AFS – Advances in Food Sciences
Continuation of CMTL founded by F. Drawert

Printed in GERMANY – ISSN 1431-7737

CONTENTS

ORIGINAL PAPERS

COMPARATIVE DEGRADATION OF D-GALACTOSE AND D-GALACTONATE BY FUNGI

Ali M. Elshafei and Osama M. Abdel-Fatah

Department of Microbial Chemistry, National Research Centre, Dokki, Cairo, Egypt

SUMMARY

Twenty filamentous fungi were screened for their abilities to grow on D-galactose and degrade D-galactonate and D-gluconate at pH 3.0 and 5.0 by cell-free extracts of D-galactose-grown mycelia. Most of the fungal strains grow fairly on D-galactose for different incubation periods. *Aspergillus niger* was selected for further studies. On studying the degradation of 17 related substrates it was noticed that cell-free extracts of D-galactonate-grown *Aspergillus niger* have the capabilities to degrade D-galactonic acid-?-lactone, potassium D-galactonate, -D-gluconate, -D-fuconate, and -L-fuconate with the formation of the corresponding 2-keto-3-deoxy-aldonate and keto compounds. D-Galac-tonate-degrading system was found to be operative in extracts of D-galactonate-grown *Aspergillus niger* and specific for D-galactonate and closely related structures. The condensation reactions of aldolases were studied by cell-free extracts of *A.niger*-grown on different related carbon sources. Optimum formation of the corresponding aldonic acid was recorded with the reaction mixtures containing cell-free extracts of D-galactonate- and D-galactonic acid-?-lactone-grown *Aspergillus niger* as compared with that containing D-galactose, D-glucose or D-gluconate. The aldolase of D-galactonate-grown mycelia exhibits relative specificity towards glyceraldehyde than glycolaldehyde. The results indicated the presence of a nonphosphorolytic pathway for the degradation of D-galactose and D-galactonate in extracts of *Aspergillus niger*.

KEYWORDS: D-galactose, D-galactonate, utilization, degradation, nonphosphorolytic pathway, *Aspergillus niger*.

INTRODUCTION

Little information is available in the literature regarding the catabolism of D-galactose and related compounds in microorganisms. A very early report showed that *Phytomonas polycolor* [1] and *Pseudomonas aeruginosa* [2, 3] are able to utilize galactose and other sugars, as the sole source of carbon for growth, and to produce acid from these substances. Many investigators demonstrated that the utilization of galactose by certain strains of *Saccharomyces cerevisiae* had been considered to be performed by an oxidative pathway and an adaptive enzyme which utilizes galactose by a unique fermentative process [4, 5]. Studies of galactose utilization in *Pseudomonas saccharophila* [6] established that Pseudomonads metabolized this sugar via a route distinct from the well-known Leloir pathway [7]. Berka & Lessie, [8] studied the enzymes related to galactose utilization in *Pseudomonas cepacia*. Many authors reported the occurrence of D-galactonate catabolic pathway in saprophytic strains of genus mycobacterium [9,10,11]. The pathway involved galactonate dehydratase, 2-keto-3-deoxy-galactonate kinase and 2-keto-3-deoxy-6-phosphogalactonate aldolase, which are the enzymes previously found in *Pseudomonas saccharophila* [6]. It appeared that these enzymes were induced in mycobacteria by galactonate and to a lesser degree by galactose [10, 12]. In previous findings we reported the utilization and degradation of D-galactose and D-galactonic acid by different species of Aspergilli. In this study we described a new nonphosphorolytic biochemical pathway for the catabolism of D-galactonate in *A. terreus* [13]. It involves the dehydration of D-galactonate to 2-keto-3-deoxy-D-galactonate (KDGal) by a D-galactonate dehydratase and the cleavage of KDGal to pyruvate and glyceraldehyde by a KDGal aldolase. Some properties of the two enzymes responsible for this degradation were then studied [14]. The aim of the present work is to study the physiological behaviour of the utilization and degradation of D-galactose and other carbon sources by different filamentous fungi with especial reference to *A. niger*.

MATERIALS AND METHODS

Microorganisms

Aspergillus allocutus, A. carneus, A. clavatus, A. egyptiacus, A. fischeri, A. niger, A. oryzae, A. stellatus, A. tamarii, A. versicolor, Cunnighamella elegans, Penicillium brevi-compactum, P. chrysogenum, P. cyclopium, P. expansum, P. notatum, P. oxalicum, P. purpurescens, P. roquefortii and P. viridicatum (obtained from the

Department of Microbial Chemistry, Division of Genetic Engineering, National Research Centre of EGYPT), were maintained on solid Czapek-Dox's medium in which 3% D-galactose is the only carbon source for growth.

Media and cultures

The organisms were grown on Czapek-Dox's liquid medium [15] containing 3% D-galactose or potassium D-galactonate as the only carbon source for fungal growth. Erlenmeyer flasks (250 ml) each containing 50 ml of sterile medium were inoculated and incubated statically at 30°C. After different incubation periods, the mycelia were harvested by filtration and washed thoroughly with cold distilled water.

Preparation of cell-free extracts

The harvested mycelia were washed with cold distilled water and blotted dry with filter paper. The blotted-dry mycelia were ground under cooling conditions with approximately twice its weight of cold sand in a cold mortar and then extracted using 0.1M potassium phosphate or Tris-(hydroxymethyl) aminomethane (Tris-HCl) buffer pH 8. The slurry so obtained was centrifuged at 5500 r.p.m. for 5 minutes. The supernatant was used as the crude enzyme preparation.

Enzyme assay

D-galactose oxidase and D-galactono-γ-lactonase activities were determined by estimation of µmoles of D-galactose and D-galactonic acid-γ-lactone disappeared from the reaction mixture according to the method described by Ashwell [16] and by Hestrin [17], respectively. D-galactonate dehydratase and 2-Keto-3-deoxy-D-galactonate (KDGal) aldolase were estimated by determination of µmoles KDGal formed from D-galactonate or pyruvate and glyceraldehyde, respectively, according to the method of Weissbach & Hurwitz [18] as previously described [19]. Enzyme activities are expressed as µmoles of product(s) formed during the reported time course of enzymatic reaction(s) which are indicated in the reaction mixture(s) contents.

Chemical methods

Reducing sugars were estimated as described by Ashwell [16]. Determination of D-galactonic acid-γ-lactone was made by the method described by Hestrin [17]. The 2-keto-3-deoxy-aldonate formed was determined by the method of Weissbach & Hurwitz [18]. Pyruvic acid was estimated by the method of Friedemann & Haugen [20]. Protein was determined by the method of Lowry *et al*. [21]. Preparations of potassium salts of aldonate were made according to the method described by Moore & Link [22].

TABLE 1 - Occurrence of the nonphosphorylative pathway for D-galactonate and D-gluconate degradation in different filamentous fungi grown on D-galactose.

Organisms	Incubation Period (days) at		Growth at		2-keto-3-deoxy aldonate (µmoles)			
					D-galactonate		D-gluconate	
	pH 3.0	pH 5.0	pH 3.0	PH 5.0	pH 3.0	pH 5.0	pH 3.0	pH 5.0
Aspergillus allocutus	15	6	- ve	+ ve	N.D.*	0.00	N.D.*	0.00
A. carneus	6	6	+ ve	+ ve	0.43	0.00	0.37	0.00
A. clavatus	15	6	- ve	+ ve	N.D.*	0.00	N.D.*	0.00
A. egyptiacus	15	6	- ve	+ ve	N.D.*	0.00	N.D.*	0.00
A. fischeri	6	6	+ ve	+ ve	0.69	0.00	0.24	0.00
A. niger	6	6	+ ve	+ ve	2.58	2.00	1.75	2.40
A. oryzae	6	6	+ ve	+ ve	1.53	0.00	0.61	0.00
A. stellatus	6	6	+ ve	+ ve	1.33	0.00	0.91	0.00
A. tamarii	6	6	+ ve	+ ve	1.50	0.00	1.01	0.00
A. versicolor	15	6	- ve	+ ve	N.D.*	0.00	N.D.*	0.00
Cunnighamella elegans	4	6	+ ve	+ ve	0.00	0.00	0.00	0.00
Penicillium brevi-compactum	5	6	+ ve	+ ve	0.00	0.00	0.00	0.00
P. chrysogenum	4	6	+ ve	+ ve	0.00	0.00	0.00	0.00
P. cyclopium	4	6	+ ve	+ ve	0.00	0.00	0.00	0.00
P. expansum	4	6	+ ve	+ ve	0.00	0.00	0.00	0.00
P. notatum	4	6	+ ve	+ ve	0.00	0.00	0.00	0.00
P. oxalicum	5	6	+ ve	+ ve	0.00	0.00	0.00	0.00
P. purpurescens	5	6	+ ve	+ ve	0.00	0.00	0.00	0.00
P.roquefortii	4	6	+ ve	+ ve	0.00	0.00	0.00	0.00
P. viridicatum	4	6	+ ve	+ ve	0.00	0.00	0.00	0.00

N.D.*: not determined (No growth was obtained at pH 3.0).

Reaction mixture contained: Substrate (potassium-D-galactonate or potassium-D-gluconate), 20 µmol; $MgCl_2$, 10 µmol; Tris-HCl buffer pH 8.0, 70 µmol; protein extracts, 5.0 mg; total volume, 2.0 ml; temperature, 40°C and reaction time, 180 minutes.

RESULTS AND DISCUSSION

D-galactonate and D-gluconate utilization: Results in Table 1 show that most of the fungal strains grow fairly on D-galactose for different incubation periods at pH 3.0 and 5.0 except four Aspergilli namely *A. versicolor, A. clavatus, A. allocutus* and *A. egyptiacus,* which could not grow at pH 3.0. The results in Table 1 indicate that extracts of all fungi failed to degrade the two hexonic acid salts (potassium D-galactonate and D-gluconate) at pH 5.0, except cell-free extract of *A. niger*, which shows almost equivalent rates for both sustrates. On the other hand, at pH 3.0 only extracts of six Aspergilli, namely, *A. niger, A. tamarii, A. oryzae, A. stellatus, A. carneus* and *A. fischeri* have the abilities to degrade D-galactonate and D-gluconate nonphosphorolytically (in the absence of ATP) at different rates. High rates of degradation were obtained when D-galactonate was used as substrate as compared with D-gluconate for these six Aspergilli which indicates that the initial pH value plays a major role in determining the form of aldonic acid prevailing in the medium under these conditions (Table 1). *A. niger* was selected for further studies to demonstrate the pathway of D-galactose and D-galactonate degradation and the enzymes responsible for this degradation.

Comparative growth rate: Medium modification was made in which the sucrose of the basal medium (Czapek-Dox medium) was replaced by a 3% of potassium D-galactonate or D-galactose. The results showed that the organism could utilize and grow on D-galactonate and D-galactose as the sole source of carbon and energy (Fig. 1). However, a lag period of about 48 hours was recorded. This lag period might be needed by the organism for denovobiosynthesis of certain inducible enzyme indispensable for the transport and/or catabolism of the two carbon sources. Maximum amount of growth obtained with D-galactonate or D-galactose medium was on the eighth day of incubation estimated as the mean value of mycelial dry weights. The value in case of growing *A. niger* on D-galaconate medium was only about 73% of the analogous amount obtained with D-galactose medium. On measuring the pH value throughout the growth period it was noticed that the pH value of D-galactose medium initially adjusted at pH 3.0 reached to pH 5.0 after 12 days, whileas the D-galactonate medium initially adjusted at pH 3.0 gradually changed throughout the growth period from pH 3.0 till reached to pH 7.0 at the twelfth day. On analyzing the broth medium (containing D-galactose or D-galactonate initially adjusted at pH 3.0) for its contents of the expected products related to the nonphosphorylated pathway of D-galactonate catabolism, 2-keto-3-deoxy-com-pound and keto compounds were detected in the medium containing D-galactonate after 8 and 10 days of incubation (Table 2). However, none of these products and intermediate were detected in the culture broth of D-galactose-grown mycelia under the same experimental conditions.

FIGURE 1 - Comparative growth of *A. niger* on D-galactonate and D-galactose-Dox´s medium.

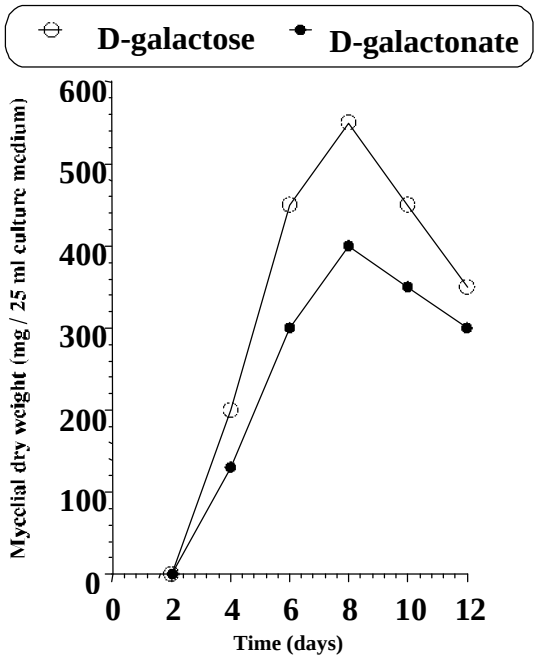


TABLE 2
Amounts of 2-keto-3-deoxy-compound and keto compounds formed in the medium of *A. niger* grown on D-galactonate.

Incubation Period (days)	Products* (µmoles / 25 ml medium) 2-keto-3-deoxy-Compound	Keto compounds (as pyruvate)**
2	0.0	0.0
4	0.0	0.0
6	0.0	0.0
8	56.25	22.0
10	25.0	12.0
12	0.0	0.0

* In case of growing *A.niger* on D-galactose Dox's medium none of these keto compounds were detected.

** 2-keto-3-deoxy compounds were not included in this determination.

Effect of initial pH value

The comparative growth rates of *A. niger* on D-galactonate medium initially adjusted to pH 3.0 and pH 5.0 show that the amounts of growth obtained in both cases are more or less the same, indicating that permeability of the D-galactonate anion, into the spores of the organism might be mediated by facilitated diffusion rather than by passive diffusion. An increase in the initial pH value of each of these media was recorded. This is due to the utilization of the D-galactonate anions that was subsequently accompanied with the accumulation of potassium cations in the culture media. This increase in the pH value did not seem to affect the uptake of D-galactonate anions by the

growing cells. It was suggested that permeability of the D-galactonate anions into the growing cells of *A. niger* is effected by a permease carrier that acts well at pH values ranging from about 5.0 to 8.0.

Degradation of different substrates

a) by extracts of D-galactonate-grown mycelia

Results in Table 3 show that cell-free extract of D-galactonate-grown mycelia of *A. niger* have the capabilities to degrade D-galactonic acid-?-lactone, potassium D-ga-lactonate, potassium D-gluconate, potassium D-fuconate, and potassium L-fuconate with the formation of the corresponding 2-keto-3-deoxy-aldonate and keto compounds. The highest activity appeared with D-galactonate, while D-galactonic acid-?-lactone, D-fuconate and L-fuconate were cleaved, but at slower rate than D-galactonate, which indicating that the degradative system responsible for the degradation of D-galactonate was more specific for this substrate and closely related structures such as D-galactonic acid-?-lactone, potassium D-gluconate, potassium D-fuconate and potassium L-fuconate.

TABLE 3 - Degradation of different substrates by cell-free extracts of *A. niger* grown on D-galactonate.

Substrate*	Products (µmoles)	
	2-keto-3-deoxy Aldonate	Keto compounds (as pyruvate)
D-Galactonic acid-?-lactone	7.38	2.59
Potassium D-galactonate	9.82	4.07
Potassium D-fuconate	3.10	1.47
Potassium L-fuconate	1.40	0.74
Potassium D-gluconate	5.74	7.64

* D-galactose, D-fucose, L-fucose, D-glucose, 1,5-gluconolactone, D-arabinose, potassium-D-arabonate, L-arabinose, L-arabonic acid-γ-lactone, potassium-L-arabonate, D-ribose, and potassium D-ribonate were not degraded under the same experimental conditions.

Reaction mixture contained:
Different substrates, 20 µmol; $MgCl_2$, 10 µmol; Tris-HCl buffer pH 8.0, 45 µmol; protein extracts, 6.8 mg; total volume, 1.0 ml; temperature, 40°C and reaction time, 180 min.

Data in Table 3 also show that in the case of using D-gluconate as substrate high amounts of keto compounds (estimated as pyruvate) were formed as compared with the amounts of the corresponding 2-keto-3-deoxy-aldonate formed in the reaction mixture. This is not the case in using D-galactonate, D-galactonic acid-?-lactone, D-fuconate or L-fuconate in the reaction mixture as 53.2%, 34%, 19.2% and 9.7%, respectively, of the µmoles formed as keto compounds (as compared with the value of D-gluconate, 100%). On the other hand, 1,5-gluconolactone, L-arabonic acid -?- lactone, D-arabo-nate, L-arabonate, D-ribonate, D-galactose, D- and L-fucose, D-glucose, D- and L-arabinose, and D-ribose were not degraded by these extracts under the same experimental conditions. The previously mentioned results may indicate that D-galactonate is dehydrated into 2-keto-3-deoxy D-aldonate. The latter compound is then cleaved into pyruvate and glyceraldehyde by an aldolase catalyzed reaction.

The results in Table 3 also indicate the specificity of this system (induced mainly by D-galactonate) toward D-galactonate and closely related substrates and the way by which the previously mentioned substrates were degraded, was completely different from that of D-gluconate by extracts of D-galactonate grown *A. niger*.

b) by extracts of related carbon sources-grown mycelia

On analyzing the data obtained in Table 4 it was concluded that cell-free extracts of D-galactonate- and D-galactonic acid-?-lactone-grown mycelia of *A. niger* (each as the only carbon source for growth) were superior for their abilities to degrade D-galctonate and D-galactonic acid-?-lactone, respectively, as substrates in the reaction mixtures. Their abilities were estimated by determining the amounts of 2-keto-3-deoxy aldonate intermediate formed under the same experimental conditions. D-gluconate as substrate was also degraded by the expected D-galactonate degrading system, but at a relatively slower rate (49.5% that of the corresponding value of D-galactonate as substrate). Introducing D-galactose as the only carbon source in the medium resulted to decreasing levels of 2-keto-3-deoxy aldonate corresponding to the values previously mentioned. On the other hand, cell-free extracts of D-gluconate-grown mycelia of *A. niger* have the ability to degrade D-gluconate (100%) more efficiently than D-galactonate (32.5%) as substrates in the reaction mixtures (Table 4). D-Glucose-grown mycelia also gave a comparatively low activity.

Concerning the other substrates under study it was noticed that D-galactose, D-glucose, 1,5-gluconolactone, D-arabinose, D-arabonate, L-arabinose and L-arabonate were not degraded under these experimental conditions by all the cell-free extracts prepared from the five carbon sources under study. From the previous results it can be concluded that the new D-galactonate degrading system (induced by D-galactonate or D-galactonic acid-?-lactone) in *A. niger* was more specific towards the degradation of D-galactonate and differs from the D-gluconate one (induced by D-gluconate) in that the µmoles of the corresponding 2-keto-3-deoxy aldonate formed from the degradation of D-galactonate exceeds 2 times the corresponding amounts in case of D-gluconate degrading system.

In case of D-galactonate degrading system the ratio of µmoles of 2-keto-3-deoxy aldonate formed from D-galactonate **:** µmoles of 2-keto-3-deoxy aldonate formed from D-gluconate. 4.50 / 2.23 = 2.01 µmoles, while the corresponding ratio in case of D-gluconate degrading system 2.50 / 7.70 = 0.32 µmoles. The previously mentioned values indicate that a new D-galactonate degrading system was operative in extracts of *A. niger*, which is completely different from the D-gluconate degrading one

TABLE 4
Degradation of different substrates by cell-free extracts of *A. niger* grown on different related carbon sources.

Substrate*	2-keto-3-deoxy aldonate (µmoles) Carbon sources				
	D-galactose	D-galactonic acid-?-lactone	D-galactonate	D-glucose	D-gluconate
D-galactonic acid-?-lactone	1.66	2.75	2.65	0.96	1.76
D-galactonate	1.95	3.25	4.50	1.73	2.50
D-gluconate	0.85	0.96	2.23	3.85	7.70

* D-galactose, D-glucose, 1,5-gluconolactone, D-arabinose, D-arabonate, L-arabinose, and L-arabonate were not degraded under the same experimental conditions.

Reaction mixture contained: Different substrates, 20 µmol; $MgCl_2$, 10 µmol; Tris-HCl buffer pH 8.0, 70 µmol; protein extracts, 3.25 mg; total volume, 2.0 ml; temperature, 40°C and reaction time, 180 min.

TABLE 5
The condensation reaction of aldolase by cell-free extracts of *A. niger* grown on different related carbon sources.

Substrate	2-keto-3-deoxy aldonate (µmoles) Carbon sources				
	D-galactose	D-galactonic acid-γ-lactone	D-galactonate	D-glucose	D-gluconate
Glyceraldehyde + Pyruvate	2.05	2.86	3.21	1.98	2.45
Glycoladehyde + Pyruvate	1.23	1.45	1.63	0.92	1.23

Reaction mixture contained:
Sodium pyruvate, 20 µmol; glyceraldehyde or glycoladehyde, 10 µmol; potassium phosphate buffer pH 7.5, 60 µmol; protein extracts, 3.25 mg; total volume, 2.0 ml; temperature, 50°C and reaction time, 12 minutes.

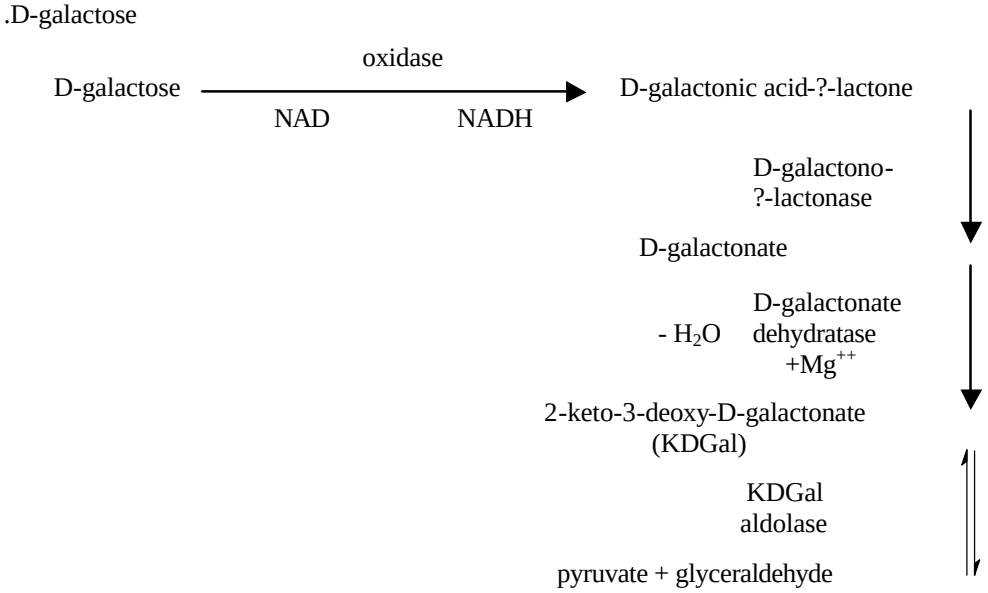
TABLE 6
Formation of D-galactonic acid-γ-lactone and KDGal from D-galactose by extracts of *A. niger* grown on D-galactose.

Time (min)	D-galactose Disappeared (µmol)	Products (µmoles)	
		D-galactonic acid-?-lactone	KDGal
Zero	0.00	0.0	0.0
60	5.00	4.5	0.3
120	8.33	7.5	0.6
180	10.00	9.0	1.0

Reaction mixture contained: D-galactose, 20 µmol; $MgCl_2$, 10 µmol; NAD, 5µmol; citrate phosphate buffer pH 6.4, 70 µmols; protein extracts, 6.76 mg; total volume, 2.0 ml; temperature, 40°C and reaction time, as indicated.

TABLE 7
Representative enzymes involved in D-galactose and D- galactonate degradation in extracts of *A.niger*.

Substrate	Products	Enzyme involved
D-galactose	D-galactono-γ-lactone	D-galactose oxidase
D-galactono-γ-lactone	D-galactonate	D-galactono-γ-lactonase
D-galactonate	2-keto-3-deoxy-D-galactonate	D-galactonate dehydratase
Pyruvate + Glyceraldehyde	2-keto-3-deoxy-D-galactonate	2-keto-3-deoxy-D-galactonate aldolase



in its specificity towards different substrates. This new system was induced greatly by incorporating D-galactonate or D-galactonic acid-?-lactone as the only carbon source in the growing medium.

Condensation reaction of aldolase

Results in Table 5 show that higher activity was observed with the reaction mixture containing cell-free extracts of D-galactonate- and D-galactonic acid-?-lactone-grown *A. niger* as compared with that containing D-galactose, D-glucose and D-gluconate. Also from the table it was observed that the aldolase exhibits relatively specificity towards glyceraldehyde than that of glycolaldehyde.

Degradation of D-galactose

Results obtained in Table 6 indicate that D-galactose was degraded by cell-free extracts of D-galactose-grown *A. niger*, in the presence of nicotinamide adenine dinucleotide (NAD), nonphosphorolytically (in the absence of ATP). No activity could be detected in case of the reaction mixture in which nicotinamide adenine dinucleotide phosphate (NADP) was added. At the same time there is a gradual increase in the amounts of D-galactonic acid-γ-lactone (Gal-γ-lac) followed by the appearance of slight amounts of 2-keto-3-deoxy-D-galactonate (KDGal)). A confirmatory experiment was conducted to demonstrate the disappearance of D-galactonic acid-γ-lactone by cell-free extracts of *A. niger* grown on D-galactose. The results show that from10 µmoles only 6.6 µmoles of the substrate were disappeared after 60 min, indicating the presence of D-galactono-γ-lactonase, which catalyzed the formation of D-galactonic acid from Gal-γ-lac in the reaction mixtures.

Based on the above mentioned findings it was concluded that D-galactose was oxidized to D-galactonic acid-?-lactone in the presence of NAD by the enzyme D-galactose oxidase. The D-galactonic acid-?-lactone was then delactonized by lactonase to give D-galactonate. The latter compound was then dehydrated to 2-keto-3-deoxy-D-galactonate (KDGal) by the action of D-galactonate dehydratase.

In the present work the data obtained from the degradation of different substrates by cell-free extracts of D-galactonate or D-galactonic acid-γ-lactone grown *A. niger* indicate the presence of a D-galactonate degrading system, which was more specific towards the degradation of D-galactonate and differs from the D-gluconate one (induced by D-gluconate) in that the µmoles of the corresponding 2-keto-3-deoxy aldonate formed from the degradation of D-galactonate (as substrate) exceeds two times the corresponding amounts in the case of D-gluconate degrading system. Doudoroff and his co-workers [6] established that *Ps. saccharophila* metabolized D-galactose via a set of reactions different from those of the well-known Leloir pathway [7]. This route was termed the De Ley Doudoroff pathway, which was similar to the Entner-Doudoroff route [23]. Our pathway for D-galactose degradation in extracts of *A. niger* was not previously reported in fungi and resembles to some extent the Entner-Doudoroff pathway and differs from this pathway in that our pathway does not contain any phosphorolytic intermediates or products.

In the light of the findings obtained, the following nonphosphorolytic pathway was suggested for the degradation of *A. niger* by the presence of four enzymes responsible for this degradation namely D-galactose oxidase, D-galactono-γ-lactonase, D-galactonate dehydratase and 2-keto-3-deoxy-D-galactonate aldolase (Table 7)

REFERENCES

[1] Clara, F.M., A comparative study of the free-fluorescent bacterial plant pathogens. Cornell Univ. Agr. Exptl. Sta. Med., 59. (1934).

[2] Gaby, W.L., J. Bact., **51**, 217 (1946).

[3] Liu Pinghui, J. Bact., **64**, 773 (1952).

[4] Spiegelman, S., Modern aspects of enzymatic adaptation. In Summer, J.B. and Myrback, K. The Enzymes. Academic Press, New York pp. 267 (1950).

[5] Sheffner, A.L. and Lindegren, C.C., J. Bacteriol., **64**, 423 (1952).

[6] De Ley, J. and Doudoroff, M., J. Biol. Chem., **227**, 745 (1957).

[7] Maxwell, E.S., Kurahashi, K. and Kalckar, H.M., Enzymes of the Leloir pathway. Methods in Enzymology, **5**, 174 (1962).

[8] Berka, T.R. and Lessie, T.G., Current Microbiology, **11**, 43 (1984).

[9] Szumilo, T. and Szymona, M., Materialy XII Zjazdu P.T. Biochem., pp. 110 Warszawa (1974).

[10] Szumilo, T., J. Bacteriol., **148**, 368 (1981).

[11] Szumilo, T., Acta Microbiol. Polonica, **32**, 47 (1983).

[12] Szumilo, T., Acta Microbiol. Polonica, **30**, 335 (1981).

[13] Elshafei, A.M. and Abdel-Fatah, O.M., Enzyme Microbial Technology, **13**, 930 (1991).

[14] Elshafei, A.M., Mohawed, S.M., Ammar, M.S. and Abdel-Fatah, O.M., Antonie Van Leeuwenhoek, **67**, 211 (1995).

[15] Difco Manual, "Difco Manual of dehydrated culture, media and reagents" 9th ED.: 245. Difco Laboratories, Detroit, Michigan, USA. (1972).

[16] Ashwell, G., Colorimetric analysis of sugars "Methods in Enzymology". (S.P.Colowick and N.O.Kaplan eds.) Vol. **3**, 73. Academic Press, New York. (1957).

[17] Hestrin, S., J. Biol. Chem., **180**, 249 (1949).

[18] Weissbach, A. and Hurwitz, J., J. Biol. Chem., **234**, 705 (1959).

[19] Elshafei, A.M. and Abdel-Fatah, O.M., Enzyme Microb. Technol., **11**, 367 (1989).

[20] Friedemann, T.E. and Haugen, G.E., J. Biol. Chem., **147**, 415 (1943).

[21] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J., J. Biol. Chem., **193**, 265 (1951).

[22] Moore, S. and Link, K.P., J. Biol. Chem., **133**, 293 (1940).

[23] Entner, N. and Doudoroff, M., J. Biol. Chem., **196**, 853 (1952).

Received for publication: August 24, 2001
Accepted for publication: September 24, 2001

CORRESPONDING AUTHOR

Ali M. Elshafei
Department of Microbial Chemistry
National Research Centre
Dokki, Cairo - EGYPT

email: alielshafei@yahoo.com

AFS – Vol 23/ No 4/ 2001 – pages 146 - 152

DEVELOPMENT INFLUENCE OF *Botrytis cinerea* ON GRAPES*

M. Dorado, E. Bermejo, J. L. González, A. Sánchez and N. Luna

Laboratorio Centro de Análisis, Asesoramiento y Control de Calidad, CAYACEA S.A. Miguel Yuste 12- 1ª. 28037 Madrid. SPAIN.

*** This research is part of the European Project: Improvement of natural resistance in fruit. Programa Europeo de Investigación D.G.XII Science, Research and Development; Standars, Measurements and Testing: call for Proposal Contract nº FAIR CT98-4211.**

SUMMARY

In most climates fruits can only be grown and harvested during a small part of the year. Yet, large consumption of fruit, and hence demand occurs throughout the year. For this reason, large parts of the fruit harvests must be stored for more or less extended periods of time before they are sold to the consumers. Obviously, such storage causes considerable losses due to pathogen attacks; and modified atmospheres and the use of synthetic pesticides are employed.

Losses caused by *Botrytis cinerea* would require the use of fungicides with expenses between 50 and 100 million ECUS in Europe per year [1].

The development of aerial mycelium and conidia of this pathogen on surfaces of infected grapes was evaluated under conditions of controlled temperature and relative humidity. Inoculated grapes were incubated at 15-23ºC and 80-85% relative humidity. Number of microorganisms were determined at 0, 3, 6 and 9 days and analysed for the total viable count of microorganisms and, more specifically, moulds and yeasts. There was a significant difference about the colonization of microorganisms in the control and infected groups. Practically all the microorganisms that grew in PCA (Plate Count Agar), SDA (Sabouraud Dextrose Agar) and PDA (Potatoe Dextrose Agar) were fungi. Other fungi, noninoculated, and bacteria grew well with the increase of the incubation time. The number of colony forming units tended to increase with the age of the lesion.

KEYWORDS:
Microorganisms, infection, production.

INTRODUCTION

The fungus *Botrytis cinerea* represents the highly variable conidial form of a series of distinct *Botryotina* species related to *B. fuckeliana*. Hansen *et al.*, 1983 [2], ascribed the variability to the existence of a mycelial and conidial basic type and used it as an example of the "dual phenomenon". Menzinger, 1966 [3], however, showed that still greater possibilities for variation existed with respect to conidium size, sclerotium formation, mycelial characters and formation of microconidia; segregation by repeated single-spore cultures led to six different forms which remained constant. The cause of variability is heterokaryosis.

Botrytis cinerea has a growth rate of 90 - 120 mm in 10 days. The cardinal temperature is -2º C, optimum 22-25º C, maximum 33º C; it grows at pH values 2-8. The minimum relative humidity for vegetative growth is 93%, and for sporulation 95%. Sporulation can be stimulated by light [4]. Air currents, water droplets or insects may disperse conidia [5].

Botrytis cinerea occurs regulary in the soil, though its proportion of the total fungus population is not high. Its distribution is on rotting plant parts and plays an important part as parasite on higher plants. It is also denominated as "gray mold" and is one of the more serious diseases, especially on certain fruits.

Figure 1 shows the life cycle of *Botrytis cinerea* [6].

Several studies have investigated the role of climatic factors on the development of *B. cinerea*. The effects of relative humidity and temperature have been determined for development, germination, release and dispersal of conidia, growth and survival of mycelium, and infection of plants [7]. It is one of the most destructive pathogens attacking greenhouse-grown crops. This situation necessitates frequent applications of fungicides, which are both costly and environmentally questionable.

Some characteristics of *Botrytis cinerea* and its infection are:

1) Parasite on superior plants (fruit, vegetables, ornamentals).

   One of the most serious diseases in agricultural products (tomatoes, cucumber, grapes, citrus fruit, etc).

2) Responsible for the post-harvest spoilage of many fruits and vegetables.

Serious problem in the field, in storage, in transit and retail.

3) Development of the disease.

- Wet years, especially if the rain occurs in the fall before harvest.
- Dispersion of conidia by wind or contact with other infected parts of the plant.
- Skin wounds not necessary, but if present, penetration is easier and more rapid.

4) Infection predisposition factors.

Maturation: produce changes in colour, pH, sugar content, starch and phenolic substances and loss of firmess.

Temperature*:* affects the maturation of the fruit and the development of the potential pathogens too.

Humidity*:* the fruit is storaged at 85 % relative humidity to prevent dehydratation. Affects the development of the pathogens.

Wounds

Gas*:* ethylene accumulation increases maturation; usually fruit is stored under a modified atmosphere of oxygen and CO_2.

The purpose of this research was to investigate the effect of inoculation of *Botrytis cinerea* on grapes, and observe the effect of microbial growth on grapes. One approach to the control of gray mold is a better knowledge of the epidemiology of the disease to manipulate it more effectively.

FIGURE 1 - Life cycle of *Botrytis cinerea* [6].

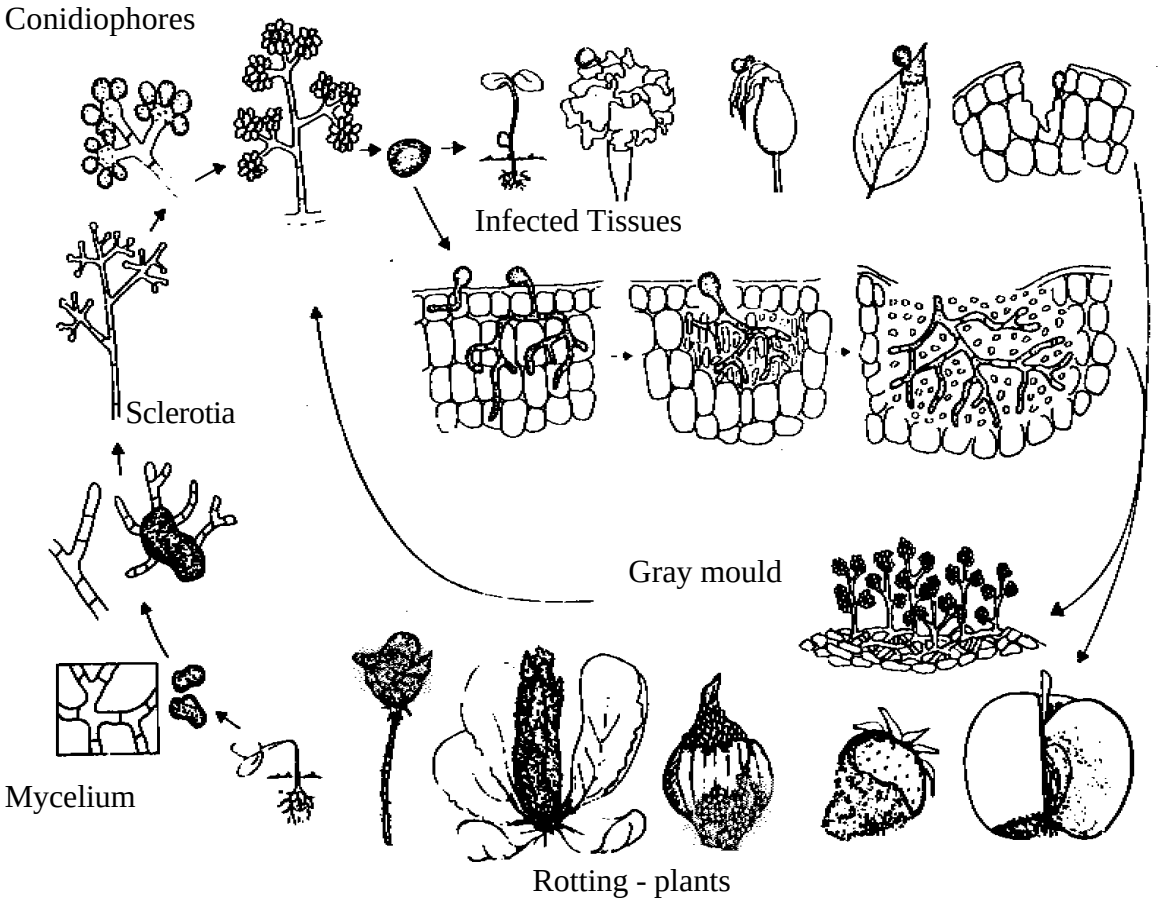


FIGURE 2 - METHODOLOGY 1

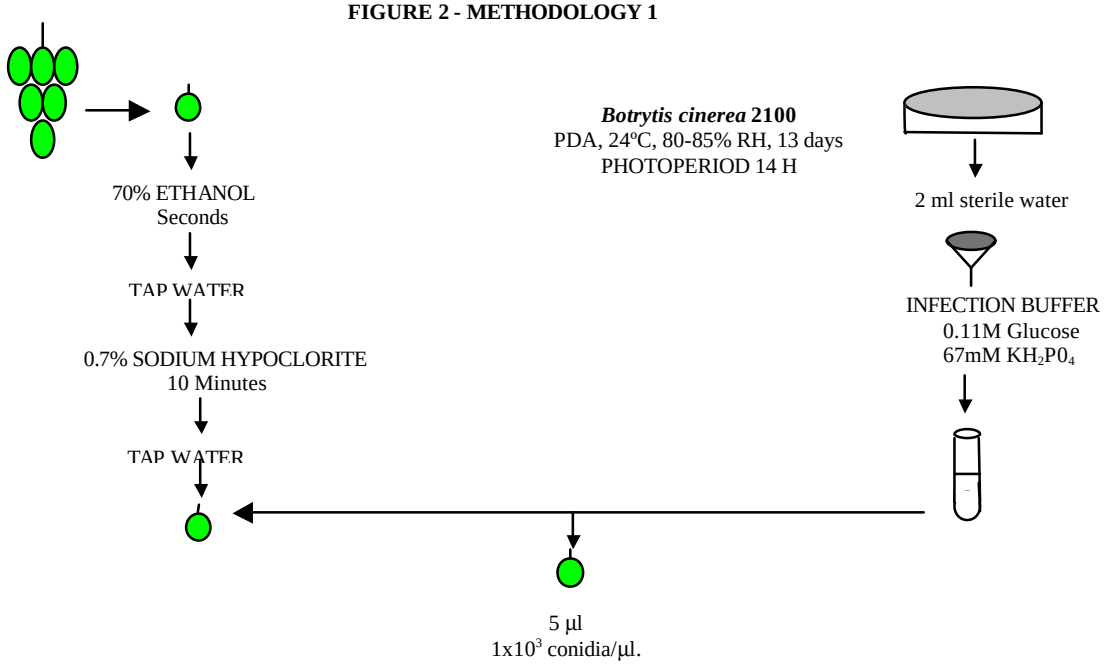
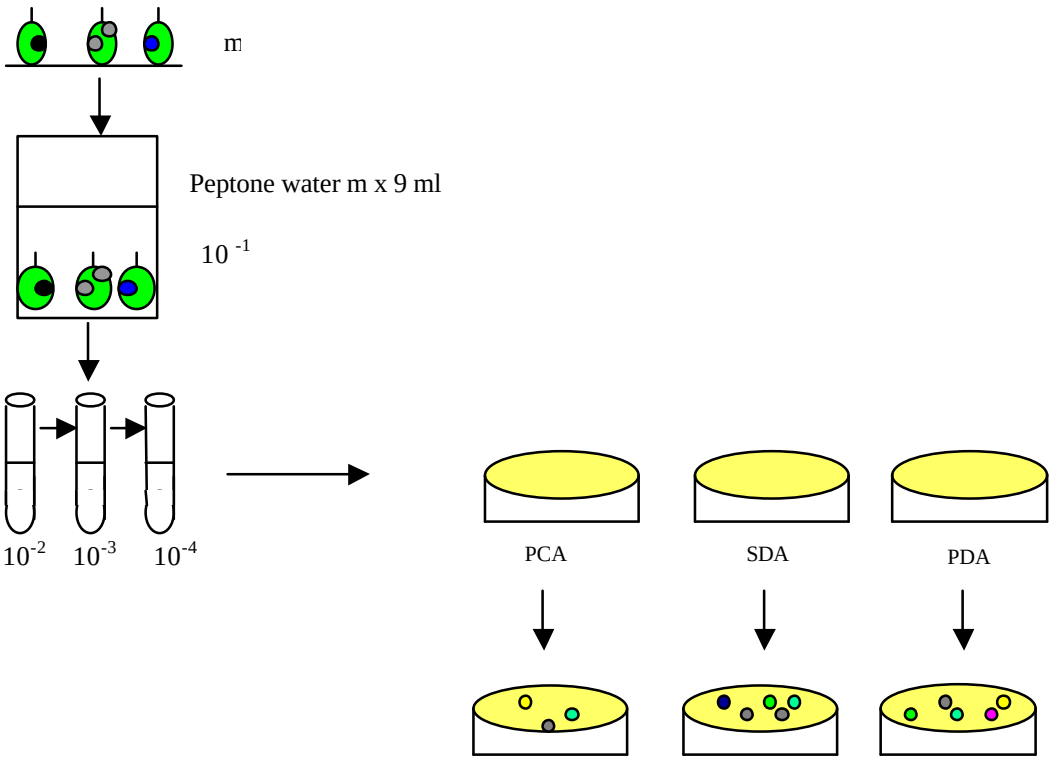


FIGURE 3 - METHODOLOGY 2



MATERIALS AND METHODS

Cultures of *Botrytis cinerea* 2100 was obtained from a Spanish Type Culture Collection, CECT 2100. *Botrytis cinerea* was grown on potato dextrose agar at 24ºC with high humidity, and the photoperiod was 14 hours. Conidial suspensions were obtained by the addition of 2 ml of sterile water to a sporulating culture of *Botrytis cinerea*. Later, the microorganisms suspension was filtered through two or three layers of gauze, to obtain a single spore suspension. The spore numbers were obtain with a Neubauer counter, and four counts were made. The spore counts were made to obtain a final concentration of $1x10^3$ conidia μl^{-1}.

The origin of the grapes was Alicante from Spain. Grapes were sterilized before fungal inoculation, and briefly dipped in 70% ethanol, washed with tap water and put in a sodium hypoclorite solution (0.7%) for 10 minutes; and then washed thoroughly. Grapes were inoculated with 5 µl of a spore suspension of *Botrytis cinerea* 2100 in infection buffer (0.11 M glucose, 67 mM KH_2PO_4), with a digital finnpipette.

On each grape a small wound was produced with a nail head in the equatorial zone. Each wound was then inoculated with only buffer in the control group, and buffer plus conidia of *Botrytis cinerea* in infected group (this methodology is summarized in Figure 2).

The inoculated grapes were incubated at 15-23ºC and 80-85% relative humidity, under natural light (approximately 10 hours/day).

The analysis frequency was 0, 3, 6 and 9 days. The samples were analysed for the total viable count of microorganisms in the fruit. Three grapes were weighted and then sterile peptone water was added until a 1:10 dilution was obtained ("initial suspension"). The sample had to be mashed and homogenized under aseptic conditions. After the preparation of decimal dilutions, 1 ml of these and the initial suspension were transferred to a sterile Petri dish. The culture media used in this analysis were: for Aerobic microorganisms and for moulds and yeast, Plate Count Agar (PCA), Sabouraud Dextrose Agar (SDA) and Potatoe Dextrose Agar (PDA) (Figure 3). The samples were incubated at temperatures recommended by the International Organization for Standardization [8-9].

Noninfected filament fungi that grew in any culture media were identified by the Saccardo System (10).

RESULTS AND DISCUSSION

EXTERNAL ASPECTS

Control group (non-infected): Figure 4 shows the grapes at 7th incubation day. During the incubation time, the tissue of the grapes was soft to the touch, with less turgidity and the skin was wrinkled. The same was observed with the infected group.

FIGURE 4 - Grapes at 7th incubated day in control group.

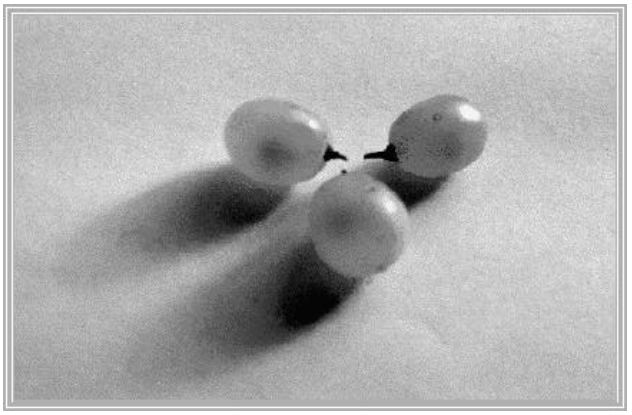
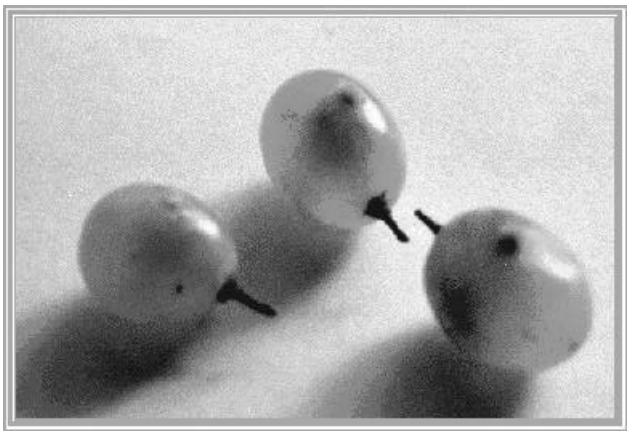


FIGURE 5 - Grapes at 9th incubated day in infected group.



Infected group: Between 0-3rd day, white mycelial growth, and light brown epidermic cells around inoculation area appeared. Between 3rd-6th day, white-gray mycelial growth, and darker brown epidermic cells around inoculation area appeared. Between 6th -9th day, mycelial with conidiophores are observed. Figure 5 shows the grapes at 9th incubation day. The grapes became totally brown.

TOTAL VIABLE MICROORGANISMS

Tables 1-5 show the results of colony forming units of microorganisms in PCA, SDA and PDA.

Results obtained in Plate Count Agar (PCA)

The count of microorganisms was greater in the infected group than in the control group. In both cases the count increased with the age of the lesion. Practically all the microorganisms that grew on PCA were fungi, and the growth of *Botrytis cinerea* could be detected from the 6th day in the majority of the samples.

Results of Moulds and Yeasts in SDA, PDA

Other fungi distinct from the inoculated *Botrytis cinerea* grew well with the increase of the incubation time. In the control group the growth of moulds and yeasts appeared too.

In general, the results obtained during the first days of the experiment are summarized as follows:

Control group

Day 0: 100% < 10 cfu/g.
Day 3: 96.7% ≤ 10 cfu/g. 3.3% > 10^2 cfu/g.

Infected group

Day 0: 50% < 10 cfu/g.
50% ≤ 10^2 cfu/g.
Day 3: 23.3 % < 10 cfu/g. 66.6 % ≤ 10^2 cfu/g.
10% > 10^3 cfu/g.

TABLE 1 - Total Viable Count of Microorganisms (c.f.u./g). Experience 1.

A) Control Group

Day	Medium	A1	A2	A3	A4	A5
6	PCA	< 10	< 10	< 10	< 10	< 10
	SDA	< 10	< 10	< 10	< 10	< 10
	PDA	< 10	< 10	< 10	< 10	< 10
9	PCA	< 10	< 10	< 10	< 10	< 10
	SDA	< 10	< 10	< 10	< 10	< 10
	PDA	< 10	< 10	< 10	< 10	< 10

B) Infected Group

Day	Medium	B1	B2	B3	B4	B5
6	PCA	$1.7x10^3$	< 10	< 10	< 10	< 10
	SDA	< 10	2.6×10^2	< 10	< 10	< 10
	PDA	5.3×10^2	1.3×10^2	< 10	< 10	< 10
9	PCA	1.8×10^2	1.4×10^3	< 10	< 10	8.4×10^2
	SDA	2×10^2	1.9×10^3	< 10	< 10	5.8×10^2
	PDA	2×10^2	1.7×10^3	< 10	< 10	4×10^2

TABLE 2 - Total Viable Count of Microorganisms (c.f.u./g). Experience 2.

A) Control Group

Day	Medium	A1	A2	A3	A4	A5
6	PCA	< 10	1.5×10^2	2.3×10^4	< 10	1.3×10^4
	SDA	< 10	1.2×10^2	4.6×10^4	7.5×10^2	< 10
	PDA	< 10	1.3×10^2	4.7×10^4	7×10^3	2×10^3
9	PCA	4.5×10^2	4.3×10^3	3.8×10^3	5.4×10^2	< 10
	SDA	1.9×10^4	5.8×10^3	7×10^3	5.3×10^2	7.3×10^3
	PDA	4.9×10^4	5×10^3	3.3×10^4	7.1×10^2	1.3×10^5

B) Infected Group

Day	Medium	B1	B2	B3	B4	B5
6	PCA	< 10	< 10	6.7×10^2	4.6×10^2	1.3×10^3
	SDA	1.2×10^3	3×10^2	6.2×10^2	7×10^2	1.1×10^3
	PDA	2.8×10^3	5×10^2	5.2×10^2	9×10^2	1.1×10^3
9	PCA	2.1×10^2	4.3×10^2	5.4×10^2	1×10^2	1.5×10^2
	SDA	2.1×10^2	3.7×10^2	4×10^2	8.9×10^4	5.3×10^4
	PDA	1×10^3	5.3×10^2	1×10^4	8.2×10^4	5.6×10^4

TABLE 3 - Total Viable Count of Microorganisms (c.f.u./g). Experience 3.

A) Control Group

Day	Medium	A1	A2	A3	A4	A5
6	PCA	3.3×10^3	< 10	< 10	6.5×10^2	< 10
	SDA	1.2×10^3	< 10	< 10	7.4×10^2	< 10
	PDA	2×10^3	< 10	< 10	2.5×10^3	< 10
9	PCA	< 10	< 10	< 10	< 10	< 10
	SDA	< 10	< 10	< 10	< 10	< 10
	PDA	< 10	< 10	< 10	< 10	< 10

B) Infected Group

Day	Medium	B1	B2	B3	B4	B5
6	PCA	< 10	< 10	< 10	1.3×10^4	< 10
	SDA	3.8×10^2	< 10	< 10	2.3×10^4	8.7×10^3
	PDA	5.8×10^2	< 10	< 10	6.2×10^4	4.1×10^4
9	PCA	< 10	< 10	< 10	1×10^4	< 10
	SDA	2.6×10^2	6.8×10^4	1×10^2	7×10^3	1.7×10^3
	PDA	3.7×10^2	8.6×10^4	2×10^2	1.4×10^4	1.7×10^3

TABLE 4 - Total Viable Count of Microorganisms (c.f.u./g). Experience 4.

A) Control Group

Day	Medium	A1	A2	A3	A4	A5
6	PCA	< 10	< 10	< 10	6.5×10^2	< 10
	SDA	< 10	4×10^3	< 10	7.4×10^2	< 10
	PDA	< 10	1.4×10^4	< 10	2.5×10^3	< 10
9	PCA	< 10	< 10	< 10	< 10	< 10
	SDA	< 10	1.4×10^4	< 10	< 10	5.7×10^3
	PDA	< 10	1.7×10^4	< 10	< 10	4.7×10^4

B) Infected Group

Day	Medium	B1	B2	B3	B4	B5
6	PCA	< 10	< 10	4.2×10^2	< 10	< 10
	SDA	3.5×10^2	< 10	4.9×10^2	< 10	1.2×10^3
	PDA	1.3×10^2	< 10	4.8×10^2	< 10	1×10^3
9	PCA	4.6×10^2	< 10	2.8×10^4	1×10^2	2.4×10^2
	SDA	9.5×10^4	5.2×10^4	9.3×10^2	1.1×10^3	2.6×10^3
	PDA	9.8×10^4	5.6×10^4	1.6×10^4	9×10^2	2.5×10^3

TABLE 5 - Total Viable Count of Microorganisms (c.f.u./g). Experience 5.

A) Control Group

Day	Medium	A1	A2	A3	A4	A5
6	PCA	< 10	< 10	< 10	< 10	< 10
	SDA	< 10	< 10	< 10	< 10	< 10
	PDA	< 10	< 10	< 10	< 10	< 10
9	PCA	< 10	< 10	< 10	< 10	< 10
	SDA	< 10	< 10	< 10	< 10	1.9×10^3
	PDA	< 10	< 10	< 10	< 10	2.8×10^3

B) Infected Group

Day	Medium	B1	B2	B3	B4	B5
6	PCA	< 10	1.7×10^3	1.9×10^3	< 10	< 10
	SDA	4.5×10^2	2.4×10^3	1.8×10^3	4.2×10^2	4×10^2
	PDA	< 10	1.2×10^3	1×10^4	< 10	< 10
9	PCA	1.4×10^3	1.2×10^3	1.3×10^3	2.4×10^5	1.4×10^3
	SDA	6.8×10^5	7.4×10^5	1.4×10^5	2.1×10^5	3.9×10^4
	PDA	1.1×10^6	6×10^5	1.4×10^5	3.6×10^5	1.2×10^5

In general, the c.f.u./g grapes obtained were fungi and yeast. Bacteria was found in the last experiments. The low growth of bacteria in these experiments can be explained by three factors:

1. Natural bacteria on the surface of the grapes was eliminated by the sterilization process.
2. Organic acidity in unripe grapes [11].
3. Production of antibiotic substances as reaction to the infection (phytoalexines) [12].

The growth of bacteria occurred in the last two experiments, possibly due to conditions similar to that of the storage at retail and greater maturity of the grapes, due to higher level of sugar.

From the time of inoculation until day 6, we observed only minimal growth in the infected group, that can be explained by different factors, according to Bourgeois *et al.*, 1994 [12]:

- Toxic compounds in the immature fruit, glycoalkaloides, tannins and phenols.
- Inactivation of the pathogen enzymes by great concentration of phenolic compounds [6]. Non-action of the enzymes of the pathogen agents on the immature fruit.
- Immature fruit does not have enough nutrients to support the parasites.

To identify the non-inoculated fungi that grew in PCA, SDA or PDA, we used the Saccardo System [10]. It is based on the morphology of the sporulating structures, as well as the morphology and colour of the conidia. The identified strains were: *Penicillium* spp.; *Aspergillus* spp.; *Alternaria* spp. Moreau, 1987 [13] described the principal microorganisms that cause alteration on the grapes: *Alternaria* sp.; *Botryosphaeria ribis*; *Botrytis cinerea; Cladosporium herbarum*; *Guignardia bidwellii*; *Penicillium* sp.; *Rhizopus stolonifer*. Temperature and humidity values applied in these experiments could be different from the real field values, for example, high relative humidity means low temperatures [14]. These conditions can be found when fruits are stored, but not when they are in the fields.

CONCLUSIONS

1. Deterioration of the grapes occur from the development of *Botrytis* as well as other microorganisms.
2. A correlation exists between the total viable count and spoilage with increased incubation time. On the 6th day, the Botrytis lesion demonstrates a few conidiophores and the appearance of the grapes make them unacceptable for consumption.
3. Microorganisms that cause the deterioration of the grapes may be yeasts, bacteria, and/ or imperfect fungi such as *Penicillium*, A*spergillus*, and *Alternaria*.

REFERENCES

[1] AOSPLANT, Project FAIR PL.97-3351: 1998-2001 (2000)

[2] Hansen, H.N., Mycologia, **30,** 442 (1983)

[3] Menzinger, W., Abteilung 2, **120,** 141 (1966)

[4] Domsch, K. H. and Gams, W. Wiley J., Halsted Press Division, New York (1972)

[5] Cole, L., Dewey, F.M. and Hawes, C.R., Mycological Research, **100 (3),** 227 (1996)

[6] Agrios, G.N., Fitopatología 2ª Edition. Limusa S.A. Uteha. Grupo Noriega (Eds.), México D.F. (1995)

[7] Thomas, C.S., Marois, J.J. and English, J.T., Phytopathology, **78 (3),** 260 (1988)

[8] ISO 4833, International Organization for Standardization, Gèneve (1991)

[9] ISO 7954, International Organization for Standardization, Genève (1987)

[10] Barnett, H.L. and Hunter, B.B., Illustrated Genera of Imperfect Fungi, 3^{h} Editon, Burgess Publishing Company, Minneapolis, (1972)

[11] Pascual, M.R., Microbiología Alimentaria. Metodología Analítica para Alimentos y Bebidas, Editorial Díaz de Santos, Madrid (1992)

[12] Bourgeois, C.M., Mescle, J.F. and Zucca, J., Microbiología Alimentaria, Tomo 1, Bourgeois C.M., Mescle J.F. and Zucca J. (Eds.), Editorial Acribia, S.A., Zaragoza, (1994)

[13] Moreau, C., Microbiología Alimentaria, Tomo 1, Bourgeois C.M., Mescle J.F. y Zucca J. (Eds.), Editorial Acribia, S.A., Zaragoza (1987)

[14] Hyre, R.A., Plant Disease Report, **56 (2),** 126 (1972)

Received for publication: October 15, 2001
Accepted for publication: November 14, 2001

CORRESPONDING AURTHOR

A. Sánchez
Laboratorio Centro de Análisis
Asesoramiento y Control de Calidad
CAYACEA S.A
Miguel Yuste 12- 1ª
28037 Madrid –SPAIN

email: a.sanchez@cayacea.com

AFS – Vol 23/ No 4/ 2001 – pages 153– 159

GC/MS ANALYSIS OF THE EXTRACTIVES BEFORE THE CONVERSION AND THE OIL FROM LOW TEMPERATURE CONVERSION (LTC) OF CORNCOB

Gilberto A. Romeiro*, Tadeu H. dos Santos*, Marcos C. de Souza*, Raimundo N. Damasceno, M. Kutubuddin*** and E. Bayer*****

* Universidade Federal Fluminense, Instituto de Química, Departamento de Química Orgânica, Niterói, CEP 24210-150, RJ, Brazil.

** Universidade Federal Fluminense, Instituto de Química, Departamento de Geoquímica, Niterói, CEP 24210-150, RJ, Brazil.

*** Institute für Organische Chemie, Universität Tübingen, Auf der Morgenstelle 18, 72076, Tübingen, Germany.

SUMMARY

Low Temperature Conversion of corncob, a very common waste material from Brazilian plantations, was accomplished. The qualitative composition of the oily phase obtained in the process was analyzed by GC/MS. This phase was determined to be basically constituted by phenols, which suggests that the oil may have been originated in the decomposition of lignin present in the sample. GC/MS technique was also used to analyze the organic extracts, which were derived from the sequential extraction of the sample taken before the conversion.

KEYWORDS:
Corncob, Low Temperature Conversion (LTC), GC/MS.

INTRODUCTION

The concept of Low Temperature Conversion (LTC 380-450 °C) is long well known[1]. Bayer and co-workers[2] in Germany and Romeiro in Brazil[3,4] have developed expressive work in the LTC area for a sort of agricultural and industrial residue.

The molecules that compose the biomass from agricultural waste material (carbohydrates, lipids, proteins, etc) are thermally degraded in the LTC process leading to the generation of liquid pyrolytic products, solid residue (char), water and gas. The yield and the composition of such products strongly depend on the kind of biomass and its dryness.

LTC became an alternative method for handling solid waste polluter materials because, unlike at the pyrolytic conditions at higher temperatures, no carcinogenic substance, such as polychlorinated dibenzodioxins and polychlorinated dibenzofurans (PCDD and PCDF), is detected.[5-7] The product from the conversion can be used not only as a potential source of energy or for economic reasons[8], but also for many other purposes, as in filters (char) or as starting material (oil) for the chemical industry[9].

Corncob was the biomass used in our work. It is normally rejected during the corn processing. Nevertheless, some companies have proposed the utilization of corncob for different purposes: as absorbent for chemical waste, as source of active coal, for smoking food preparation, as plywood, in the card-bond manufacture, etc.

Corncob is basically constituted of lignin and polysaccharides (~83%), which are responsible for the chemical composition of the products from the conversion.

The oil from the conversion was characterized as a complex mixture. Attempts to elucidate the composition of this oily phase were made by GC/MS technique.

MATERIALS AND METHODS

The sample corncob was obtained from house maize-field (Cachoeiras de Macacu, Rio de Janeiro-Brazil) and stored at room temperature before drying at 60 °C to constant weight. The sample was triturated to different granulation (Granutest® of 2.0, 1.0, 0.5, 0.75, 0.125 and 0.053 mm).

The dried corncob (50 g) was extracted with 200ml each of different solvents in the increasing polarity order, hexane, dichloromethane and acetone. After refluxing during 48 h the extracts were dried with 5 g of anhydrous $MgSO_4$, filtered and then concentrated. They were thereafter analyzed by GC/MS.

LTC was carried out batchwise in a laboratory reactor under exclusion of oxygen at 380 °C for two hours. 250 g of sample was used for each run. Details about the process and equipment are described elsewhere. The yields of LTC oil, water and char were determined by weight balance, while the gas yield is calculated from 100 – yield (char + water + oil) [%].

The samples were injected on a capillary column Ultra-2 (25m x 0.2mm x 0.33µm), injection volume 1-3 µL, split ratio 1:100, injection temperature 260 °C, and Flame Ionization Detector (FID) 280 °C. The temperature program for the analyses was: started at 80°C and held for 10 min, then at heating rate of 10°C/min up to 280 °C and held for 20 min.

Mass selective detector (MSD): Mass spectra were recorded on a Hewlett Packard, 5988A instrument, using EIMS source at 70 eV and 230 °C, quadrupole analyzer at 106 °C and electronic detector at 1682V, GC/MSD interface at 240 °C, range of mass 30-400 amu.

RESULTS AND DISCUSSION

The biomass was sequentially extracted with different solvents before the conversion and the extracts were analyzed by GC/MS. The results of the analysis are shown in Table 1. Thus, hydrocarbons, carboxylic acids, aldehydes and phenols revealed to be the main substances identified.

TABLE 1
Substances found in the extract of corncob before the LTC process [a] in n-hexane, [b] in dichloromethane and [c] in acetone.

Substance	Retention Time[a] (min.)	Retention Time[b] (min.)	Retention Time[b] (min.)	Quality (%)
heptadecane	18.907			95
hexadecanoic acid[a]	24.223			97
eicosane	24.937			91
octadecane	26.755			91
linoleic acid	27.357			97
9,17-octadecadienal-Z	27.443			95
n-docosane	28.517			98
octacosane	36.412			98
phenyl acetaldehyde		9.154		90
1,3-benzodioxol-5-methanol		11.898		91
4-vinyl phenol			7.123	90
4-vinyl-2-methoxy phenol			7.634	95
4-hydroxy benzaldehyde			7.798	95
4-hydroxy-3-methoxy benzaldehyde			8.054	96
hexadecanoic acid			13.400	99
linoleic acid			16.036	97
tricosane			18.668	90
tetracosane			20.218	94
pentacosane			21.728	95
hexacosane			23.204	93
heptacosane			24.715	91

FIGURE 1 - Schematic diagram and yield of products from LTC process.

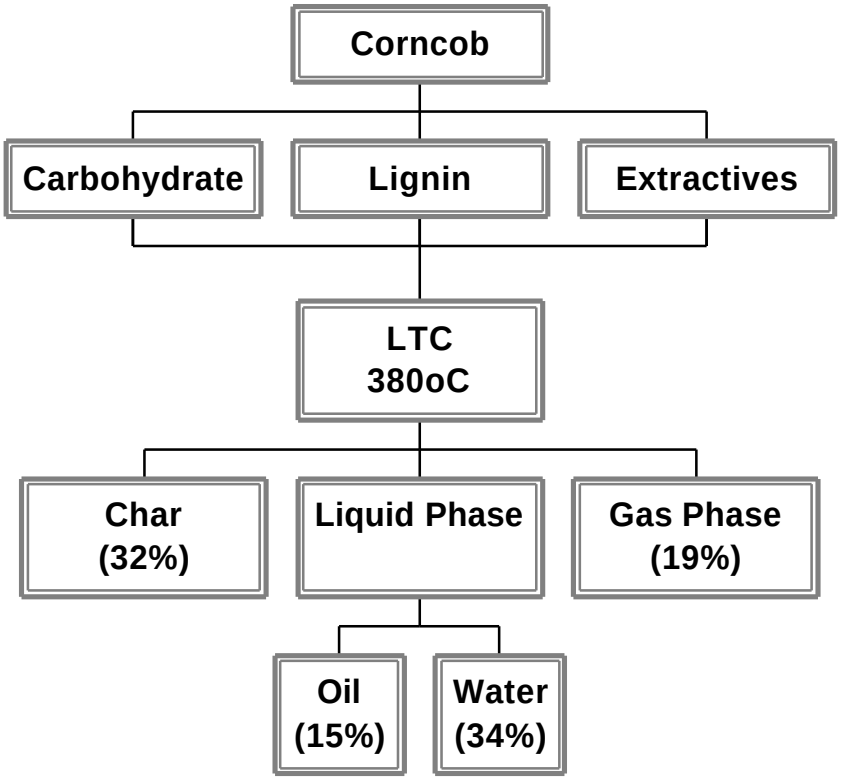


FIGURE 2 - GC of the oil from LTC of corncob.
(column: Ultra-2, 25 m x 0.2 mm x 0.33 m; split ratio 1 : 100; injector: 260 °C; FID: 280 °C; 10°C/min to 280 °C)

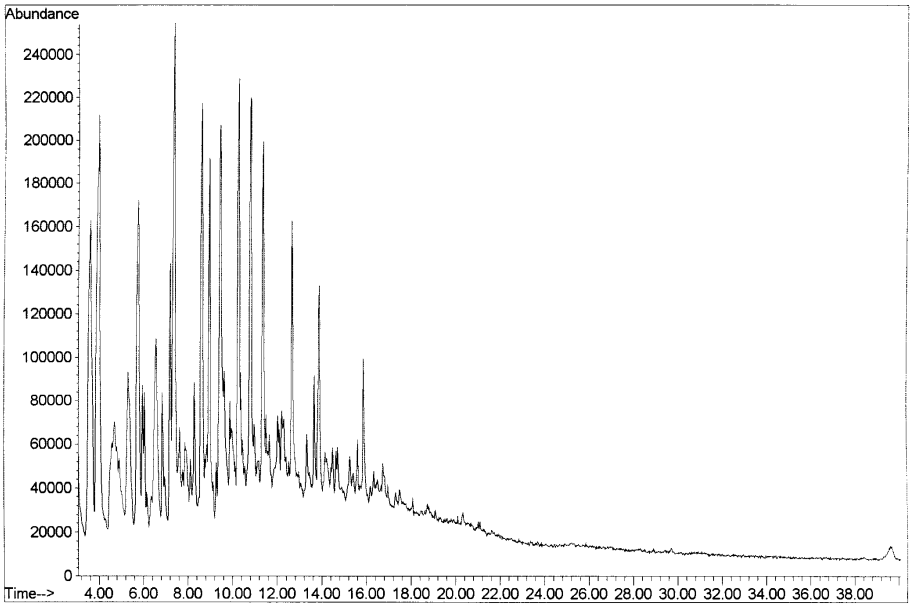


TABLE 2 - Composition of the oil from LTC of corncob.

Compound	Retention Time (min)	Quality (%)
phenol	5.767	91
corilone	6.544	95
2-methyl phenol	6.856	95
3-methyl phenol	7.199	94
2-methoxy phenol	7.381	94
2,5-xylenol	8.268	91
4-ethyl phenol	8.611	91
2-methoxy-4-methyl phenol	8.954	97
4-ethyl-2-methoxy phenol	10.266	94
4-vinyl-2-methoxy phenol	10.810	81
2,6-dimethoxy phenol	11.375	96
4-hydroxy-3-methoxy benzaldehyde	12.202	94
2,6-dimethoxy-4-(2-propenyl) phenol	15.854	89

When applied to corncob, LTC process produced a solid phase (char), a liquid phase (oil and water) and a gas phase. The yields (by weight) and a schematic diagram of the process are represented in Figure 1.

Oil Analysis

The oil from LTC was properly separated and analyzed by gas chromatography. The total ion chromatogram (TIC) is shown in Figure 2. Comparison with the library of the mass spectrometer allowed the elucidation of the composition of the oil (Table 2).

During the process of Low Temperature Conversion the different kinds of molecules that constitute the biomass undergo fragmentation and de-polymerization reactions caused by temperature increasing[10].

Phenolic compounds predominate in the oil composition. This fact suggests that the oil may have been originated from the lignin fraction of corncob, which is constituted by a great variety of methoxy substituted aromatic compounds arranged aleatory[11]. Alternatively, the aromatic compounds may have been originated from an aldol-like rearrangement of the carbohydrate[12] present in corncob. However, the major amount of methoxy substituted phenols found in the oil extract[11] indicates that lignin is one of the main constituents in the final composition of the oil.

CONCLUSIONS

The organic extracts collected before LTC process for corncob sample showed a high variety of chemicals, most of them aromatic compounds; the major components, however, being cellulose, hemicellulose and lignin[11].

When applied to corncob, LTC process afforded an oily fraction in nearly 14% yield by weight, which is basically composed by substituted phenols. The complex composition of that oil makes unattractive and time consuming any attempt for getting purified phenols. Nevertheless, mixtures of that kind, sometimes called "bio-oil", are worthwhile when used as fertilizers, in the adhesive industry, as upgrade in the combustible industry, etc.

ACKNOWLEDGEMENTS

We are thankful to Claudete Taniguchi e Miriam Sanctos from INT for the GC/MS analysis and to CNPq for the financial support for our work.

REFERENCES

1. Shibata, S., Procede de Fabrication d´une Huille Combustible a Partir de Boue Digeree. Fr. Pat., 838.063, **1939**.

2. Bayer, E. and Kutubuddin, M., Thermocatalytic Conversion of Lipid-rich Biomass to Oleochemicals and Fuels, Presented at the *International Conference on Research in Thermochemical Biomass Conversion*, Phoenix, USA, **1988**.

3. Romeiro, G. A., Bayer, E., Vieira, G. G., Santos, M. C., Kunigami, C. G., Kutubuddin, M. and Damasceno, R. N., Low Temperature Conversion of Beverage Industry, *Advances in Food Sciences*, **2000**, 22 (5/6), 170-177.

4. Romeiro, G. A., Bayer, E., Damasceno, R. N., Lutz, H. and Kutubuddin, M., Identification of Fatty Acids in Oil From Low Temperature Conversions (LTC) Using Fast Atom Bombardment (FABMS) and Field Desorption (FDMS), *Fresenius Environmental Bulletin*, **1999**, 9, 163-170.

5. Hagenmaier, H., Kraft, M., Brunner, H., Haag, R., Catalytic Effects of Fly Ash From Waste Incineration Facilities on the Formation and Decomposition of Polychlorinated Dibenzo-p-dioxins and Dibenzofurans, *Environ. Sci. Technol*., **1987**, 21, 1085-1094.

6. Stieglitz, L., Vogg, H., On Formation of PCDD/PCDF in Fly Ash from Municipal Waste Incinerators, *Chemosphere*, **1987**, 16, 1317-1322.

7. Stegen, M. Th., Fate of Chlorinated Organic Compounds During Thermal Conversion of Sewage Sludge, *Wat. Sci. Tech*., **1990**, 26, 2261-2264.

8. Bridle, T. R., Hertle, C. K., Oil from Sludge: A Cost Effective Sludge Management System, *Water*, **1988**, 32-36.

9. Bayer, E., Becker, G., Kutubuddin, M. and Maurer, A., Recovery of Activated Carbon from Wastes via Low Temperature Conversion Part I: Preparation and Determination of Pore Structure, *Fresenius Environmental Bulletin*, **1995**, 4, 533-538.

10. Shafizadeh, F. and DeGroot, W. F., *Thermal Uses and Properties of Carbohydrates and Lignins*, Academic Press, **1976**.

11. Goldstein, I. S., *Organic Chemicals from Biomass*, CRC Press Inc., **1992**.

12. Nelson, D. A.; Hallen, R. T.; Theander, O., *Pyrolysis Oils from Biomass - Producing Analyzing and Upgrading*, American Chemical Society, Washington DC, **1988**.

Received for publication: October 15, 2001
Accepted for publication: November 14, 2001

CORRESPONDING AURTHOR

Marcos C. de Souza
Universidade Federal Fluminense
Instituto de Química
Departamento de Química Orgânica
Niterói
CEP 24210-150, RJ - BRAZIL

email: gqomarc@vm.uff.br

MICROFLORA AND PROXIMATE COMPOSITION OF *OKPEHE*, A FERMENTED CONDIMENT FROM *PROSOPIS AFRICANA* SEEDS

F. A. Oguntoyinbo[1*], A. A. Onilude[2] and A. I. Sanni[2]

[1] Department of Botany and Microbiology, University of Lagos, Akoka, Nigeria.

[2] Department of Botany and Microbiology, University of Ibadan, Nigeria.

SUMMARY

Studies were conducted on **okpehe,** a Nigerian alkaline fermented condiment used as a seasoning agent. 141 strains of bacteria were identified as *Bacillus subtilis* (36.17%); *Bacillus pumillus* (19.86%); *Bacillus licheniformis* (15.60%); *Bacillus megaterium* (13.48%) and *Enterococcus* (14.89%). *Bacillus* species were most dominant with average counts of $2.20 \times 10^7 - 8.40 \times 10^8$ cfu /g while *Enterococci* range from $5.10 \times 10^6 - 1.40 \times 10^7$. Yeasts and Gram-negative bacteria were not detected in all the samples.The moisture content of **okpehe** from different market ranges varied from 40.70 – 60.00%, while the protein content ranged from 32.83 – 34.92%, fat content 1.23–143%, fiber 1.29–1.56%, ash 3.08–4.41%, starch 13.59 – 14.20% and pH values from 6.80 – 7.81.

KEYWORDS:
Bacillus, Enteroccus, okpehe, fermentation, Prosopis africana

INTRODUCTION

Okpehe is a strong smelling traditional fermented soup condiment produced by fermentation of *Prosopis africana* seeds (*Guill., Perrott*. & *Rich*.) Taub. a leguminous seed [1, 2]. The people of the middle belt and eastern region of Nigeria consume the condiment and use it as seasoning agent that produces a pleasing aroma and serves as tasty, low cost protein source in soups and sauces.

Fermentation of vegetable proteins generally results in complete hydrolysis of moisted cotyledons with enhancement of nutritional profile [3]. Production of most condiments in West Africa is by moist solid-state fermentation, mainly by chance inoculation brought about by various species of microorganisms [4]. Different species of *Bacillus, Lactobacillus, Staphylococcus, Leuconostoc* and *Pseudomonas* have been reported [5, 6].

Production of **okpehe** in Nigeria is still a traditional family art practiced in homes in crude manners with rudimental utensils, starter cultures are not used, the fermentation process is uncontrolled and variation in the associated microflora from one region to the other is imminent. Also, spoilage and pathogenic microorganisms cannot be totally ruled out in some fermentation batches. Knowledge of microflora of **okpehe** is essential in order to select the appropriate microorganisms that can bring about the desirable product.

Barminas *et al*. [7] reported on the nutritional content of *Prosopis africana* seeds. Leguminous seeds are not consumed in their natural state due to one anti-nutritional factor or the other [8], although the seeds are cheap and readily available. They can support the nutritional and dietary intake of rural and urban people when fermented to products such as **okpehe**. In West Africa, information is available on similar products such as **iru**, **ugba** and **ogiri** [4, 9, 10]. But there is still need of information on **okpehe** and its nutritional potential. Therefore, this paper provides information on the microflora and the proximate composition of **okpehe**.

MATERIALS AND METHODS

Sample collection: Fifty samples of okpehe were collected randomly from different retail markets in Lagos, Nnsuka, Ankpa, Makurdi and Ibadan, Nigeria. They were aseptically transported to the laboratory in sterile polyethylene bags and stored at 4 °C. Microbiological analyses were conducted immediately on the samples.

Laboratory preparation of okpehe: Viable seeds of *Prosopis africana* were boiled at 100 °C for 15-24 hrs until they become soft and dehulled by pressing between palms. Then the cotyledons were washed and boiled in an autoclave at 121 °C for 15 min and spread on calabash trays already lined with pawpaw leaves. The trays were sticked together and incubated at 37 °C for 48 hrs [25]. The final fermentation product is okpehe with its characteristic pungent aroma. Samples were taken for analysis.

TABLE 1 - Characteristics of *Bacillus* species isolated from okpehe.

Tests	*Bacillus* species			
	A	**B**	**C**	**D**
Gram's reaction	+	+	+	+
Endospore	+	+	+	+
Catalase	+	+	+	+
Methyl red test	+	+	+	+
Voges-Proskauer	+	+	+	-
Anaerobic growth	-	+	-	-
Acid from sugars				
D-Glucose	+	+	+	+
L- Arabinose	+	+	+	+-
D-Xylose	+	+	+	+-
D-Mannitiol	-+	+	+	+-
Hydrolysis of				
Casein	+	+	+	+
Starch	+	+	-	+
Gelatine	+	+	+	+
Utilization of				
Citrate	+	+	+	+
Propionate	-	+	+	w+
Growth in NaCl				
5 %	+	+	+	+
7 %	+	+	+	+
10 %	+	+	+	+
Growth at				
10 °C	+	+ -	-	+
30 °C	+	+	+	+
50 °C	+-	+	+	-
60 °C	-	-	-	-
	Bs	**Bl**	**Bp**	**Bm**

Bs = *B. subtilis*; Bl = *B. licheniformis*; Bp = *B. pumilus*; Bm = *B. megaterium.*
Positive = (+); Negative = (-); Positive / negative = (+ -); Weak positive = (w+)

Isolation procedure: 10 g each of market samples of okpehe and laboratory samples of fermenting *Prosopis africana* seeds were aseptically placed in sterile stomacher bags (A. J. Seward, London) and 90 ml of sterile distilled water was added and homogenized by stomaching 90 s in a Colworth Stomacher (A. J. Seward, London), and 1 ml was diluted ten-fold serially. The pour plate method of Harrigan and McCance [11] was adopted, 0.1 ml of 10^{-6} cfu/g was transferred into sterile petri dishes and molten nutrient agar (NA, oxoid), de Man Rogosa Sharpe agar (MRS, oxoid), Potato dextrose (PDA) agar and Maconkey agar were, respectively, cooled off to 47-50 °C and poured on the suspension. The plates were gently rotated to obtain dispersion of inoculum before incubation at 30 °C. MRS plates were incubated under anaerobic conditions in jars at 30 °C. Different colonies of distinct morphology were counted using a colony counter (GallenKamp). Finally, colonies were randomly picked and further subcultured on nutrient agar and MRS agar, respectively. Uncountable growth of *Bacillus* species was observed on NA and MRS agar and no growth on Maconkey and PDA agar using the spread plate method for isolation.

Characterization of isolates: Cells of the pure culture were stained using the method of Collins and Lyne [12] and observed microscopically. Catalase tests were performed on 24 hrs old cultures. Fermentation of sugars was determined using microtiter plates and hydrolysis of starch by the method of Harrigan and McCance [11]. Proteolytic activity was assessed using hydrolysis of casein and hydrolysis of gelatine [13]. Growth at different pH values, temperature and NaCl were determined as described by Gordon et al. [14]. Voges-Proskauer (V.P.) test and anaerobic growth were carried out as described by Claus and Berkeley [15]. Non endospore-forming bacteria were not characterized further, while selected strains of endospore-forming rods were further characterized with API 50 CHB and API 20E (Biomerieux France).

pH measurement: 10 g of each of the unfermented cotyledon, and market and laboratory fermented okpehe samples were homogenized with 90 ml of distilled water in a Colworth Stomacher. The pH of the homogenate was determined using a digital pH meter (WTW pH 526).

Proximate analysis: Crude protein, fibre and ash were determined by the procedures of the A.O.A.C. [16]. The fat content was determined by ether extraction in a Rose-Gottlieb extractor. Carbohydrate content was calculated by difference [Standal, [17]). Moisture content was determined by drying the well-mixed sample at 130 ± 1 °C to constant weight (A.O.A.C. [16]). Free fatty acids were calculated by titrating the filtrates with 0.1 N sodium hydroxide to the end point of phenolphthalein 0.1% w/v in 95% ethanol. Unfermented samples served as control in all tests.

RESULTS

A total of 141 strains of bacteria isolated from 50 samples of **okpehe** were grouped on the basis of their colony morphology, cell shape, spore formation, catalase activity, motility, oxygen requirement and sugar fermentation (Tables 1 and 2). The spore-forming bacteria were assigned to the genus *Bacillus* [15] and non spore-forming cocci to *Enteroccoci* [18].

In the isolates *B. subtilis* occurred most frequently (36.17%) while *B. megaterium* was found in the lowest percentage (13.48%, Table 3). The average mean counts of the microbial load of the condiments are presented in Table 4. The range obtained for the colony forming units/g was 10^6 to 10^8. The proximate composition of *Prosopis africana* and **okpehe** is shown in Table 5. The protein, fat, fiber, ash and starch content of **okpehe** samples was less than that of *Prosopis africana* cotyledon, while the sugar and free fatty acid content of substrate was similar to that of the fermented samples. The mean pH of *Prosopis africana* was 6.68, higher pH was found for **okpehe**. The moisture content of *Prosopis africana* was 30% and there was appreciable difference in the moisture content of samples from different sources.

DISCUSSION

The preparation of **okpehe** is very similar to that of **daddawa** in West Africa, **soumbala** in French speaking African countries, **kinema** in India and **natto** in Japan [19, 20, 21]. The dominance of *Bacillus* species in the fermented **okpehe** is in agreement with earlier microbiological information available on similar condiment in West Africa and Asia [22]. These bacteria may have originated from the air or the fermentation vessels (calabash), a symbolic ubiquitous nature of these organism [23], However, *Enterococci* detected in some samples in this study are often isolated from plant materials. They were unexpected in the microflora, although Sarkar *et al*. [24] isolated *Enterococci* during **kinema** production. The possible role of *Enterococci* during fermentation, its probiotic potential and virulence factors needed to be investigated.

The high population rate of *B. subtilis* is an indication of being the main fermentation microflora. Similar trend was reported during **iru**, **ogiri** and **kinema** production [25, 26]. All other *Bacillus* strains demonstrated their unique characteristics and were proved to be non-variants of *Bacillus subtilis* as confirmed by API 50 CHB combined with API 20 E.

Okpehe production is a moist solid state fermentation and a high moisture content is expected as observed in this study in agreement with earlier work on condiment.

TABLE 2 - Biochemical characteristics of non endo spore, catalase negative isolates.

Isolate #	Cell morphology	Growth at / in								Sugar fermentation																			
		Co2/Glucose	10°c	15°c	45°c	pH3.9	pH9.6	NaCl 6.5%	Arginine hydrolysis	Arabinose	Ribose	Xylose	Galactose	Trehalose	Rhamnose	Mannitol	Sorbitol	Esculine	Salicine	Cellobiose	Maltose	Lactose	Melbiose	Raffinose	Melezitose	Inulin	Glucose	Identification
1	**cocci in pairs**	-	+	+	+	-	+	+	+	w+	+	-	+	+	-	-	-	w+	+	+	+	w+	+	+	-	-	+	Enterococci spp.
2	**cocci in pairs**	-	w+	+	+	-	+	+	+	+	+	-	+	+	-	+	-	w+	+	+	+	w+	+	+	-	-	+	Enterococci spp.
3	**small cocci**	-	w+	+	+	-	+	+	+	w+	+	-	+	+	-	-	-	w+	+	+	+	w+	+	+	-	-	+	Enterococci spp.
4	**cocci in pairs**	-	w+	+	+	-	+	+	+	w+	+	-	+	+	-	+	-	w+	+	+	+	w+	w+	-	-	-	+	Enterococci spp.
5	**cocci in pairs**	-	w+	+	+	-	+	+	+	w+	+	-	+	+	-	+	-	w+	+	+	+	w+	+	+	-	-	+	Enterococci spp.
6	**cocci in pairs**	-	w+	+	+	-	+	+	+	w+	+	-	+	+	-	+	-	w+	+	+	+	w+	+	-	-	-	+	Enterococci spp.
7	**cocci in pairs**	-	w+	+	+	-	+	+	+	w+	+	-	+	+	-	+	-	w+	+	+	+	w+	+	-	-	-	+	Enterococci spp.
8	**cocci in pairs**	-	w+	w+	w+	-	+	+	+	w+	+	-	+	+	-	+	-	w+	+	+	+	w+	+	-	-	-	+	Enterococci spp.
9	**cocci in pairs**	-	w+	+	+	-	+	+	+	w+	+	-	+	+	-	+	-	w+	+	+	+	w+	w+	-	-	-	+	Enterococci spp.
10	**cocci in pairs**	-	w+	+	w+	-	+	+	+	w+	+	-	+	+	-	+	-	w+	+	+	+	w+	w+	-	-	-	+	Enterococci spp.
11	**cocci in pairs**	-	w+	+	+	-	+	+	+	w+	+	-	+	+	-	+	-	w+	+	+	+	-	+	+	-	-	+	Enterococci spp.
12	**cocci in pairs**	-	w+	+	w+	-	+	+	+	+	+	-	+	+	-	+	-	w+	+	+	+	-	+	+	-	-	+	Enterococci spp.
13	**cocci**	-	w+	+	w+	-	+	+	+	w+	+	-	+	+	-	+	-	w+	+	+	+	-	+	+	-	-	+	Enterococci spp.
14	**cocci in pairs**	-	w+	+	+	-	+	+	+	w+	+	-	+	+	-	+	-	w+	+	+	+	w+	+	+ +	-	-	+	Enterococci spp.
15	**cocci in pairs**	-	w+	+	w+	-	+	+	+	+	+	-	+	+	-	+	-	w+	+	+	+	-	+	+	-	-	+	Enterococci spp.
16	**cocci in pairs**	-	-	-	+	-	+	+	+	w+	+	-	+	+	-	-	-	w+	+	+	+	-	+	+	-	-	+	Enterococci spp.
17	**cocci in pairs**	-	w+	+	+	-	+	+	+	w+	+	-	+	+	-	-	-	w+	+	+	+	-	+	+	-	-	+	Enterococci spp.
18	**cocci in pairs**	-	w+	+	+	-	+	+	+	w+	+	-	+	+	-	-	-	w+	+	+	+	-	+	+	-	-	+	Enterococci spp.
19	**cocci in pairs**	-	w+	+	+	-	+	+	+	w+	+	-	+	+	-	-	-	w+	+	+	+	-	+	+	-	-	+	Enterococci spp.
20	**cocci in pairs**	-	w+	+	+	-	+	+	+	w+	+	-	+	+	-	-	-	w+	+	+	+	-	+	+	-	-	+	Enterococci spp.
21	**cocci in pairs**	-	w+	+	+	-	+	w+	+	w+	+	-	+	+	-	+	-	w+	+	+	+	+	+	+	-	-	+	Enterococci spp.
22	**cocci in pairs**	-	w+	+	+	-	+	w+	+	w+	+	-	+	+	-	+	-	w+	+	+	+	+	+	w+	-	-	+	Enterococci spp.

TABLE 3 - Frequency of occurrence of okpehe microflora.

Microorganism	Frequency of occurence: *Prosopis africana*	Nnsuka	Makurdi	Ankpa	Lagos	Ibadan	Number of species	Percentage rate
Bacillus subtilis							51	36.17%
Bacillus licheniformis							22	15.60%
Bacillus pumilus							28	19.86%
Bacillus megaterium							19	13.48%
Enterococci spp							21	14.89%
Total							141	100 %

TABLE 4 - Microbial load of dehulled *Prosopis africana* and okpehe from different soures

Media	Samples: Nnsuka	Makurdi	Ankpa	Lagos	Ibadan	Laboratory
Bacillus spp.	8.40×10^8	1.20×10^7	2.60×10^7	2.20×10^7	2.80×10^7	4.60×10^7
Enterococcus spp.	1.40×10^7	-	6.00×10^6	5.10×10^6	-	-

Data reperesent mean values of five dilutions

TABLE 5 - Proximate composition of unfermented *Prosopis africana* seeds and okpehe samples.

Parameters	*Prosopis africana*	okpehe					
		Nnsuka	Makurdi	Ankpa	Lagos	Ibadan	Laboratory
Protein %	46.42	33.64	34.26	33.95	34.82	32.83	34.82
Fat %	3.59	1.23	1.25	1.30	1.43	1.63	1.43
Fiber %	1.85	1.35	1.29	1.38	1.49	1.56	1.49
Ash %	6.37	3.08	3.10	3.05	4.21	3.40	4.21
Starch %	24.62	13.85	13.70	13.90	13.86	13. 59	13.86
Sugar %	6.63	6.51	6.54	6.60	6.65	6.50	6.65
FFA %	0.73	0.84	0.85	0.86	0.94	0. 86	0.85
Moisture %	30.00	52.80	53.92	40.70	60.00	56.83	53.92
pH %	6.68	7.01	7.11	6.94	7.10	7.00	7.20

Data represent the mean values of five batches of each sample

This is due to hydrolytic decomposition of the fermenting substrate. The moisture content of the different samples differs from one source to the other, this may be due to different soaking and boiling time of the cotyledons before fermentation. Also, the rate of exposure of the samples and the different humidity regime in the different regions of production might have affected the moisture content [27]. High moisture content may not be favorable since it may contribute to rapid microbial spoilage.

The reduction in protein content of **okpehe** may be due to leaching during cooking and hydrolysis of protein in the cotyledons leading to production of amino acids which eventually support growth of the fermentation microorganism and subsequent loss of ammonia [28]. In spite of the reduction in protein content of **okpehe**, it is still a good source of dietary protein.

Leguminous seeds contain high levels of fat and oil and the percentage is equally high in **okpehe**, although lipase activity has been reported to be very minimal during fermentation [25]. This is desirable since high lipase activity may result into rancidity and subsequent objectionable taste [26, 27, 28].

The high pH of the condiment is in line with the pH reported for similar condiments and it is due to production of amines and ammonia by the hydrolytic activity of microorganism [29].

To be able to modernize **okpehe** production and maximize the functional properties of associated microorganisms it is important to study the physiology and genetics of the associated microorganism to aid starter culture development. Also, the optimum fermentation conditions needed to be determined for small scale industrial take-off and product consistency.

Okpehe has enormous potentials to support the nutritional intake of people, especially in West Africa where there is protein malnutrition and deficiency of essential fatty acids. The results in this work have been proved and support the much needed data base for industrialization of indigenous fermented foods in Nigeria.

ACKNOWLEDGEMENT

Award of scholarship by ICSC World Laboratory to Oguntoyinbo Folarin Anthony is gratefully acknowledged.

REFERENCES

[1] Eze, J. M. O. and Obadan, A. O., Inter. J. Trop. Agric. (India) **11**, 88-92 (1993)

[2] Tchoundjeu, Z., Weber, J. and Guarino, L., Agroforestry systems. The Netherlands **39**, 91-100 (1997)

[3] Eka, O. V., Food Chem. **5,** 303-308 (1980)

[4] Odunfa, S. A., Die Nahrung **25**, 811 (1981)

[5] Odunfa, S. A., African fermented foods, in Microbiology of fermented foods vol 2 (ed. Wood B. J. B.), pp. 155-191, London - New York, Elsevier Applied Science Publishers (1985)

[6] Sanni A. I., Onilude, A. A. and Oguntoyinbo, F. A., Adv. Food Sciences (CMTL) **20** 5/6, 163-167 (1998)

[7] Barminas, J. T., Maina, H. M. and Ali, J., Plant Foods for Human Nutrition **52,** 325-328 (1998)

[8] Liner, I. F., Toxic constituents of plant foodstuffs. New York Academic Press (1969)

[9] Odunfa, S. A. and Adewuyi, E. Y., Chem. Mikrobiol. Technol. Lebensm. (CMTL) **9**, 6 (1985)

[10] Odunfa, S. A. and Oyeyiola, J., Plant Food **6**, 155-163 (1984)

[11] Harrigan, W. F. and McCance, M. E. R., Laboratory Methods in Food and Dairy Microbiology. Academic Press, New York 342 pp.(1976)

[12] Collins, C. H. and Lyne, P. M., Staining methods, in Microbiological Methods (ed. Collins, C. H. and Lyne, P. M.), London, Butterworths. (1984)

[13] Seeley, Jr., H. W. and Van Demark, F. J., Microbes in action. A laboratory manual of Microbiology 2nd edn., 361pp., San Francisco, CA W.A. Freeman and Co. (1972)

[14] Godon, R E., Hayunes, W. C. and Pany, C. H., The genus *Bacillus*. USDA Agricultural Handbook No. 427 Washington, D.C. (1973)

[15] Claus, D. and Berkeley, R. C. W., Genus *Bacillus* Cohn 1872, in Sneath, P. H. A., Mair, N. S., Sharpe, M. E. and Holts, J. G. (eds.), Bergeys's Manual of Systemic Bacteriology vol 2, Baltimore Willams and Wilkins 1105-1139 (1986)

[16] A. O. A. C. Official Methods of Analysis 15th edn., Virgina Association of Official Analytical Chemists (1990)

[17] Standal, B. R., J. Nutr. **81,** 279 – 285 (1963)

[18] Devriese, L. A. and Pot, B., The genus *Enterococcus* in the General of Lactic Acid Bacteria (eds. B. J. B. Wood and W. H. Holzapfel), Blackd Academic and Professional (Imprint of Chapman and Hall, London, vol **2**) (1995)

[19] Ogbadu, L. J. and Okagbue, R. N., J. Applied Bacteriol. **65**, 353-356 (1988)

[20] Ohta, T., Natto in Legume based fermented foods (eds. Raddy, N. R, Pierson, M. D. and Salunkhe, D. K.), pp. 85-95, Boca Raton, Florida CRC Press (1986)

[21] Sarkar, P. K., Tamang, J. P., Cook, P. E. and Owens J. D., Food Microbiology **11**, 47-55 (1994)

[22] Campbell-Platt, G., Ecol. Food Nutr. **9**, 123 – 132 (1980)

[23] Reva, O. N., Sorokulove, I. B. and Smirov, V.V., Int. Journal of Systematic and Evoluationary Microbilogy **51**, 1361-1371 (2001)

[24] Sanni, A. I., Ayernor, G. S., Sakyi Dawson, E. and Sefa Dedeh, S., Plant Foods for Human Nutrition **55**, 111-118 (2000)

[25] Sanni, A. I., Chem. Microbiol. Technol. Lebensm. (CMTL) **15,** 97-100 (1993)

[26] Shibasaki, K. and Hesseltine, C. W., Eco. Bot. **16,** 180-195 (1962)

[27] Odunfa, S.A., Plant Food Hum. Nutr. **32,** 11-18 (1983)

[28] Wang, H. I. and Hesseltine, C. W., Can. J. Microbiol. **11,** 727-732 (1965)

[29] Whitaker, J. R., Food Technol. **32,** 175 (1978)

Received for publication: October 15, 2001
Accepted for publication: November 14, 2001

CORRESPONDING AUTHOR

Oguntoyinbo Folarin Anthony
Institut für Hygiene und Toxikologie
Bundesforschungsanstalt für Ernährung
Haid- und Neu-Str. 9
76131 Karlsruhe, Germany.

email: foguntoyinbo@yahoo.com/
anthony.oguntoyinbo@bfe.uni-karlsruhe.de

A NEW ENVIRONMENT-FRIENDLY APPROACH TO THE TREATMENT OF LIGNOCELLULOSE RESIDUES

Mateos, E.[1]; Elías, A.[1]; González , J. M.[2]

[1] Departamento de Ingeniería Química y del Medio Ambiente. University of the Basque Country

[2]Departamento de Filología Inglesa y Alemana. University of the Basque Country

ABSTRACT

Straw from cereals constitutes one of the lignocellulose residues that in great quantities is generated within the agricultural sector, this causing serious environmental disruptions. For this reason, different treatments capable of degrading the fibrous fraction of this by-product have been investigated, their main purpose being its conversion into usable stuff as food complement in the diet of cattle. This research work studies the effect of the alkaline treatment, using NaOH at different concentrations (0.25, 1.0 and 2.0 N) and different substrate-liquid ratios (200, 250, 350 and 1000 g straw/l), on the hemicellulosic fraction (%E-hem) of barley straw samples.

KEYWORDS:
lignocellulose residues, alkaline treatment, hemicellulose.

INTRODUCTION

Straw products with a high cellulose content are available in immense quantities all over the world. Approximately 1kg of residue is generated per each kg of grain harvested. The grain-residue ratio is translated into an excess of 400 million tons of crop residue produced each year in the USA and 330 million tons in Europe (Agricultural Statistics, 1988). Most of this crop waste ought to be tilled back into the soil to promote soil health, tilth, fertility, and nurturing of the organisms remaining within the soil. When this is impractical, crop residues should be composted or recycled into paper products, but not destroyed in incinerators, when they form dioxins from pesticides applied to crops (Ewall, 2000). Only pesticide-free "energy crops" such as barley straw should be burnt in biomass facilities. While carbon dioxide is emitted during combustion, an equal amount of carbon dioxide is adsorbed from the atmosphere during the growth phase of crops, thus crops fuels "recycle" atmospheric carbon, minimizing global warming impacts (http://ww.nrel.gov/research/industrial_tech/biomass2.html, Borcher, 1998). Barley straw packs are also used as pond pads floating near the water surface to destroy algae.

Furthermore, it is not only interesting to use these residues as raw material for making chemical and energy products but also utilize them as food for cattle after proper treatment.

Cereal straws represent a large potential source of dietary energy for ruminants. The structural polysaccharides which comprise the carbohydrate fraction are, however, only partially degraded by rumen microorganisms thus, severely limiting the value of unmodified straw as food component. The digestibility of those materials is limited by the presence of lignin, which prevents the access of hydrolytic enzymes to cellulose and hemicellulose (Chesson, 1981).

Many different chemicals have been used for upgrading poor-quality crop residues [sodium hydroxide: Jackson (1977); ammonia: Sundstöl (1978); alkaline hydrogen peroxide (AHP): Gould (1984), Cameron et al., 1991)]. Only methods involving the use of alkali and, in particular, the use of sodium hydroxide have achieved a measure of commercial acceptance (Wanapat et al., 1985). Alkali is able to produce multiple effects on the intact cell wall of cereal straws. An assessment of the influence of alkali treatment on the rate of degradation of hemicellulose is the subject of the present research work.

A large number of researchers have carried out cereal straw treatments using NaOH with the lowest possible soda concentration which together with the highest solid-liquid ratio produces the expected effect, that is, an increase in the solubility of straw (Lázaro et al., 1994).

The general purpose of this work is to optimize substratum treatment by selectively solubilizing the hemicellulose fraction to render a cellulose-enriched fraction for further fermentation or enzyme hydrolysis. The influence of different treatments with NaOH concentrations ranging between 0.25 N and 2N in a 50-90ºC temperature range, utilizing different solvent-substratum relations and different treatment times has been experimentally studied.

In this research work, the non-wasting processes of residues have been reviewed, cereal straw being included, by studying the different alternatives in treatment for the valuing of this kind of residue.

MATERIALS AND METHODS

Characterization

The barley straw samples used in the experiments were taken from previously chopped bulk samples. Barley straw, from the North of Spain, was hammer-milled to pass a 1mm screen milled straw. Eighteen samples (40g) of the barley straw were used in this study for chemical analysis of crude protein, gross fat and ash by means of the methods provided by the A.O.A.C. (1990). For gross energy using a ballistic bomb calorimeter calibrated with benzoic acid, for neutral detergent fiber or cell wall constituents (NFD), acid detergent fiber or cellulose + lignin content (ADF), and permanganate lignin, cellulose content was estimated as ADF minus lignin, hemicellulose content as NFD minus ADF and residual ash by the methods of Goering & Van Soest (1970), and in vitro dry matter digestibility (IVDMD) values were determined by the method with "cellulases" (DMD-cellulases) (Aufree 1982).

For the analysis of a straw sample to reflect faithfully its nutritional value and chemical composition, a sample that represents all the straw must be taken in order to produce meaningful information; besides, this must not suffer any kind of alteration till the analysis has been completed. Taking this into account, eight different types of sampling were accomplished within this working stage, all of them having been taken from the same bale and their names being SS1,…, SS8. The eight processes of taking a sample of barley straw were all subjected to the same preparation treatment prior to the analysis, according to the First Community Directive of June 15, 1971 (17/250/CEE): "*Diario Oficial de las Comunidades Europeas*" no. L 155, (12/6/71).

Digestion

The experimental part of digestion consists of carrying out a series of chemical treatments, these using the proof barley straw as a substratum through a solution of NaOH.

The substratum utilized has been ground and sieved (2-5mm) barley straw, and it has been characterised according to the sequential method proposed by Goering & Van Soest (1970).

To begin with, the treatment temperature was studied in two test series carried out by submerging the samples in 1N alkali dissolution for 17 hours. In order to accomplish this, the solubility of compounds from the cellular wall (hemicellulose and lignin) and the enzymatic digestibility of the organic matter fibre (D_{oc}) were investigated by means of the enzymatic method FND-cellulase (Aufree, 1982). The treatments were accomplished at temperatures of 50ºC and 90ºC, and solid-liquid concentration ratios ranging between values of 150g and 250g of straw per litre.

Afterwards, the NaOH concentration influence upon the solubility of the material hemicellulose fraction was investigated. For this reason, the following tests were carried out with NaOH aqueous solution at different concentrations (0.25 N, 1.0 N, and 2.0 N). Such treatments were concluded in a thermostatic bath at 50ºC by using water as thermostatic liquid.

Tests were carried out in 500ml flasks, in which 40g of straw samples were introduced. Immediately afterwards, the NaOH solutions with the selected concentration and volume for each experiment, were added. Then, the treated sample was stirred up until a homogeneous mixture was obtained and this was maintained for 20 minutes at room temperature, so that the sample remained impregnated in a homogeneous way with the alkaline reagent. The digestion time was started to be measured once the fixed temperature for the treatment in the thermostatic bath had been reached.

In order to avoid liquid evaporation the flask was connected to a reflux condenser and then introduced into the thermostatic bath for the predetermined time in each treatment. Once the straw had been digested, the residue removal was concluded by vacuum filtration using no. 2 porous glass filtering plates. Once digestions had finished, the treated straw was dried in a heating cabinet at 103ºC till a steady weight was reached, keeping it later on within watertight vessels until its chemical composition was determined.

The variables considered in the alkaline treatment were temperature, NaOH concentration, g straw-litre ratio, and treatment time. Due to the existing relationship between these variables, different treatments were carried out with the aim of determining the most adequate values of such operating variables that provide an optimum yield with a maximum substratum delignification.

RESULTS

The fiber compositions of the untreated samples of barley straw are given in Table 1.

Considering the analysis carried out on the different samples, it was observed that their chemical composition did not significantly depend on the different ways utilized for obtaining the representative sample. Due to this circumstance, the research work was continued with one of the samples and in this case, the sample chosen was the one given no. 1 (SS 1).

TABLE 1 - Fiber composition of untreated barley straw as a percentage of dry matter.

Samples	SS 1	SS 2	SS 3	SS 4	SS 5	SS 6	SS 7	SS 8
Ash (%)	7.38	7.30	7.03	7.31	7.29	7.47	7.45	7.12
Crude protein (%)	3.47	3.34	3.23	3.47	3.33	3.48	3.56	3.15
Ether extract (%)	1.71	1.78	1.56	1.78	1.53	1.58	1.77	1.56
Crude fiber (%)	45.63	45.68	47.05	46.51	45.94	46.75	46.01	48.67
N-free extracts (%)	41.80	41.89	41.13	40.93	41.90	40.72	41.20	39.50
ADF (%)	50.54	50.83	51.91	51.25	50.95	51.57	51.98	52.33
NFD (%)	79.74	79.60	81.21	80.80	80.16	83.00	82.91	82.62
Permanganate lignin (%)	4.51	4.94	4.81	5.12	5.04	5.10	5.10	5.18

Effect of temperature

Two test series of four samples (SS 1) from the same substratum were carried out in triplicate. All the samples from the barley straw were treated for 17 hours with an alkaline solution of 1N NaOH, utilizing values of 150 and 250g of straw per litre. Moreover, digestion temperatures of 50 ºC and 90 ºC were selected. Table 2 shows the temperatures at which the different samples were subjected.

The results of the experiments indicated that there was an improvement in the percentage of lignin elimination (%E-lignin) of approximately 20% with a temperature increase of 40 ºC. Regarding D_{oc}, straw digestion at a temperature of 90 ºC generated a slight increase of enzymatic digestibility (D_{oc}), this being figured at 6-8 units above the previous value at 50 ºC, for a similar proportion of straw in grams per litre of reagent. However, owing to the increased costs of the process at high temperatures, the experiments were continued at a steady temperature of 50 ºC.

Effect of NaOH concentration

Samples of barley straw from the same variety were treated with NaOH concentrations 0.25N, 1.0N and 2.0N, the treatment being carried out always on 40g of sample at a steady temperature of 50 ºC. The influence of the solid-liquid relationship in digestion was studied concluding the experimentation with variable volumes of the attacking solution, thus obtaining relationships ranging between 150-1000g of straw/litre. Digestion times in the tests ranged between 6 and 48 hours.

Treatment with 0.25N NaOH

Digestions were carried out on three samples from the same barley straw, with alkaline solution of 0.25 N NaOH at 50 ºC for times ranging between 17 and 48 hours. Solid/liquid concentrations of 250 and 350g of straw per litre of solution were utilized. Three test series were accomplished in all experiments under the same conditions and the corresponding mean values were determined. The effects on barley straw samples treated with 0.25N NaOH are given in Figure 1.

TABLE 2 - Lignin solubility (%E-lignin) and enzymatic digestibility (D_{oc}).

Temperature	g straw/litre	%E-lignin	D_{oc}
50ºC	150	53.2 ± 4.5	75.0 ± 4.2
50ºC	250	48.5 ± 2.9	73.1 ± 3.8
90ºC	150	72.3 ± 4.8	83.1 ± 4.5
90ºC	250	69.2 ± 3.5	79.0 ± 3.7

FIGURE 1 - Digestion of barley straw with 0.25N NaOH at 50 °C.

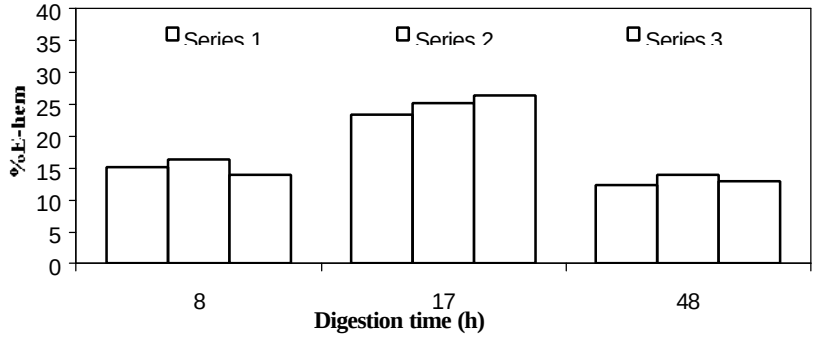


FIGURE 2 - Barley straw digestion with 1N NaOH at 50 °C.

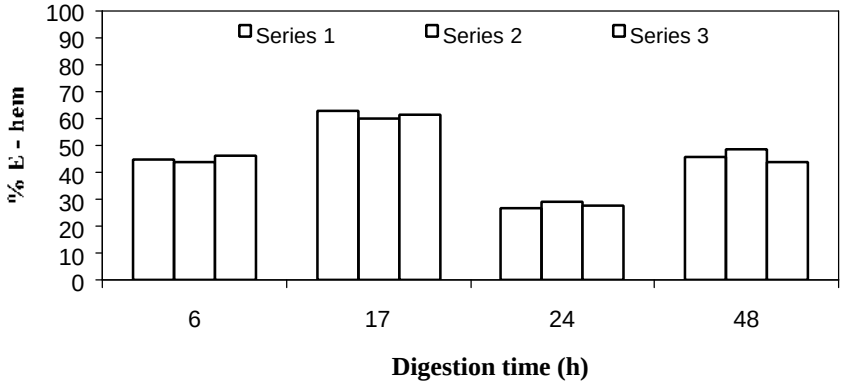


FIGURE 3 - Barley straw digestion with 2N NaOH at 50°C

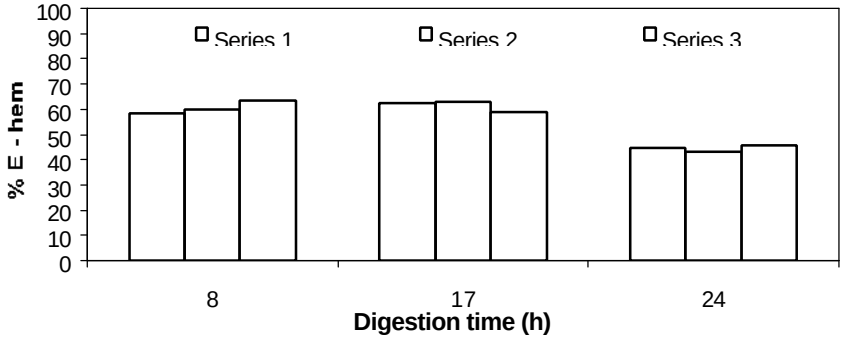


TABLE 3 - Percentage of degraded hemicellulose in different alkaline treatments.

Figure No.	Quartet (°C, NaOH, h, g straw/l)	% E – hem	Time (h)	g straw/litre
1	(50°C, 0.25N, t, g straw/l)	(15.2 ± 1.5– 24.9 ± 1.7)	(17 – 48)	(250 – 350)
2	(50°C, 1N, t, g straw/l)	(27.6 ± 1.1– 59.9 ± 2.3)	(6 – 48)	(150 – 350)
3	(50°C, 2N, t, g straw/l)	(45.9 ± 2.3– 62.6 ±2.9)	(8 – 24)	(200 – 1000)

These results show a low solubility of hemicellulose in the straw treated with the concentration under study. This fact may be due to the low concentration of the alkaline reagent, observing degrading hemicellulose values (%E-hem) not greater than 25% even for digestion times of 48 hours. The results obtained in the treatments carried out at 17 and 48 hours with 250g of straw per litre show that the solubility of the hemicellulose fraction (%E-hem) increases with treatment time from 15 to 25%, not being profitable to increase this parameter for the digestion values obtained. It is also observed that below 24 hours of digestion, the grams-of-straw-per-litre proportion hardly influences the solubility of the hemicellulosic fraction.

Treatment with 1N NaOH

Taking the same quantity of barley straw sample, different amounts of 1N alkaline solution have been added in order to reach grams-of-straw-per-litre values of 150, 200, 250 and 350. Digestions were carried out with times ranging between 6 and 48 hours. The results of the treatments are shown in Figure 2; digestion having been carried out at 50 ºC, similar to the previous tests.

As it can be observed in Figure 2, the percentage of degraded hemicellulose (%E-hem) of the samples treated varies according to the different treatments within the following mean values 27.6 ± 1.1 and 60.0 ± 2.3.

These results indicate that below 17 hours of treatment the loss of the hemicellulosic fraction (%E-hem) is below 50 %, although the solid-liquid concentration ratio utilized may have values of the following order: 150 g of straw per litre.

The degradation of the hemicellulose reached values above 60% in the treatments concluded with operating times of 17 hours and 200g of straw per litre. This result is logical, since the lower the relation of g of straw per litre of the alkaline solution is, the higher the moisture increase in the treated samples and the more homogeneous the mixing of the straw with the reagent. However, it is not interesting to obtain excessively moist straw after the treatment, since this damages its subsequent drying.

The increases of digestion time to 24 hours and g of straw per litre to 350 diminished significantly the degradation percentage of hemicellulose %E-hem $\leq$ 30%. By increasing the g of straw per litre from 200 to 250 and the digestion time from 17 to 48 hours a drop in hemicellulosic solubility figured at 16 points was observed.

Treatment with 2N NaOH

A test was concluded on barley straw samples with 2N NaOH at a constant temperature of 50ºC. The treatment times varied between 8, 17 and 24 hours and the solid-liquid ratio among 200, 250 and 1000 g of straw/litre. The results obtained are shown in Figure 3.

These results show a good solubility of hemicellulose, this being included within the following interval: 45.9 ± 2.3 and 62.6 ± 2.9 %. As it can be observed in Figure 3, that digestion carried out with a solid-liquid concentration of 250g of straw per litre for 8 hours gave a high digestion of the hemicellulosic fraction (%E-hem = 58.1 ± 1.9%). This value did not increase considerably when the concentration was dropped to 200g of straw per litre and the treatment time was increased to 17 hours (%E-hem = 62.6 ± 2.9 %). The treatment carried out with a concentration of 1000g of straw per litre and maintaining a digestion time of 24 hours solubilized a lower percentage of hemicellulosic fraction (%E-hem = 45.9 ± 2.3%). This result seems to confirm the higher influence of the g of straw-litre ratio with respect to the treatment time in its efficiency.

The results obtained in these three series of experiments are gathered in Table 3.

After analysing the results obtained through this research, it can be observed that the treatments carried out with 0.25N NaOH provide very low yields concerning the degradation of the hemicellulose. Therefore, it will be advisable to utilize higher concentrations of alkaline reagent in order to have a higher percentage of hemicellulose solubilized.

CONCLUSIONS

By analysing the results obtained from the research work carried out, the following conclusions were reached:

1. *Ceteris paribus*, the percentage of solubilized hemicellulose increases with respect to treatment time, reagent concentration and process temperature, but decreases when the g straw-litre ratio increases.
2. For obtaining high values of degraded hemicellulose (%E-hem $\approx$ 60%), it is advisable to carry out the treatment at 50 ºC with 1N NaOH and a concentration of solid of 200g of straw/litre of alkaline dissolution. A homogeneous mixing of the sample treated will be necessary in order to favour a better contact between straw and caustic reagent.
3. The results obtained show that the most efficient alkaline treatment of barley straw shows the following features: T = 50 ºC, N = 1N, t = 17h, g straw/litre = 200.

ACKNOWLEDGEMENTS

We would like to acknowledge Sarmiento Fernandez, M. and Rodriguez Loperena, M. A. for their technical assistance, as well as the technical staff of *Agroalimentario* [Laboratory of the *Ministerio de Agricultura y Pesca*. (Santander - Spain)], for their valuable experimental assistance.

REFERENCES

Agricultural Statistics. 1988 U.S. Dep. of Agriculture. Washington, DC.

A.O.A.C. (1990). Official methods of analysis. Association of Official Analytical Chemists. (15th Ed), Arlington, USA.

Aufrere J. (1882). Etude de la prevision de la digistibilité de fourages par une methode enzymatic. Ann. Zootechnn., 31(2): 111-130.

Borcher, J. 1998. Strom und Wärme aus Biobrennstoffen lohnt sich, BMELF-Informationen 36, 07.09. 1998

Cameron., M.G., Fahey. J.R., Clark, J.H., Merchen, N.R & L.L. Berger (1991). Effects of feeding alkaline hydrogen peroxide-treated wheat straw-based diets on intake, digestion, ruminal fermentation and production responses by mid-lactation dairy cows J. Anim. Sci. 69: 1775-1787.

Chesson, A. (1981). Effects of sodium hydroxide on cereal straws in relation to the enhanced degradation of structural polysaccharides by rumen microorganisms. Journal of Agricultural Science, 32: 745-758.

Ewall, M. 2000. The Burning Issues with Biomass, Part IV of Pennsylvania Enviromental Network's series on Green Energy, March 2000.

Goering H.K. & P.J. Van Soest (1970). Forage Fiber Analysis (Aparatus, Reagents, Procedure and some Applications). Agricultural Handbook, nº. 379. USDA, 1-20

Gould, J.M. (1984). Alkaline peroxide delignification of agricultural residues to enhance enzymatic sacharification. Biotchnology and Bioengineering, 26: 46-52

Jackson, M.G. (1977). Review article: The alkali treatment of straws. Anim. Feed Sci. Technol., 2: 105-130.

Lázaro, L., Mastral, J. & J. Arauzo (1994). Pretratamiento con NaOH de paja de cereal para su transformación por hidrólisis enzimática. Química e Industria, 3: 13-18.

National Renewable Energy Laboratory, "Biomass – Nature's Renewable Storehouse of Solar Energy and Chemical Resources, Biomass Power Program,

http://ww.nrel.gov/research/industrial_tech/biomass2.html

Sundstöl, F., Coxworth, E. & D. N. Mowat (1978). Improving the nutritive value of straw and other low quality roughages by treatment with ammonia. World Anim. Rev., 26: 13-21

Wanapat, J.M., Sundstöl, F. & T. H Garmo (1985). A comparison of alkali treatment methods to improve the nutritive value of straw. I. Digestibility and metabolizability. Anim. Freed. Sci. Technol., 12: 295-309.

Received for publication: May 20, 2001
Accepted for publication: November 14, 2001

CORRESPONDING AUTHOR

Mateos, E.
Departamento de Ingeniería Química y
y del Medio Ambiente
University of the Basque Country
Plaza La Casilla. 3
48012 Bilbao – SPAIN

email:iapmasae@lg.ehu.es

AFS – Vol 23/ No 4/ 2001 – pages 172 - 177

AFS Book Reviews - Bücherschau

Chiral Environmental Pollutants – Trace Analysis and Ecotoxicology

Roland Kallenborn, H. Hühnerfuss

209 pp., numerous tables and figures; Springer-Verlag Berlin – Heidelberg – New York – Barcelona – Hong Kong – London – Milano – Paris – Singapore – Tokyo, **2001**; **ISBN 3- 540-66423-8**; Hardcover **DM 249.00**.

In the last decade, new analytical methods have been developed to separate and detect the enantiomers of persistent organic pollutants in environmental samples at different trophic levels. Especially enantioselective gas chromatographic methods play an important and major role in assessing enzymatic transformation processes of anthropogenic and natural organic pollutants in the environment.

The authors have tried to give a comprehensive survey of the state-of-the-art of enantioselective trace analysis and also to reflect the future of this promising new field of analytical research. As experts in this special analytical field "Chirality of Xenobiotics" they mainly focus on the principal questions:

- Why does chirality play such an outstanding role when biochemical transformation and accumulation processes of chiral organic pollutants in organisms (from bacteria up to human beings) are being discussed?

- How can chirality as a basic selector for enzymatic proceesses be documented and proven for organic chemicals which are transformed and/or accumulated in ultra-trace concentrations?

This monograph is employable to environmental trace analysts, analytical chemists, ecotoxicologists, food scientists, and also experienced laboratory personnel.

FROM THE CONTENTS

Introduction (Links Between Chirality and Life Processes; General Principles of Chirality; Chiral Environmental Pollutants with a Stereogenic Centre; Environmental Pollutants with Axial Chirality; Asymmetry of Cyclic Environmental Pollutants; Chiral Environmental Pollutants with Two or More Stereogenic Centres; References.

Enantioselective Chromatographic Methods for the Analysis of Chiral Environmental Pollutants (General Considerations; Enantioselective High Performance Liquid Chromatography (HPLC); Indirect Methods; Direct Methods; The Evolution of Chiral Stationary Phases for Liquid Chromatography; Other Experimental Approaches for Enantioselective Liquid Chromatography; Liquid Chromatography as a Measurement Tool for Chiral Interactions; Recent Reviews on Enantioselective HPLC; Enantioselective High Resolution Gas Chromatography (HRGC); The Evolution of Chiral Stationary Phases for Capillary Gas Chromatography; Enantioselective Multidimensional Capillary Gas Chromatography (MDcGC); Other Experimental Approaches for Enantioselective Capillary Gas Chrornatography; Possible Sources of Error of Enantioselective cGC; Recent Reviews on Enantioselective HRGC; Capillary Electrophoresis (CE); General Considerations; Enantioselective CE; Other Experimental Approaches for Enantioselective CE; Recent Reviews on Enantioselective CE; Supercritical Fluid Chromatography (SFC); General Considerations; Enantioselective SFC; Recent Reviews on Enantioselective SFC; Other Methods for the Elucidation of Molecular Structures and Mechanistic Details of Chiral Pollutants; X-ray Crystallography; Nuclear Magnetic Resonance Studies; References.

Chiral Xenobiotics in the Environment (Microbiological Transformation of Chiral Environmental Xenobiotics; Laboratory Experiments; *In Situ* Investigations in Marine and Limnic Waters; Transformation/ Accumulation of Chiral Xenobiotics in Biota; Enzymatic Transformation Processes; Marine and Limnic Ecosystem; Terrestrial Ecosystem; Enantio-selective Permeation Through the Blood-Brain Barrier; Transformation/ Accumulation of Xenobiotics in Water, Sediment and Biota of Wastewater Treatment Plants; Separation of the Standard Substances of Polycyclic Musks; Sampling Locations and Samples; Enantiomeric Ratios of ATII in Water and SPMD Samples; Enantiomeric Ratios of HHCB, ATII, AHTN, and AHDI in Biota Samples; Comparison of Tench and Crucian Carp Samples; Implications of the Observed Enantioselectivity for Risk Assessment Studies; A New Challenge: HHCB Metabolites; Other Enantioselective Studies Including Sewage Water or Sludge; Photochemical Conversion Processes; Transformation/ Accumulation of Chiral Xenobiotics in Sediments and Soils; Chiral Xenobiotics in Sediments; Chiral Xenobiotics in Soils and Ambient Air; Air/Water Gas Exchange and Atmospheric Long-Range Transport; Air/ Water Gas Exchange Studies in Lakes; Air/ Sea Gas Exchange Studies; References.

Enantioselective Toxic and Ecotoxic Effects of Drugs and Environmental Pollutants (Differential Toxic Effects of Drug Enantiomers and the Role of Enantioselective Chromatography; Differential Toxic Effects of Chiral Environmental Pollutants and the Role of Enantioselective Chromatography; Acute and Chronic Toxicity of Chiral Environmental Pollutants; Enantioselective Toxic Effects of Other Industrial Chiral Chemicals; Ecotoxic Effects of Biogenic Chiral Compounds: a Challenge for Enantioselective Chromatography; Differential

Mortality of Test Animals Induced by Chiral Environmental Pollutants; References.

Perspectives of Enantioselective Analyses (Concern About Chiral Environmental Pollutants and the Legal Implications; Regulation of Chiral Drugs in the USA; Regulations on Chiral Drugs in the European Community; Regulations on Chiral Drugs in Japan and Other Countries; The Role of Enantioselective Analyses for Model and Mechanistic Studies of Enantioselective Phenomena; Models for the Prebiotic Formation of Homochirality; Innovative New Enantioselective Detectors; References.

Subject Index

Plasmids for Therapy and Vaccination

M. Schleef (Ed.)

287 pages, numerous tables and figures; WILEY-VCH Weinheim – New York – Chichester – Brisbane – Singapore – Toronto, **2001**; **ISBN 3-527-30269-7**; Hardcover **DM 198.00/ € 101.24.**

Gen therapy and vaccination with nucleic acids is one of the most impressive innovations in medical and veterinary sciences. The pharmaceutical application of genetic material – modified to become the so-called active pharmaceutical ingredient (API) – requires development and optimization in biotechnology and pharmacy to obtain systems for transfer and expression of the API resulting in appropriate effects on the organism or cell. Therefore, geneticists have been working for years with plasmids and recent successes in the fields of vector design and manufacturing point to potent therapeutics and vaccines.

The editor was assisted by 45 coauthors and specialists from academia and pharmaceutical industry to elucidate the "state of the art" in this interesting, important and innovative field of plasmids for gen therapy and vaccination.

In chapters 1 (Biology of Plasmids) and 2 (Structures of Plasmid DNA) the background of plasmids and their structure, which is important for their function, are summarized. In chapters 3 (Genetic Vaccination with Plasmid Vectors) and 5 (Immunotherapy of Chronic Hepatitis B by pCMV-S2.5 DNA Vaccine), recent overviews on DNA vaccination are presented. Examples of clinical application are described comprehensively in chapters 4 (A Liposomal iNOS-Gene Therapy Approach to Prevent Neointimal Lesion Formation in Porcine Femoral Arteries) to 6 (pSG.MEPfTRAP – A First Generation Malaria DNA Vaccine Vector). Chapters 7 (Polyvalent Vectors for Coexpression of Multiple Genes), 8 (Form Follows Function: The Design of Minimalistic Immunogenically Defined Gene Expression (MIDGE®) Constructs) and 9 (Synthetic Genes for Prevention and Therapy: Implications on Safety and Efficacy of DNA Vaccines and Lentiviral Vectors) inform about modified vector systems based on plasmids and also the potency of genomic research and vector design by informatics. The link between genomics and genomic information requires nucleic acids. Chapter 10 (Plasmids in Fish Vaccination) reports one example of veterinary health care. Chapters 11 (Plasmid Manufacturing – An Overview) and 12 (Quality Control of pDNA) illustrate the necessity of quality assurance in manufacturing and quality control when characterizing an API made from DNA. Regulatory and quality assurance aspects of new drugs in the application of nucleic acid vaccines are summarized in chapter 13 (From Research Data to Clinical Trials). Chapter 14 guides through the history of gene drug development and expectations in pharmaceutical markets.

This recommendable overwiew on the field offers the option to have major improvement in comparison to conventional drugs.

The Dictionary of Gene Technology – Genomics, Transcriptomics, Proteomics.

G. Kahl

2nd edition; **942** pages, numerous figures and structural formulas; WILEY-VCH Verlag GmbH Weinheim, **2001**; **ISBN 3-527-30100-3**; Hardcover **DM 298.00/ € 152.00**.

Genomics, transcriptomics and proteomics are fields of gene technology, a modern and extremely rapidly evolving part of life sciences. The author has, therefore, added more than 2200 new terms in this edition to the already existing 4000 terms in the first edition of this dictionary. This is a dramatic increase and underlines the literally explosive development of new technologies, new insights into genic and genomic biology, and world-wide marketing of achievements. Furthermore, these new terms witness current tendencies to nanotechnologies, automation, chip technologies, and the increasing involvement of bioinformatics.

Multiple cross-references are of interest for a broad readership to form networks linking the various terms in context and understand relationships between Gene Technology, Genetics, Molecular Genetics, Biochemistry, Biotechnology, Microbiology and Applied Sciences.

This recommendable Dictionary of Gene Technology from A to ZOO is a must for researchers, students and officials engaged in this exciting field of life sciences.

Split and Splitless Injection for Quantitative Gas Chromatography – Concepts, Processes, Practical Guidelines, Sources of Error

Konrad Grob

4th, completely revised edition; **460** pages, **175 f**igures, **33** tables; WILEY-VCH Weinheim – New York – Chichester – Brisbane – Singapore – Toronto, **2001**; **ISBN 3-527-29879-7**; Hardcover **DM 298.00/ € 152.36**.

GCs are still evaluated by means of a few injections of some alkanes in a simple solvent. Real evaluation is far more demanding. Even today instruments differ significantly in their essential parts. Therefore, critical details of injector design are a subject of this book. Most of the problems arising are important only for certain types of sample and conditions. Capillary GC in general and injection techniques in particular are full of pitfalls, but also rich in possibilities for a creative analyst. It is a fact that GC injection techniques are still far from being optimized to the point which could be reached.

The author has decided to update his classical handbook (Classical Split and Splitless Injection, 1986; Split and Splitless Injection in Capillary GC, 1993). The new material, primarily on sample evaporation, necessitates a new structure and a large part of this fourth edition was rewritten. A CD-ROM with videos on the processes occurring in devices imitating injectors was added. This handbook will help thousands of analysts to perfect their injection techniques.

FROM THE CONTENTS

Syringe injection into hot vaporizing chambers (Introduction; Syringes; Evaporation inside the needle; How much is really injected? Syringe needle handling minimizing discrimination; Dependence of discrimination on sample volume; Solvents and solutes; Injector temperature; Plunger-in-needle syringes; Possibilities of avoiding evaporation in the needle; Summarizing guidelines; References); Sample Evaporation in the Injector (Introduction; Solvent evaporation – heat transfer; Solvent evaporation – visual observation; Solute evaporation; Sample degradation in the injector; Retention and adsorption in the vaporizing chamber; Deactivation of linears and packing materials; Cleaning of injector lines; References); Split Injection (Introduction; The split ratio; Sample concentration suitable for split injection; Initial band widths; Split injection for fast analysis; Analysing requiring maximum sensitivity; High split ratios for reducing the sample size; Problems concerning the split ratio; Problems concerning linearity of splitting; Techniques for improving quantitative analysis; General evaluation of split injection; References); Splitless Injection (Introduction; How to perform splitless injection; Sample volumes suitable for splitless injection; Injection Problems with quantitative analysis; Reconcentration of initial bands; Related injection methods; References); Injector Design (Vaporizing chamber; Surroundings of the vaporizing chamber; Autosamplers; The gas regulation systems; References); Appendices 1 to 3 and Subject Index.

Wasseranalysen – richtig beurteilt; Grundlagen, Parameter, Wassertypen, Inhaltsstoffe, Grenzwerte nach Trinkwasserverordnung und EU-Trinkwasserrichtlinie.

Walter Kölle

Trinkwasser ist unser wichtigstes Lebensmittel. Wasseranalysen müssen regelmäßig durchgeführt werden und geben Aufschluß über den ökologischen Zustand der Wasserressourcen, die Möglichkeiten der technischen Aufbereitung und auch über die Leistungsfähigkeit der Laboratorien. Der blanke Vergleich von Messergebnissen reicht zur Beurteilung der Wasserqualität längst nicht mehr aus, zumal derzeit die Umsetzung der EU-Trinkwasserrichtlinie stattfindet.

Dieses empfehlenswerte Buch ist ein geeignetes Hilfsmittel zur richtigen Beurteilung von Wasseranalysen. Der Autor hat seine jahrzehntelange Erfahrung eingebracht und den gesamten Themenkomplex systematisch und praxisorientiert vorgestellt. Zusätzlich wertvoll ist die Untermauerung der Theorie durch zahlreiche Tabellen und konkrete Fallbeispiele mit vielen nützlichen Daten.

AUS DEM INHALT

Vorwort; Grundlagen; Wasser-Typen; Physikalische, physikalisch-chemische und allgemeine Parameter; Anorganische Wasserinhaltsstoffe, Hauptkomponenten und Spurenstoffe; Organische Wasserinhaltsstoffe; Calcitsättigung; Mikrobiologische Parameter und Desinfektionsmittel; Radioaktivität; Kürzel und Begriffe; Tabellenanhang; Analysenanhang; Literatur und Register.

Quality in Chemical Measurements – Training Concepts and Teaching Materials

Bernd Neidhardt, Wolfhard Wegscheider (Eds.):

177 pages, **15** tables, **40** figures; Springer-Verlag Berlin – Heidelberg – New York – Barcelona – Hong Kong – London – Milan – Paris – Singapore – Tokyo, **2001**; ISBN 3-540-65994-3; Hardcover **DM 98.00/US$ 49.95**.

The concepts of "Analytical Quality Assurance (AQA)" and "Analytical Quality Management (AQM)" developed in the wake of the harmonization of the European market have now been formally established via the appropriate directives and norms (ISO 25, EN 45001, and

recently ISO 17025). These developments have become widely accepted as market-regulating elelments by both the chemical industry and independent laboratories for routine chemical analysis and are practised extensively in the form of accreditation. But this has taken place without any perceptible participation on the part of the universities. But quality of chemical measurements must become a sustaining element of modern research and teaching in the chemistry departments of universities. The prerequisite is an improvement in the teaching and training in Analytical Chemistry via changes in content, concepts and organization of teaching in foundation, undergraduate and graduate courses in Chemistry, the introduction of Analytical Chemistry as a compulsory subject or intensive support for the new generation of academics in Analytical Chemistry. Academic freedom in teaching and research involves a responsibility to adapt oneself to changed conditions, to prepare students for new tasks, to face the competition from other universities and to give priority to fulfilling duties, if necessary, at the expense of one's own scientific interests.

The 2nd EURACHEM Workshop on Current Issues in Teaching Quality in Chemical Measurements (27-29 Oct. 1998) was a meeting of 50 experts from 14 European countries to fill the gap between theory and reality in this field. The output is published in this textbook comprising a collection of transparencies on a CD-ROM. This helpful material will assist in reducing the activation barrier associated with the preparation of lectures and seminars on this topic.

Nahrungsergänzungsmittel

Andreas Hahn

(unter Mitarbeit von Maike Wolters, Sandra Marohn, Günther Hanke; Band 41 der Paperback APV-Reihe – herausgegeben im Auftrag der Arbeitsgemeinschaft für Pharmazeutische Verfahrenstechnik e.V., Mainz)

286 Seiten, **60** Abbildungen, **35** Tabellen; Wissenschaftliche Verlagsgesellschaft mbH Stuttgart, **2001**; **ISBN 3-8047-1789-6**; Kartoniert **DM 122.00/€ 62.38**.

Nahrungsergänzungsmittel sind immer häufiger in vielerlei Formen zu finden und werden häufig mit gewagten Werbemaßnahmen angepriesen. Man versucht sie als Jungbrunnen, Fettfresser oder Flügelverleiher oder mit durchaus vorstellbarem Nutzen für die Gesundheit an den Mann zu bringen. Es ist nicht leicht einzuschätzen, was von diesen Mitteln eigentlich zu halten ist. Daher ist erklärbar, dass diese Produktgruppe "Nahrungsergänzungsmittel" im Gesundheitsmarkt in den letzten Jahren - sowohl ein ungeheures Interesse hervorgerufen hat als auch massiv kritisiert worden ist. In diesem boomenden Markt werden neben sinnvollen Produkten auch viele nutzlose angeboten. Dies verunsichert die Konsumenten, ruft Protest der Verbraucherschützer hervor und stellt, als letzte Konsequenz, diese Produkte und Präparate insgesamt in Frage.

Das vorliegende Buch versucht, das Thema in all seinen Facetten aufzuarbeiten, Nahrungsergänzungsmittel (rechtlich betrachtet: Lebensmittel, faktisch: im Grenzbereich Lebens- und Arzneimittel) sachlich zu bewerten und auf eine rationale Ebene zu bringen. Es ist klar, auch aus Sicht der Autoren, dass ihre Darstellung nicht von allen Interessengruppen akzeptiert werden kann. Wissenschaftliche Meinungen lassen sich durch gegensätzliche Studien widerlegen.

Dieses Buch ist als äußerst interessante, möglichst objektive Studie und Bestandsaufnahme der Nahrungsergänzungsmittel für alle einschlägig tätigen Wissenschaftler und Studenten (Pharmazie, Lebensmitteltechnologie, Lebensmittelchemie, Ernährungswissenschaft, usw.) an Hochschulen, Ämtern und in der Industrie gleichermaßen empfehlenswert.

AUS DEM INHALT

Abkürzungsverzeichnis; Definition und rechtliche Einordnung von Nahrungsergänzungsmitteln (Derzeitige Rechtssituation in Deutschland; Abgrenzungsprobleme; Ausnahmeregelungen beim Inverkehrbringen von Nahungsergänzungsmitteln; Kennzeichung von Nahrungsergänzungsmitteln; Lösungsmöglichkeiten für die Zukunft; Nahrungsergänzungsmittel - Versuch einer Definition aus ernährungsphysiologischer Sicht; Werbung für Nahrungsergänzungsmittel); Nahrungsergänzungsmittel - eine Bestandsaufnahme (Sicht des Verbrauchers; Vertriebswege für Nahrungsergänzungsmittel; Tendenzen bei neuen Nahrungsergänzungsmitteln; Das Problem der „Pseudoprodukte"; Was ist besser – Nahrungsergänzungsmittel oder Arzneimittel?); Nahrungsergänzungsmittel aus ernährungsphysiologischer und präventivmedizinischer Sicht (Physiologische Bedeutung der Ernährung; Der Paradigmenwechsel: Ernährung heißt auch Prävention; Anforderungen an eine gesunderhaltende Ernährung; Nährstoffbedarf/ Empfehlungen für die Nährstoffzufuhr; Toxikologische Grenzwerte; Besondere Ernährungsanforderungen; Freie Radikale und Antioxidanzien); Substanzen zur Nahrungsergänzung (Vitamine; Mineralstoffe; Vitaminoide; Sekundäre Pflanzenstoffe; Mehrfach ungesättigte Fettsäuren; Phospholipide; Proteine, Aminosäuren und Derivate; Pro- und Prebiotika; Ballaststoffe; Hormone; Sonstige); Fazit und Perspektiven (Anhang A: Würzburger Deklaration; Anhang B: DGE-Richtlinien Nährstoffzufuhr; Anhang C: Schätzwerte Vitamin- und Spurenelementezufuhr); Literatur; Sachregister.

AFS - GUIDE FOR AUTHORS

General

AFS accepts original papers, review articles, short communications, research abstracts from the entire sphere of chemistry, microbiology, technology, biotechnology and flavours/ aromas of food and, furthermore, about residue analysis/ ecotoxicology of food (from raw material to final product).

Acceptance or nonacceptance of a contribution will be decided, as in the case of other scientific journals, by a board of reviewers.

Papers are processed with the understanding that they have not been published before (except in form of an abstract or as apart of a published lecture, review or thesis); that they are not under consideration for publication elsewhere; that their publication has been approved by all co-authors, if any, as well as- tacitly or explicitly- by the responsible authorities at the institute where the work has been carried out and that, if accepted, it will not be published elsewhere in the same form, in either the same or another language, without the consent of the copyright holders.

Language

Papers must be written in English. Spelling may either follow American (Webster) or British (Oxford) usage but must be consistent. Authors who are less familiar with the English language should seek assistance from proficient colleagues in order to produce manuscripts that are grammatically and linguistically correct.

Size of manuscript

Review articles should not exceed 30 typewritten pages. In addition up to 5 figures may be included.

Original papers must not exceed 14 typewritten pages. In addition up to 5 figures may be included.

Short-Communications should be limited to 4 typewritten pages plus not more than 1 illustration.

Short descriptions of the authors, presentation of their groups and their research activities (with photo) should together not exceed 1 typewritten page.

Short research abstracts should report in a few brief sentences (one-forth to one page) particularly significant findings.

Short articles by relative newcomers to the chemical innovation arena highlight the key elements of their Master and PhD-works in about 1 page.

Book Reviews are normally written in-house, but suggestions for books to review are welcome.

Preparation of manuscript

Dear Authors,

AFS is available both as printed journal and as online journal on the web. You can now e-mail your manuscripts with an attached file. Save both time and money! To avoid any problems handling your text, please follow the instructions given below.

When preparing your manuscripts have the formula ***KISS*** (Keep It Simple and Stupid) in mind. Most word processing programs such as MS-Word offer a lot of features. Some of them can do serious harm to our layout. So please do not insert hyperlinks and/or automatic cross-references, tables of contents, references, footnotes, etc.

1. **Please use the standard format features of your word processor (such as standard.dot for MS Word).**
2. **Please do not insert automatisms or secret link-ups between your text and your figures or tables. These features will drive our graphic department sometimes mad.**
3. **Please only use two fonts- for text or tables "Times New Roman" and for graphical presentations "Arial".**
4. **Stylesheets, text, tables and graphics in shade of grey**
5. **Turn on the automatic language detection in English (American or British)**
6. **Please - check your files for viruses before you send them to us!!**

Thank you very much!

Structure of manuscript

1) Title page

The first page of the manuscript should contain the following items in the sequence given:

A concise *title* of the paper (no abbreviations)

The *names*[1] of all authors with at least one first name spelled out for every author.

The [1]*names of* Universities with Faculty, City and Country of all authors.

2) Summary

The second page of the manuscript should start with an abstract that summarizes briefly the contents of the paper (except short communications). Its lenght should not exceed 150-200 words. The abstract should be as informative as possible. An extended repetition of the paper's title is not considered to be an abstract.

3) Key words

Below the Summary up to 6 key words have to be provided which will assist indexers in crossindexing your article.

4) Introduction

This should define the problem and, if possible, the frame of existing knowledge. Please ensure that people not working in that particular field will be able to understand the intention. The word length of the introduction should be 150 to 300 words.

5) Material and Methods

Please be as precise as possible to enable other scientists to repeat the work.

6) Results

Only material pertinent to the subject must be included. Data must not be repeated in figures and tables.

7) Discussion and Conclusion

This part should interpret the results in reference to the problem outlined in the introduction and of related observations by the author/s or others. Implications for further studies or application may be discussed. A conclusion should be added if results and discussion are combined.

8) Acknowledgements

Acknowledgements of financial support, advice or other kind of assistance should be given at the end of the text under the heading "Acknowledgements". The names of funding organisations should be written in full.

9) References

Responsibility for the accuracy of references rests with the authors. References are to be limited in number to those absolutely necessary.

References should appear in numerical order in brackets and in order of their citation in the text. They should be grouped at the end of the paper in numerical order of appearance. Abbreviated titles of periodicals are to be used according to Chemical or Biological Abstracts, but names of lesser known journals should be typed in full.

References should be styled and punctuated according to the following examples:

ORIGINAL PAPERS:

1. AUTHOR, N.N. AND AUTHOR, N.N. (Year) Full title of the article. Journal and Volume, first and last page.

BOOK OR PROCEEDING:

2. AUTHOR, N.N. AND AUTHOR, N.N. (Year) Title of the contribution. In: Title of the book or proceeding. Volume (Edition of Editor-s, ed-s) Publisher, City, first and last page

DOCTORAL THESIS:

3. AUTHOR, N.N. (Year) Title of the thesis, University and Faculty, City

UNPUBLISHED WORK:

Papers that are unpublished but have been submitted to a journal may be cited with the journal's name followed by "in press". However, this practice is acceptable only if the author has at least received galley proofs o f his paper. In all other cases reference must be made to "unpublished work" or "personal communication".

10) Corresponding Author

The name of the corresponding author with complete postal address and E-mail address.

Tables

Every table should be numbered in Arabic numerals in the sequences in which they occur. They are to be included in the manuscript. Every table must begin with a caption that starts with, for example, "Table 2". The caption must explain precisely the contents of the table. The table itself must be written so that it can be read and understood without reference to the text. Every column and every line of a table must be labeled unambiguously and indicate units wherever data are reported. References to a table are to be handled in the same way as references to the text (see Section References). Footnotes to a table should be indicated by lower-case letters in parentheses and typed directly under the table.

Figures

The figures should be numbered consecutively in Arabic numerals in order of mention in the text. Every figure must be accompanied by a legend that begins with, for example, "Figure 4".

Photographs

Black-and-white photographs are to be submitted in TIF-format (shade of gray) or as JPEG black-and-white-format (shade of gray). Glossy prints with soft contrasts are also acceptable.

SI metric system

SI units are to be used for all data (exceptions: L, g, bar, h, ppm, ppb, ppt), e.g. c (NaOH) = 0.1 mol L^{-1}. Greek/ unusual symbols/ abbreviations should be defined in the text at their first occurrence.

Submission of manuscript

The manuscripts should be sent directly to:

PSP Publishing,
Angerstr.12, 85354 Freising GERMANY.

Email: parlar@psp-parlar.de

Authors are requested to submit manuscripts in electronic form (as an E-Mail attachement). Electronic manuscripts eliminate the need for re-keying and thereby introduction of new errors. They must be in exact journal format and identical to the final hard copies. The manuscript should be saved in the native format of the word processor used (please use *Microsoft Word*). Authors should keep copies of everything submitted.

Offprints

Precondition for publishing:
A minimum number of 25 Offprints must be ordered and prepaid.

They are purchased at cost price [1-4 pages = 150 €; 5-8 pages = 250 €, additionally postage/ handling (Germany 10 €, Europe 15 €, International 20 €) and VAT in Germany and EU member countries (if you do not have a VAT-No.)]. Granting of 50% discount for members of MESAEP, SECOTOX, contributors from developing countries, and students. 25 offprints will be provided free of charge only to subscribers of AFS.

Copyright

Abstracted/ Indexed in:

CA, FSTA, BIOSIS, CAB

SUBJECT INDEX

subject-index

AUTHOR INDEX

author-index

SUBJECT INDEX for Advances in Food Sciences 2001

AUTHOR INDEX for Advances in Food Sciences 2001

author-index

AFS - ORDER FORM

Yes, I wish to order **AFS** starting with Volume 24/ 2002

☐ Printed journal
120 EURO plus postage/ handling
(Germany 15 EURO/ Europe 30 EURO/
International 60 EURO)

☐ Online journal
96 *EURO*

Minimum subscription period: 1 year
Cancellation must be generally effected
3 months before end of subscription period.

☐ Will be paid in full

☐ 50% will be paid (postage/ handling full) because…
- ☐ Member of MESAEP/ SECOTOX
- ☐ contributor from developing country

Special-Offer to Subscribers- back issues at reduced rates!

Subscribers in 2002 are entitled to receive back issues at reduced rates (while stocks last).
(6 double issues/ less 50%:Euro 125,00 + postage and handling).

To take advantage of this offer please tick the boxes below when ordering a subscription to AFS for 2002.

☐ 1996 ☐ 1997 ☐ 1998
☐ 1999 ☐ 2000 ☐ 2001

Invoice address

Name: .....
Position: .....
Organization: .....
Address: .....
.....
.....
E-Mail: .....

Delivery address (if different)

Name: .....
Position: .....
Organization: .....
Address: .....
.....
.....
E-Mail: .....

Methods of Payment

☐ Please send me a pre-payment invoice

☐ I enclose a cheque made payable to PSP

Value Added Tax

In certain circumstances we may be obliged to charge Value Added Tax (VAT) on sales to other EU member countries. To avoid this, it is therefore essential to provide us your VAT number if you have one.

☐ I am not registered for VAT

☐ My VAT number is

Signature:…….

Date:……

Please complete this form and return to:

AFS – Advances in Food Sciences
c/o PSP – Parlar Scientific Publications
Angerstr. 12 - 85354 Freising – GERMANY

Tel: ++ 49 (0) 8161 48420
Fax: ++ 49 (0) 8161 484248
email: parlar@psp-parlar.de