a ± 15.5

1 Culture conditions: carbohydrate concentrations of vinasse medium: 8 gl-1; C:N:P: ratio = 20: 3.0: 0.1; T = 30°C; N = 250 rpm; pH = 4.6; time of incubation = 48 hours.
Means values (n=4) followed by the same letters in the columns did not differ statistically at the 1% significance level according to the SNK or NMC tests.

plain the increase in pH of the vinasse medium as culture progressed. The highest carbohydrate consumption was obtained at pH 4.6. No statistically significant differences were observed between mean values for BOD reduction, although they varied substantially (Tabela 4).

Physical characteristics of the environment can also influence microbial growth. The highest biomass production was observed at 25°C, a result which could be related to fungal aerobic life. *Cr. laurentii*, for example, is exclusively air-dependent (11) and lower temperatures may affect it indirectly by increasing solubility and thus oxygen supply (6). Protein content, final pH and carbohydrate consumption , on the other hand, were highest at 30°C and 25°C (Table 5). During *Phanerochaete chrysosporium* cultivation in vinasse, carbohydrate consumption at 45°C was reported to be almost similar to that at 30°C, but for a shorter period of time (3). The higher values for carbohydrate consumption at 30°C and 35°C described in this study could also be related to increased protein content of biomass. Mean BOD reduction values did not differ significanlty; however, large standard deviations were experienced with these determinations.

Classical studies on fungal physiology show that members of the genus *Cryptococcus* grow slowly in liquid medium (11), and our results obtained in vinasse medium after 72 hours of incubation corroborate these observations (Figure 1).

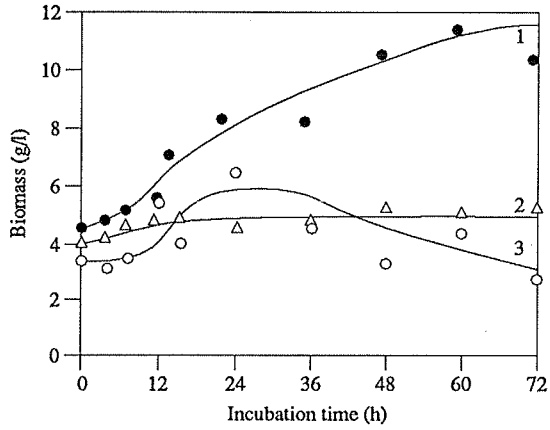


FIGURE 1 - Biomass production (g/l) of pure cultures of *A. niger* (1, ●), *Cr. laurentii* (2, Δ) and mixed culture of *A. niger* and *Cr. laurentii* (3, ○) in vinasse medium with a C:N:P ratio of 20:3.0:0.1, 8 g/l of carbohydrate, pH 4,6, at 30°C, on a rotary shaker at 250 rpm.

TABLE 5 - Effect of incubation temperature on growth of mixed cultures of *A. niger* and *Cr. lauretii* in vinasse medium[1].

Incubation temperature	Biomass (gl^{-1})	Protein (%)	Final pH	Carbohydrate consumption (%)	BOD reduction (%)
25°C	10.31 a ± 2.77	32.1 a ± 2.1	4.2 a ± 0.9	85.1 a ± 2.1	43.0 a ± 15.5
30°C	4.02 b ± 0.32	40.0 b ± 2.1	5.8 ab ± 0.7	93.1 b ± 1.3	50.0 a ± 14.4
35°C	6.47 ab ± 0.06	37.4 b ± 2.3	6.8 b ± 0.9	90.6 b ± 1.7	47.7 a ± 9.5

1 Culture conditions: initial carbohydrate concentration of vinasse medium: 8 g l^{-1}; C:N:P: ratio = 20: 3.0: 0.1; N = 250 rpm; pH = 4.6; time of incubation = 48 hourd.
Means values (n=4) followed by the same letters in the columns did not differ statistically at the 1% significance level according to the SNK or NMC tests.

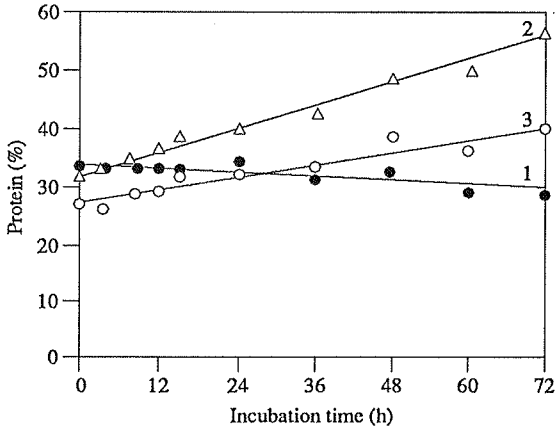


FIGURE 2 - Protein content (%) of pure cultures of *A. niger* (1, ●), *Cr. laurentii* (2, Δ) and mixed culture of *A. niger* and *Cr. laurentii* (3, ○) in vinasse medium with a C:N:P ratio of 20:3.0:0.1, 8 g/l of carbohydrate, pH 4,6, at 30°C, on a rotary shaker at 250 rpm.

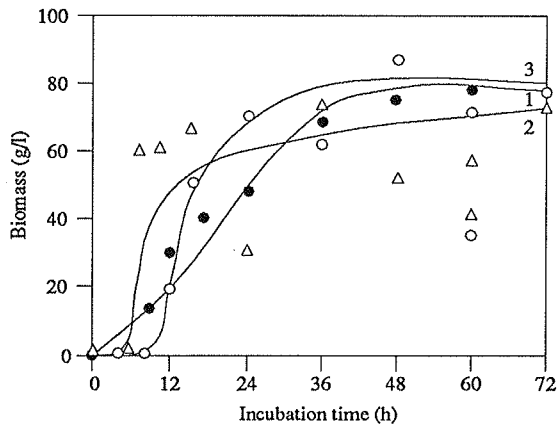


FIGURE 4 - Carbohydrate consumption (%) of pure cultures of *A. niger* (1, ●), *Cr. laurentii* (2, Δ) and mixed culture of *A. niger* and *Cr. laurentii* (3, ○) in vinasse medium with a C:N:P ratio of 20:3.0:0.1, 8 g/l of carbohydrate, pH 4,6, at 30°C, on a rotary shaker at 250 rpm.

However, there was a high BOD reduction (around 70%) after 24 hours (Figure 5); an explanation for this marked reduction may relate to the fact that organic matter can be eliminated as carbon dioxide (CO_2), which does not participate in cell mass biosynthesis. In fact, the final pH of vinasse medium decreased significantly after 24 hours of culture (Figure 3), probably through $C0_2$ release into the medium, or nitrogen utilization. There was an efficient carbohydrate uptake (around 80%) resulting in high protein content of biomass (aproximately 60%) and generation of CO_2, ATP and water as final products of carbohydrate metabolism (Figures 2, 4).

A. niger grown singly produced very high yields of biomass in vinasse medium, as previously described for cultures in wood vinasse (12). However, protein content varied only slightly during 72 hours, ranging from 30% to 34% (Figure 2). Very low pH values were recorded in vinasse medium at the end of incubation (final pH) probably due to the ability of *A. niger* to produce citric acid (7); since the latter is an organic acid, an increase in soluble BOD would be expected, a pre-

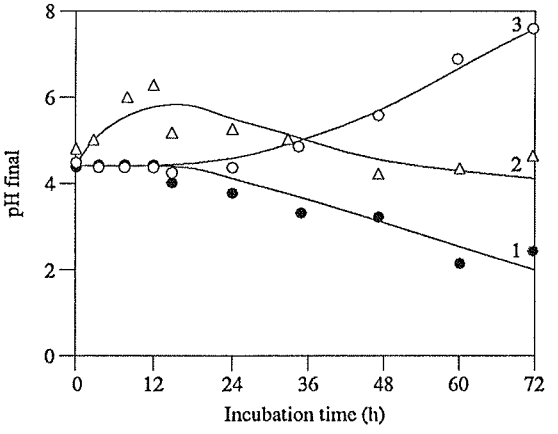


FIGURE 3 - Final pH of pure cultures of *A. niger* (1, ●), *Cr. laurentii* (2, Δ) and mixed culture of *A. niger* and *Cr. laurentii* (3, ○) in vinasse medium with a C:N:P ratio of 20:3.0:0.1, 8 g/l of carbohydrate, pH 4,6, at 30°C, on a rotary shaker at 250 rpm.

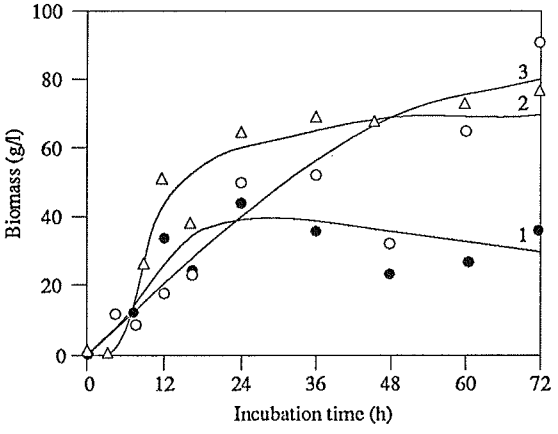


FIGURE 5 - BOD reduction (%) of pure cultures of *A. niger* (1, ●), *Cr. laurentii* (2, Δ) and mixed culture of *A. niger* and *Cr. laurentii* (3, ○) in vinasse medium with a C:N:P ratio of 20:3.0:0.1, 8 g/l of carbohydrate, pH 4,6, at 30°C, on a rotary shaker at 250 rpm.

diction that held true as shown by the fall in BOD reduction detected ((Figure 5).

Considering now the concomitant growth of *A. niger* and *Cr. laurentii* in mixed cultures, the highest biomass production was reached after 24 hours of incubation (Figure 1). It is likely that the first 12-hours of culture represent an adaptation period of the microorganisms to the substrate (lag phase). Probably, *A. niger* growth contributed more to this fast increase in biomass production than *Cr laurentii*. After 24 hours, autolysis of hyphae may have occurred, resulting in higher pH values (from 4.5 to 5.2). The yeast grew sequentially though not leading to increased biomass production. BOD reduction was aproximately 80% at the end of incubation. The accepted BOD value for effluent release into water is 0.6 g $O_2 l^{-1}$ according to Brazilian laws, and to attain such value BOD reduction should be of the order of 96%.

Results from a previous work on microorganisms grown in association in vinasse medium showed that mixed cultures presented higher amino acid contents than pure cultures, with a profile comparable to that of conventional protein sources and to FAO protein standards (5). However, the relatively low values for biomass production detected in mixed cultures in the present work do not favour the combined use of *A. niger* and *Cr. laurentii* for SCP production in cane sugar vinasse medium yet, despite the adequate level of BOD reduction attained under optimized conditions. Further studies will be carried out to obtain higher biomass concentrations concomitant with low BOD reduction values.

RESUMO

Otimização das condições para produção de SCP a partir do cultivo mixto de *A. niger* e *Cr. laurentii em* meio de vinhaça

Foi estudado o crescimento da cultura mista de *Aspergillus niger* e *Cryptococcus laurentii* em vinhaça, em frascos agitados, pata a otimização das proporções ideais de carbono, nitrogênio e fósforo (20 a 30:3.0:0.1); concentração de carboidrato (3 a 32 g/l); pH inicial (3,5 a 5,5); temperatura (25 a 35°C) e tempo de incubação, objetivando produção de biomassa e depuração biológica da vinhaça, um resíduo das destilarias. A adição de nitrogênio e fósforo ao meio de vinhaça alterou significativamente a produção de biomassa, o consumo de carboidrato e a redução de DBO. No entanto, com concentrações maiores que 3 g/l de carboidrato no meio de cultura, o conteúdo protéico da biomassa aumentou. O pH inicial do meio de cultura e a temperatura de incubação não alteraram a redução de DBO, que permaneceu por volta de 50%. O pH da vinhaça esteve sempre perto de 7.0 depois de 48 horas de cultivo. O crescimento do cultivo misto alcançou a máxima produção de biomassa depois de 24 horas, com os melhores resultados para redução de DBO (cerca de 80%) e teor de proteína (40%) após 72 horas em meio otimizado (20:3.0:0.1 para C:N:P, 8g/l de carboidrato, pH 4,6 e 30°C para incubação). Os resultados obtidos, quando comparados com os dados dos cultivos puros, não sugerem ainda a utilização do cultivo misto de *A. niger* + *Cr. laurentii* para a produção de SCP a partir de vinhaça, pois a concentração em biomassa foi baixa.

Palavras-chave: vinhaça, fungos, SCP.

ACKNOWLEDGEMENTS

The authors wish to thank Prof. Dr. Miguel Petrere Junior, Departamento de Ecologia, IB/ UNESP-Campus de Rio Claro, for his helpful assistance in the statistical analysis.

REFERENCES

1. AOAC. Association of Official Agricultural Chemists. Official methods of analysis, 12ª ed., Washington, 1970.
2. APHA. American Public Health Association. Standard Methods for the examination of water and wastewater. 14ª ed., Washington, 1975.
3. Cardoso, M.B. & Nicoli, J.R. Single - cell protein from the thermotolerant fungus *Phanerochaete chrysosporium* grown in vinasse. I. Production and composition. *Nutr. Rep. Int.*, 24(2): 237-247, 1981.
4. Ceccato, S.R. Produção de biomassa e depuração biológica da vinhaça de cana-de-açucar por cultura mista de fungos filamentosos e leveduras. Rio Claro, UNESP. 1988 (MSc Dissertation).
5. Ceccato-Antonini, S.R. & Tauk, S.M. Amino acid composition of single and mixed fungal cultures grown in sugar cane vinasse. *Rev. Microbiol.*, 23(1): 43-47, 1992.
6. Christophersen, J. Basic aspects of temperature action on microorganisms. In: Precht, H.; Christophersen, J.; Hensel, H. & Larcher, W. *Temperature and life*. Berlim, Springer - Verlag, 1973.
7. Cochrane, V.M. *Physiology of fungi*. New York, John Wiley & Sons, 1958.
8. Dawson, P.S.S. Continuous cultivation of microorganisms. *CRC Crit. Rev. Biotechnol.*, 2(4): 315-372, 1985.
9. Ferraz, C.A.M.; Aquarone, E.; Krauter, M.; Balloni, W. & Florenzano, G. Utilização de subprodutos da

indústria alcooleira na obtenção de biomassa de *Spirulina maxima*. Parte II. Emprego do resíduo da destilação do mosto fermentado (vinhaça). *Rev. Microbiol.* 17(1): 15-21, 1986.

10. Gonzalez, I.M. & Murphy, N.F. Potencial use of rum distillery slops as animal feed supplement. III. Effect of pH, composition and dilution in mold growth and BOD reduction of slops. *J. Agric. Univ.* PR, 63(3): 138-147, 1979.
11. Kreger-van Rij, N.J.W. *The yeasts*. A taxonomic study. 3ª ed. Amsterdam, Elsevier, 1984.
12. Nalin, D. & Kiyan, C. Cultivo de *A. niger* e *A. oryzae* em vinhaça de madeira para a produção de biomassa. *Rev. Microbiol.*, 20(2): 233-239, 1989.
13. Peres, C.S.; Figueiredo, M.G.; Vitoratto, E. & Perego Junior, L. Crescimento de bactéria fotossintetizante (BF) anaeróbica em meio constituído de vinhaça. *Rev. Microbiol.*, 17(1): 1-9, 1986.
14. Rosalem, P.L.; Tauk, S.M. & Santos, M.C.N. Efeito da temperatura, pH, tempo de cultivo e nutrientes no crescimento de fungos imperfeitos em vinhaça. *Rev. Microbiol.*, 16(4): 299-304, 1985.
15. Serzedello, A. A vinhaça como substrato para produção de proteína alimentar. *Boletim Instituto Zimotécnico* (ESALQ), 2: 303-312, 1962.
16. Snedecor, G.W. & Cochran, E.G. *Statistical methods*. Ames, The Iowa State University Press, 1976.
17. Sokal, R.R. & Rohlf, F.J. *Biometry. The principles and practice of statistics in biological research*. San Francisco, WH Freeman, 1969.
18. Srur, A.V.O.S. & Aquarone, E. Depuração de vinhaça pela produção de biomassa de *Rhodotorula gracilis*. *Revista ST AB*, 2(1): 34-37, 1983.
19. Tauk, S.M. Efeito do tratamento com ácidos minerais em vinhaça no desenvolvimento de *Candida utilis*. *Cienc. Cult.*, 30(3): 350-353, 1978.
20. Tauk, S.M. Alternative uses for vinasse. An alcohol industry residue. A review. *Environ. Technol. Lett.*, 3: 411-414, 1982.
21. Tauk, S.M. & Gambale, V. Efeito da adição de H_3PO_4 em cultura mista de levedura em vinhaça. *Bras. Açucareiro*, 5:9-14, 1978.
22. Waehner, R.S.; Giulietti, A.M. & Fraile, E.R. Producción de biomasa y depuración biológica de vinaza de destileria en un proceso de dos etapas. *Rev. Argent. Microbiol.*, 15(1): 47-50, 1983.
23. Weiner, J. Determination of total carbohydrate in beer. *J. Inst. Brew.*, 84: 222-223, 1978.
24. Zar, J.H. *Bioestatistical analysis*. New Jersey, Prentice-Hall Inc., 1974.