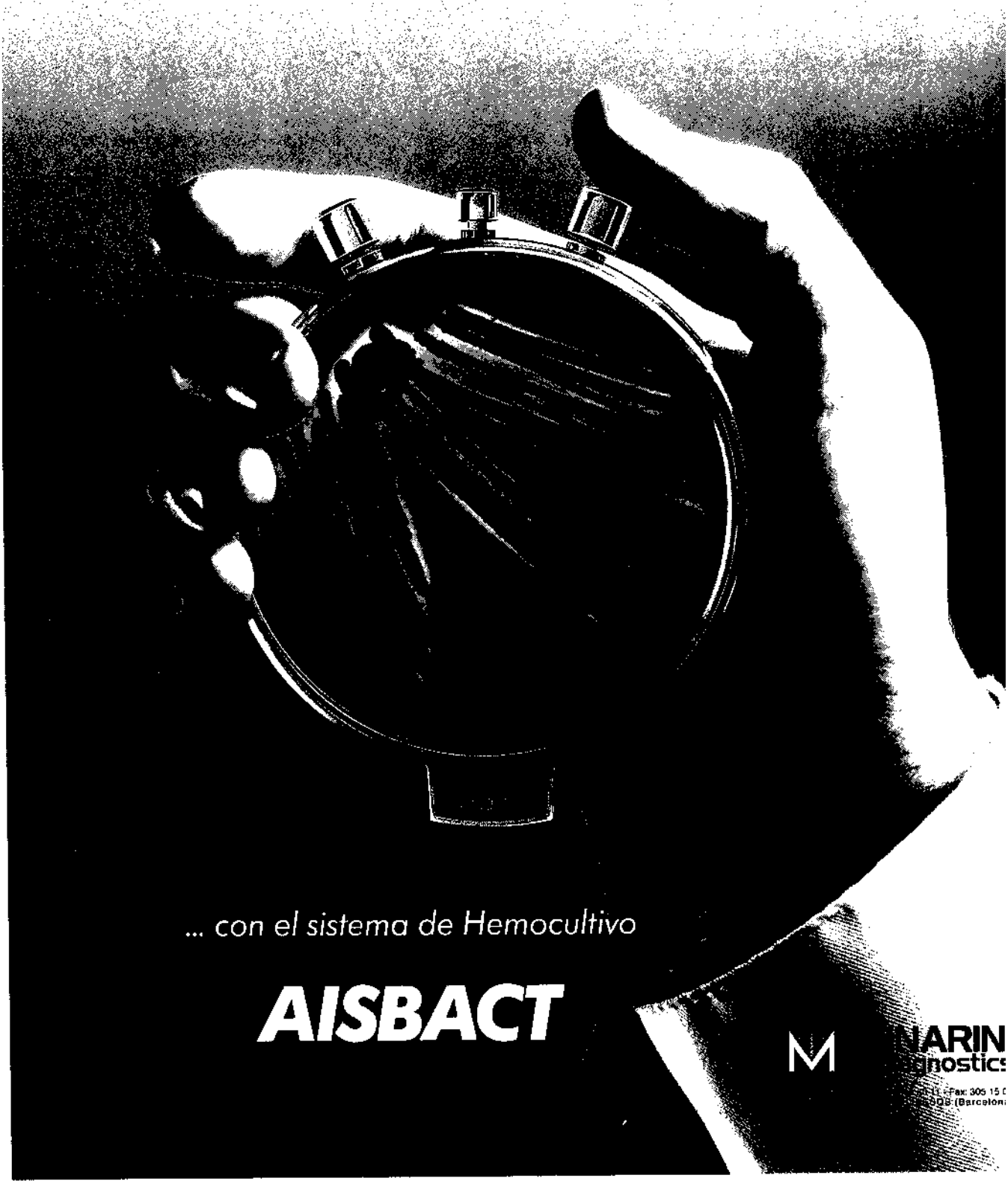
Volumen 8. N.° 1
Abril 1992
ISSN 02 13-4101

PUBLICACION DE LA SOCIEDAD
ESPAÑOLA DE MICROBIOLOGIA

Microbiología

SEM

Superamos el factor tiempo...



... con el sistema de Hemocultivo

AISBACT

M

NARIN
gnostics

Fax: 305 15 0
08 (Barcelon

BIOMÉRIEUX: LOS SISTEMAS ...

ATB Expression

Automatización experta en bacteriología

ATB Expression, es un sistema automático de identificación y antibiograma que simplifica el trabajo del laboratorio de bacteriología, optimizando la **calidad** y la **rapidez** de respuestas.

Flexibilidad de utilización para el laboratorio: permite organizar libremente el trabajo diario eligiendo identificaciones y antibiogramas rápidos o en 24 horas.

Validación de resultados: el sistema Expert pone a su disposición el conocimiento de los expertos en antibiograma, para que pueda estar seguro de los resultados en cualquier circunstancia.

Sistema de gestión y explotación de resultados: permite tratar los resultados por informe-paciente, realizar estudios estadísticos y la conexión con la informática central.

Si desea información más amplia, tenemos documentación completa a su disposición.

bioMérieux

69280 Marcy-l'Etoile. Francia. Tel. 78 87 20 00 / Télex 330967
España: Manuel Tovar, 24. 28034 Madrid. Tel. (91) 358 11 42 / Télex 46620
Otras filiales en Alemania, Bélgica, Holanda, Italia, Japón, Portugal, Reino Unido, Suiza.

MICROBIOLOGIA SEM

Publicación de la Sociedad Española de Microbiología

Dirección: Sociedad Española de Microbiología. Vitrubio, 8.
28006 Madrid (España). Tel. (91) 261 98 00. Ext. 211.
Aparecen dos números al año (1990), que se integran en un volumen.
Precio de suscripción anual. Año 1991: España, 5.900 ptas. (IVA incluido);
Europa, 60 $; resto países, 72 $.

FOTOCOMPOSICION: Lasercom, S. A.
IMPRIME: Graesal. Madrid.
DEPOSITO LEGAL: M-30455-1985.

EDITORIAL GARSI, S.A.

Edita: EDITORIAL GARSI, S. A. Publicidad: Sociedad para la Publicidad Especializada (SPE, S. L.). Londres, 17. 28028 Madrid. Teléfono 726 08 00. Delegación Barcelona: Numancia, 85-87. 08029 Barcelona. Teléfono 993) 322 99 11. Teléfono para cambios de domicilio y suscripciones: 255 55 87.

Miembro de la asociación española de prensa técnica
Sección española de la Federación Internacional de la Prensa Periódica
FIPP

Guidelines to authors

«Microbiología» (Published by the Spanish Society for Microbiology) publishes original research papers, research Notes and ocassionally reviews covering all aspects of Microbiology. All submissions should be written in Spanish or in English. The decision to accept manuscripts is made by the Editorial Board.

Submission of a paper to this Journal is understood to imply that it has not previously been published and that it is not being considered for publication elsewhere. Consent is given for reproducing publication of this Journal if acredite as the source.

ORGANIZATION AND FORMAT OF THE MANUSCRIPTS. Type every portion of the manuscript double-space with a wide margin at the left on UNE A-4 format sheets. Only one side of the sheet should be used and the pages should be numbered sequentially. Papers must be restricted to a maximum of 15 printed pages including figures and tables (this corresponds to approximately 25 typewritten pages).

The front page should include title, name(s) of the author(s), institution affiliation(s) and complete address(es). Three to five keywords would also be included.

Papers should be divided into: Abstracts in English and in Spanish (not exceeding 250 words). Introduction. Materials and Methods. Results. Discussion. Acknowledgements and References. Results and Discussion can be combined.

Abbreviations and symbols'should follow the recommendation of the IUPAC-IUB Commission and the Metric System is to be used throughout.

Cite each listed reference by numbers in the text. References should be numbered and arranged in alphabetical order as indicated in the following examples:

Miller, J. H. (1972). Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y.

Seeberg, E., Nissez-Meyer, J. and Stricke, P. (1976), *den* V gene of bacteriophage T4 determines a DNA glucosilate specific for pyrimidine dimers in DNA. J. Viriol. 35, 790-797.

Tomasz, A. (1984). Building and breaking in the cell wall of bacteria - The role for autolysins. *In:* C. Nombela (ed.) Microbial Cell Wall Synthesis and Autolysis. pp. 3-12. Elsevier Science Pub. B. V. Amsterdam.

References to thesis, manuscripts not accepted for publication or Meetings should be indicated in the text as follows: (García, P. *et al.* 1985, in preparation), (Smith, T. 1985. Ph. D. thesis, University of Colorado, Colorado) or (Suárez, A. y González, F. 1975). V Congr. Nac. Microbiol, p. 1845).

Only those photographs which are strictly necessary for the understanding of the paper should be submitted. Fotoprints must be of sufficient quality to ensure good reproduction. There should be numbered on the back and identified with the first author's name written in pencil. Legends for linedrawings and photoprints must be typed double-space on a separate sheet. The size of the photographs should not exceed the printing area (13 x 20 cm). All elements in the drawing should be prepared to withstand reductions. Drawings and line figures should be drawn in black ink on tracing paper and should be prepared as indicated for the photographs. Colored illustrations are not accepted.

Tables should be compiled on separate sheets with a descriptive title and numbered independently of the figures using Arabic numerals.

Please indicate with a soft pencil the approximate location of tables and figures in the left margin of the page.

NOTES. Notes should be restricted to 6 typewritten pages and are intended to present experimental observations and descriptions of techniques or methodological changes of interest. They should be written according to the guidelines given for papers, but wuthout the heading divisions, and their abstracts should not exceed 50 words. Figures and tables should be restricted to a maximum of 2 figures and 1 table or vice versa.

MINIREVIEWS. Minireviews articles should deal with microbiological subjects of broad interest. Specialists will be called upon to write them. However, if some authors are interested in publishing minireviews, these will be submitted for publication. They shoul not be longer than approx. *twelve* to twenty double-spaced typewritten pages *including* the space needed for figures and tables.

PROOFS. On acceptance of the paper, one galley proof will be sent to the nominated author to check for typesetting accuracy. The corrected proofs should be duly returned within one week's time. If delays were observed, the proofs will be corrected by the editorial staff and published. Broader changes implying recomposition of the text will be at the author's expense. Twenty-five offprints of each paper are supplied free of charge. Additional reprints will be billed at cost price if requested upon returning the corrected galley proofs.

Contributions, in duplicate, may be sent to the Chief Editor or to the editor whose special field is the most closely related to the subject matter.

Normas para los autores

«Microbiología» (Publicaciòn de la SEM) acepta trabajos y Notas de investigación originales dentro del campo de la Microbiología y, ocasionalmente, artículos de revisión. Textos en castellano o en inglés. La aceptación corresponde al Consejo Editorial.

Sólo se admitirán trabajos inéditos que no estén pendientes de publicación en cualquier otra revista. Los originales publicados en «Microbiología» podrán ser reproducidos siempre que se indique su origen.

PRESENTACION DE LOS MANUSCRITOS. Los trabajos, por duplicado, estarán escritos a máquina, a doble espacio, en hojas UNE A-4 por una sola cara, numeradas correlativamente y con un amplio margen en la parte izquierda y no deberán exceder de 15 páginas impresas incluyendo tablas y figuras (lo que corresponde aproximadamente a 25 hojas mecanografiadas).

Los trabajos incluirán una primera página en la que se indicará por este orden: Título del trabajo, nombre y apellido del autor o autores, centro en el que se ha realizado el trabajo y dirección completa del mismo, así como de tres a cinco palabras clave. En los artículos en castellano se deberá incluir una versión inglesa del título.

Los trabajos constarán de: Resúmenes en inglés y en castellano (de no más de 250 palabras), Introducción, Materiales y Métodos, Resultados, Discusión, Agradecimientos y Bibliografía. Las secciones de Resultados y Discusión se podrán fusionar en una sola.

Las abreviaturas deberán seguir las recomendaciones de la Comisión IUPAC-IUB sobre nomenclatura bioquímica. Las unidades de medida serán las correspondientes al Sistema Métrico Decimal.

La bibliografía será citada en el texto mediante números y se preparará numerada y en orden alfabético de acuerdo con los ejemplos que se ofrecen a continuación:

Miller, J. H. (1972). Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y.

Seeberg, E., Nissez-Meyer, J. and Strike, P. (1976). *den* V gene of bacteriophage T4 determines a DNA glycosilate specific for pyrimidine dimers in DNA. J. Viriol. **35,** 790-797.

Tomasz, A. (1984). Building and breaking in the cell wall of bacteria - The role for autolysins. *In:* C. Nombela (ed.) Microbial Cell Wall Synthesis and Autolysis. pp. 3-12. Elsevier Science Pub. B. V. Amsterdam.

Las referencias a tesis doctorales, manuscritos no aceptados a comunicaciones presentadas a Congresos, deben incluirse en el texto del trabajo de acuerdo con los siguientes ejemplos: (García, P. *et al.* 1985 in preparation), (Smith, T. 1985. Ph. D. Thesis, University of Colorado, Colorado)_ or (Suárez, A. y González, F. 1975. Res. V. Congr. Nac. Microbiol. p. 1845).

Las fotografías, que deberán estar preparadas para su reproducción directa, se limitarán a las estrictamente necesarias para la comprensión del trabajo y serán de calidad suficiente para asegurar una buena reproducción. Deberán estar numeradas al dorso indicando el apellido del primer autor a lápiz. Los textos de las mismas irán mecanografiados a doble espacio y en hoja aparte. En los trabajos en castellano las figuras incluirán asimismo un texto en inglés. El tamaño de las fotografías no excederá de 13 × 20 cm. Las dimensiones de los rótulos deberán ser las adecuadas para ser legibles en caso de que se reduzca la fotografía. La presentación de dibujos en tinta china y papel vegetal seguirá las mismas normas. No se admitirán fotografías en color.

Las tablas se enviarán en hojas aparte, numeradas independientemente de las figuras, con números arábigos y deberán llevar el correspondiente título explicativo.

Los autores deberán indicar a lápiz en el margen la situación aproximada en donde deben aparecer las tablas y las figuras.

NOTAS. Las Notas, que no deberán exceder de seis páginas mecanografiadas, incluyendo figuras y tablas, tienen por objeto la presentación de observaciones experimentales, descripción de técnicas o modificaciones metodológicas de interés. Su redacción se efectuará ateniéndose a las Normas previamente descritas para los trabajos, pero suprimiendo las divisiones con encabezamiento y con resúmenes no superiores a 50 palabras. Sólo incluirán, como máximo, dos figuras y una tabla, o viceversa.

ARTICULOS DE REVISION. Los artículos de revisión versarán sobre temas de microbiología de gran interés, y su redacción se solicitará a especialistas. Sin embargo, si algún autor está interesado en publicar artículos de revisión, éstos tendrán que ser supervisados. Los manuscritos deberán comprender aproximadamente de 12 a 20 páginas (incluidas figuras y tablas) mecanografiadas a doble espacio.

PRUEBAS. Los autores recibirán pruebas que deberán devolver en plazo no superior a una semana. Transcurrido dicho plazo sin devolución de las pruebas, éstas serán corregidas por la revista y publicado el trabajo. Las correcciones se limitarán a errores tipográficos, gramaticales o de datos incorrectos. Modificaciones más importantes que impliquen recomposición del texto, deberán ser abonadas por el autor. Se enviarán 25 separatas gratuitas por artículo; si se desearan más, deberá indicarse por escrito cuando se devuelvan las pruebas corregidas. Las separatas adicionales serán facturadas a precio de coste.

Dos copias de cada manuscrito se enviarán al editor-coordinador o al editor de la especialidad más relacionada con el contenido del trabajo.

CONTENTS

(*) Corresponding author.

INDICE

(*) A quien debe dirigirse la correspondencia.

MICROBIOLOGIA SEM **8** (1992), 1-8 **MINIREVIEW**

Are there salvage routes within the general secretory pathway in yeast?

Daniel Gozalbo, José P. Martínez, and Rafael Sentandreu*

Sección de Microbiología. Facultad de Farmacia. Universitat de València. Avda. Blasco Ibáñez, 13. 46010-València, Spain.
(Received May 10, 1991)

Summary

It is generally accepted that both extracellular protein secretion and plasma membrane expansion in yeast occur basically as in higher eukaryotic cells. In addition to the constitutive (default) secretory pathway, some specialized mammalian cells possess a regulated route which at present has not been detected in yeast. However, there is a body of experimental results suggesting that under certain circumstances export of integral plasma membrane and exocellular proteins may take place through alternative (salvage) pathways. The existence of these latter routes would enable the yeast cells to adapt more efficiently to distinct or adverse conditions requiring the secretion of discrete amounts of specific sets of proteins.

Key words: Saccharomyces cerevisiae, *yeast mutants, secretion, cell wall material, plasma membrane expansion, adaptative response.*

Resumen

Hasta el momento presente está generalmente aceptado que la secreción de macromoléculas y la expansión de la membrana plasmática tienen lugar en levaduras mediante mecanismos básicamente semejantes a los que han sido caracterizados en las células eucariotas superiores. Determinados tipos de células animales especializadas presentan, además de la ruta de secreción constitutiva, una vía regulada que aún no ha sido encontrada en aquéllas. Sin embargo, existen diversos hallazgos experimentales que sugieren la posibilidad de que ante determinadas situaciones la secreción de proteínas integrales de membrana y de material exocelular en levaduras sea llevada a cabo a través de rutas alternativas o de salvamento. En este contexto la existencia de un mecanismo de secreción en el que participasen rutas secretoras alternativas implicaría una mayor plasticidad, lo que permitiría que las células de las levaduras pudieran adaptarse con mayor eficiencia a nuevas condiciones ambientales que requerirían la secreción de cantidades definidas de determinados tipos de proteínas.

(*) Corresponding author.

Introduction

Intracellular transfer of secretory proteins in *Saccharomyces cerevisiae* from the endoplasmic reticulum (ER) to their final delivery at the cell surface is mediated by different types of membrane organelles (40, 41). Temperature sensitive mutants which are defective for distinct steps in the export pathway but not for the synthesis of secreted enzymes (*sec* mutants) have demonstrated clearly that the organelles involved in the secretory pathway in *S. cerevisiae* are similar to those found in higher eukaryotes. Studies performed with the *sec* mutants have provided evidence that both protein secretion and plasma membrane expansion in yeast occur basically as envisioned by Palade (30) in higher eukaryotic cell systems, and that at least 25 different gene products are required for successful secretion, integration of plasma membrane proteins and plasma membrane expansion (5, 6, 24-27). During transit from the ER to the cell surface many export proteins undergo further modifications being one of the most significant the addition of the carbohydrate moiety (43).

A large assortment of exocellular materials having different nature (enzymes, wall components, pheromones, etc.) are secreted by yeast cells. Release of some of these molecules occurs constitutively, whereas in other instances some materials are secreted only in response to changes in the environmental conditions, so as the cells will rapidly adapt to diverse nutricional, physical or chemical shifts. Although the basic regulatory mechanisms occur at the level of gene expression, the involvement of alternative or salvage routes could be suggested. The existence of these routes that under normal growth conditions do not operate would represent an additional way to ensure secretion of appropriate amounts of specific molecules. A somewhat similar situation exists in higher eukaryotes in which a regulated secretory pathway, different from the so-called constitutive one, has evolved in specialized secretory cells (13, 35).

It is generally accepted that in yeast all major plasma membrane surface and periplasmic proteins require the standard constitutive secretory pathway for export, being coupled both cell surface growth and secretion (25, 27, 41). Coordination of secretion and membrane assembly has been shown by biochemical characterization of secretory vesicles from *sec1* mutant which transport plasma membrane ATPase and the secretory enzyme acid phosphatase in a single vesicle species (12).

However, there is a body of experimental data suggesting differences between the mechanisms of secretion for some extracellular and integral plasma membrane proteins in yeast.

Does secretion occur in yeast through a single pathway?

Different mechanisms may be involved in the export of integral plasma membrane and exocellular proteins

Tschopp *et al.* (44) reported incorporation of proteins into the plasma membrane in the *sec* mutants incubated at the non-permissive temperature of 37° C. Blocking of the secretory process at early stages (i. e., as in *sec18* mutant which accumulates ER vesicles) avoids more efficiently the assembly of integral plasma membrane proteins than a block in later steps of the secretory pathway (i. e., as in *sec1* mutant which accumulates secretory vesicles). Although contamination of plasma membrane preparations examined by these authors either with soluble cell components or membranous structures cannot be ruled out, one possible explanation is that under normal and/or stress conditions some proteins may branch off during transport along the secretory pathway, being inserted into the plasma membrane by a mechanism independent of the late-acting SEC gene products (44).

Furthermore, Atkinson and Ramírez (1) described that secretion of invertase and acid

phosphatase can proceed independently from net plasma membrane expansion in *S. cerevisiae* inositol auxotrophs (*inol* mutants) under inositol-starvation conditions, which caused cessation of plasma membrane growth when incubated in glucose-free medium (1). As cessation of plasma membrane assembly has not been demonstrated under these conditions, it may be possible that phospholipid deprivation would result in an increase of plasma membrane turnover thus balancing the insertion of new membrane material. A plausible mechanism to explain the peculiar physiological behavior of *ino* mutants could be that two different types of Golgi-derived vesicles are produced in yeast cells (some requiring sustained lipid synthesis for their formation whereas some others do not [1]). Similar observations (invertase secretion continues in inositol-starved yeast after surface growth stopped) were also reported by Novick and Schekman (26). It has to be noted that in all instances, uncoupling of secretion and plasma membrane expansion occurs under non-optimal growing conditions, which in turn may suggest the involvement of a salvage secretory route.

Further results may support the contention that secretion of some exocellular glycoproteins is not balanced with cell growth or net plasma membrane expansion. In this way, inhibition of protein synthesis does not apparently affect secretion of invertase in *S. cerevisiae*, as release of the intracellular pool of glycosylated enzyme continues (20, 21), whereas in *C. albicans* yeast cells delivery of vesicles containing zymogenic chitin synthase (a likely plasma membrane-related enzyme, chitosomes [2]) and secretion of certain wall mannoproteins does not take place ([10]; unpublished observations). It has also been found that in the slime variant of the filamentous fungus *Neurospora crassa* chitosomes and secretory vesicles containing invertase and acid phosphatase represent different populations of microvesicules (37). A similar observation has been reported for chitin synthetase and acid phosphatase activities in *C. albicans* (9). Finally, secretion of invertase occurs in yeast cells arrested in mitosis (i. e., by treatment with inhibitors of microtubule assembly or by using temperature-sentivite cell division cycle *[cdc]* mutants incubated at the non-permissive temperature, [17] as well as in clathrintdeficient *S. cerevisiae* mutants (see below) that grow two-three times slower than wild-type cells (31, 32). Although absence of data on plasma membrane turnover makes difficult to evaluate results in some cases, it can be suggested that under certain distress conditions secretion of exocellular material proceeds uncoupled from plasma membrane growth.

Not all exocellular proteins appear to be secreted through a single common mechanim

In addition to differences in the mechanisms responsible for secretion of integral plasma membrane and exocellular proteins, distinct experimental observations also suggest the existence of possible alternative routes for maturation and secretion of the latter molecules.

In this way, yeast *sec* mutants which accumulate invertase and exoglucanase when incubated at the non-permissive temperature, secrete much faster invertase than exoglucanase when switched to permissive conditions (11). Although different models may account for the distinct rates of secretion observed for different exocellular proteins (35), the possibility exists that invertase secretion in yeast involves a receptor mediated step, whereas that of exoglucanase would occur through a passive flow. The existence of a specific receptor for invertase secretion may be additionally supported by the observation that invertase-β-galactosidase hybrid proteins are not exported, and that certain SUC2 point mutations result in a failure in invertase secretion (4, 39), probably due to the inability of the receptor to recogniz the hybrid or the mutated proteins. In any case, it has to be noted that these altered proteins may exhibit a modified folding pattern which in turn could be responsible for an impairment in their secretion, since correct assembly and folding of proteins seems to be an essential prerequisite for their secretion (35). Further support for the existence of these alternate routes between the ER and Golgi arises from the analysis of the car-

TABLE 1
POSTULATED CHARACTERISTICS OF ALTERNATIVE ROUTES IN THE YEAST SECRETORY PROCESS
Experimental support for some of the characteristics mentioned below has been reported (see text). In the case of the salvage route evidences arised from studies mainly performed with invertase.

Characteristic	Route	
	Constitutive	Salvage
Lipid synthesis/effect of cerulenin	Required/inhibited by	Not-required/not inhibited by
Requirement of fatty acyl-Coa	Yes	Not
Relation with cell growth (plasma membrane and wall expansion).	Yes	Not
Requirement of microtubules/effect of nocodazole.	Yes/inhibited by	Not/not-inhibited by
Type of vesicles involved.	Non-clathrin coated	Clathrin-coated
Participation of specific receptors	Not	Yes
Relative flow rate	Slow (passive flux)	Fast
Material exported	Plasma membrane proteins, cell wall related molecules, others (i. e., exoglucanase, α-galactosidase?)	Invertase acid phosphatase
Main role	Cell growth support	Adaptative response to environmental changes and stress situations

bohydrate moiety of glycoproteins in *mnn9* mutants (8), and from the fact that a single protein (exoglucanase) do possess distinct glycosylation patterns (15). This may reflect the pasage of the protein through different membranous compartments in which different glycosylation reactions would occur.

On the other hand, a notorious increase (5 to 50-fold) in the amount of secreted heterologous proteins has been observed in yeast cells carrying the *ssc1-1 (pmr1)* mutation with respect to the wild-type cells, thus suggesting that loss of PMR1 product (a P-type ATPase) function leads to bypass a segment of the normal secretory pathway, which allows increased secretion of heterologous proteins (36, 42). It has to be stressed that yeast proteins secreted from *pmr1* mutants lack the outer carbohydrate chain, whose addition normally occurs at the Golgi level.

In addition, a route independent of the classical secretory pathway involves the translocation of the protein (*a*-factor) from the cytosol to the exocellular space by means of a membrane-spanning protein (STE6 product [14]) that acts as a transmembranal translocator. This kind of proteins (ATP-driven transport ATPases) might be involved in translocation of proteins lacking hydrophobic sequences (22). Furthermore, these proteins could be also responsible for the transport of polypeptides between all the intracellular compartments (14).

Finally, cerulenin (an antibiotic which specifically inhibits lipid synthesis [28, 29]) partially prevented secretion of α-galactosidase (concomitantly, intracellular accumulation of the enzyme was observed at the level of the plasma membrane and other internal membranous structures

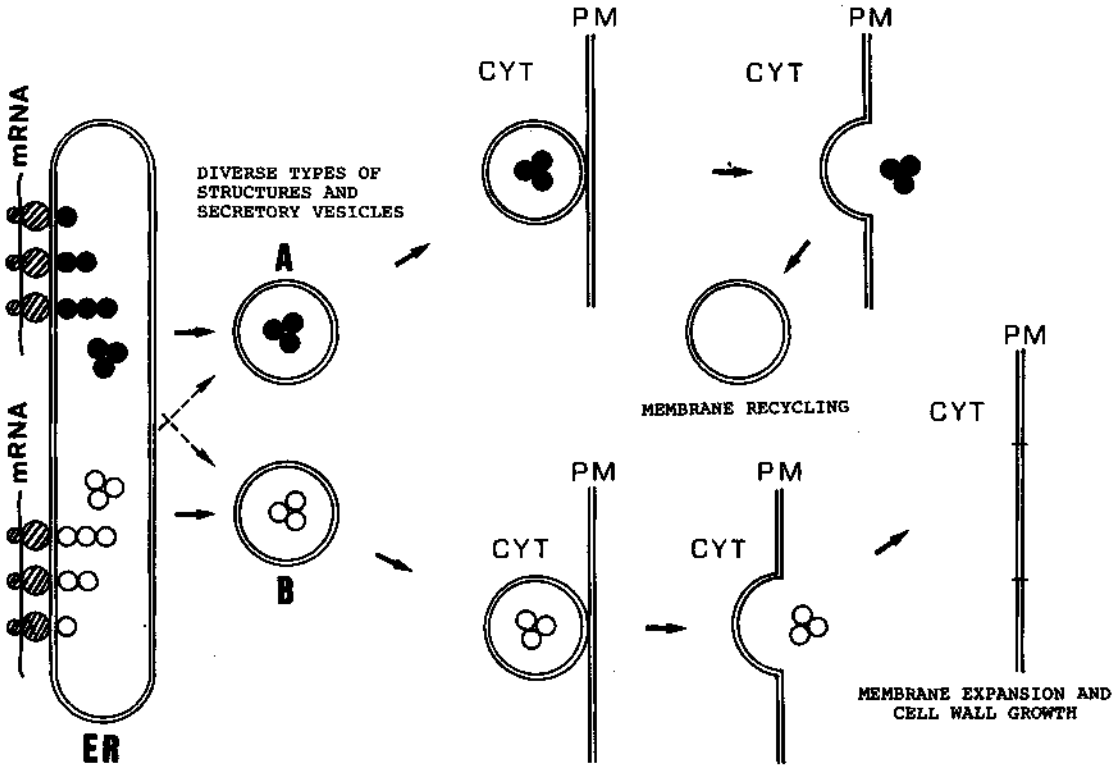


Fig. 1. Simplified model for the secretion of exocellular yeast mannoproteins through alternative (constitutive and salvage) routes. Secretion of certain mannoproteins (i. e., invertase; ♣) would occur uncoupled to net plasma membrane expansion, by means of vesicles which do not require sustained lipid synthesis (route A), whereas export of some others (i. e., α-galactosidase;) would take place concomitantly with plasma membrane growth through vesicles (route B) which call for sustained lipid synthesis. Abbreviations used: ER, endoplasmic reticulum; CYT, cytoplasm; PM, plasma membrane.

[19]), whereas neither inhibition of secretion nor intracellular accumulation of invertase was observed in cerulenin-treated cells (18). These results suggest that the secretory pathway for α-galactosidase may differ, at least in some particular steps (protein processing, presence of specific receptors...), from that responsible for invertase secretion. A possible candidate could be the step in which vesicles are budding from the Golgi stacks. In higher eukaryotes fatty acyl-CoA is required for budding of coated vesicles from Golgi cisternae (7, 34), and hence formation of such vesicles would be sensitive to cerulenin. In this context, it has to be indicated that clathrin appears to aid in the production of regulated secretory vesicles in many cells (7). However, clathrin-coated vesicles formation appears to be not absolutely essential for protein transport in yeast (i. e., invertase and α-factor precursor are efficiently secreted in clathrin-deficient *[chc1]* mutants (31-33, 45), though clathrin is required for normal growth in these organisms (16). In yeast, clathrin may be involved in some other physiological functions not well defined yet (i. e., the intracellular retention at the Golgi stacks of the Kex2p endoprotease which is responsible for initiating proteolytic maturation of the α-factor precursor; [33]). Nevertheless, some recent observations suggest that clathrin-like coated vesicles may be involved in the secretion of yeast invertase (3); hence, biogenesis of such vesicles would not be affected by cerulenin according to the hypothesis raised in this paper (Table 1). Some other general processes such as calcium compartmentalization could be alternatively affected by cerulenin (23), resulting in an impaired secretion as calcium is known to play an important role in membrane stabilization during vesicle biogenesis (38).

Final remarks

It is difficult to understand how secretion of heterogeneous exocellular materials may occur through a single mechanism. The efficiency of cellular processes would require the regulated (qualitatively and quantitatively) secretion of the distinct cell surface-related materials, as differences in regulation of gene expression cannot explain by themselves some results above described.

The existence of at least two overlapping secretory pathways which are functional under different physiological or environmental conditions may be hinted. In normally growing cells one pathway is functional, and both structural material of plasma membrane and cell wall and other exocellular proteins are secreted (Fig. 1; Table 1). In conditions in which cellular growth is blocked (i. e., inhibition of protein synthesis, adaptation to new nutritional sources, treatment with inhibitors of microtubule formation, etc.), the transport of vesicles carrying molecules directly involved in cell growth (plasma membrane expansion, cell wall constituents, etc.) is inhibited, being also blocked secretion of other molecules that eventually may be transported in this kind of vesicles (Fig. 1, route B; constitutive pathway?). On the contrary, proteins functionally more important in these conditions (i. e., proteins involved in adaptation to environmental changes, for instance invertase) may be secreted through different routes (Fig. 1, route A; salvage?). In any case, both pathways may share several common steps but differ in other(s), and some kind of interregulation among them could be also hypothesized, in such way that inhibition of one of the pathways may result in an activation of the other one. In this context, a bypass in some steps of the secretory process has been postulated to explain the phenotype exhibited by yeast *pmr1* null mutants (36). This interregulation could also explain the fact that, under inositol starvation conditions, *ino1* mutants secrete invertase and acid phosphatase (1), only when incubated with sucrose as carbon source (route A will be functionally prevalent), whilst acid phosphatase secretion is blocked in glucose-containing medium (route B will be functionally prevalent here). Model shown in Fig. 1 and Table 1 would also explain the opposite results reported in the literature on the role played by clathrin in the invertase secretory process (it has to be stressed that the many diverse experimental conditions used to perform such studies could also account for the different results obtained). Since the alternative routes are interconnected and interregulated, *chc1* yeast mutants would secrete invertase by the constitutive pathway, whilst in wild-type cells invertase could be exported through both pathways depending on environmental conditions.

The existence of these seemingly plastic secretory pathway in yeast cells may be envisaged as an efficient way to avoid death caused by disadvantageous environmental changes, by allowing the secretion through the salvage pathway of specific molecules required to adapt to the new conditions. However, it has to be stressed here that under an evolutionary point of view the existence of a specialized secretory pathway in yeast, similar to that existing in certain mammalian cells, would not imply any biological advantage, as yeast have to face many different environmental shifts, contrary to higher cells which are subjected to stringent homeostasis characteristic of complex pluricellular organisms. Hence it is more useful for yeast to possess a ductile secretory process able to respond to variations in their habitat.

Acknowledgments

This work was partially supported by grant (PB90-0424) of the Dirección General de Investigación Científica y Técnica (Spain) and the Commision of the European Communities (EEC Group on *Candida albicans* cell Biology and Pathogenicity and CI 1*. 0631 M).

References

1. Atkinson, K. D. and Ramírez, R. M. (1984). Secretion can proceed uncoupled from net plasma membrane expansion in inositol-starved *Saccharomyces cerevisiae*. J. Bacteriol. **160,** 80-86.

2. Bartnicki-García, S. (1981). Role of chitosomes in the synthesis of fungal cell walls. In D. Schlessinger (ed.) Microbiology pp. 238-241. American Society for Microbiology, Washington.
3. Casanova, M., Miragall, F., Parets-Soler, A., Martínez, J. P. and Sentandreu, R. (1990). Polypeptide composition of invertase containing vesicles of *Saccharomyces cerevisiae* Mycol. Res. **94,** 1026-1030.
4. Emr, S. D., Schauer, I., Hansen, W., Esmon, P. and Schekman, R. (1984). Invertase β-galactosidase hybrid proteins fail to be transported from the endoplasmic reticulum in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **4,** 2347-2355.
5. Ferro-Novick, S., Hansen, W., Schauer, I. and Schekman, R. (1984). Genes required for completion of import of proteins into the endoplasmic reticulum in yeast. J. Cell. Biol. **98,** 44-53.
6. Ferro-Novick, S., Novick, P., Field, C. and Schekman, R. (1984). Yeast secretory mutants that block the formation of active cell surfaces enzymes. J. Cell. Biol. **98,** 35-43.
7. Fine, R. E. (1989). Vesicles without clathrin intermediates in bulk flow exocytosis. Cells, **58,** 609-610.
8. Gopal, P. K. and Ballou, C. E. (1987). Regulation of the protein glycosylation pathway in yeast: structural control of N-linked oligosaccharide elongation. Proc. Natl. Acad. Sci. USA **84,** 8824-8828.
9. Gozalbo, D., Dubón, F., Schwencke, J. and Sentandreu, R. (1987). Characterization of chitosomes in *Candida albicans* protoplasts. Exp. Mycol. **11,** 331-338.
10. Gozalbo, D., Dubón, F. and Sentandreu, R. (1991). Chitin synthetase activity in *Candida albicans*: subcellular distribution in yeast cells and protoplasts. Mycol. Res. **95,** 513-520.
11. Hernández, L. M., Ramírez, M., Olivero, I. and Larriba, G. (1986). Accumulation and secretion of exoglucanase activity in yeast secretory mutants. Arch. Microbiol. **146,** 221-226.
12. Holcomb, Ch. L., Hansen, W. L., Etcheverry, T. and Schekman, R. (1988). Secretory vesicles externalize the major plasma membrane ATPase in yeast. J. Cell. Biol. **106,** 641-648.
13. Kelly, R. B. (1985). Pathways of protein secretion in eukaryotes. Science **230,** 25-31.
14. Kuchler, K., Sterne, R. E. and Thorner, J. (1989). *Sacharomyces cerevisiae* STE6 gene product: a novel pathway for protein export in eukaryotic cells. EMBO J. **8,** 3973-3984.
15. Larriba, G., Basco, R. D., Hernández, L. M., Muñoz, M. D. and Ramírez, M. (1990). Variations in the number and elongation of N-linked oligosaccharides lead to the secretion of three exoglucanase (β-glucosidase) isoenzymes in *Saccharomyces cerevisiae*. Yeast **6** (Special issue), S500.
16. Lemmon, S. K. and Jones, E. W. (1987). Clathrin requirement for normal growth of yeast. Science **238,** 504-509.
17. Makarow, M. (1988). Secretion of invertase in mitotic yeast cells. EMBO J. **7,** 1475-1482.
18. Martínez, J. P., Herrero, E., González, C., Gozalbo, D., Tomás, F., Pastor, F. I. J., Rico, H., Casanova, M., Mormeneo, S., Dubón, F., Elorza, M. V. y Sentandreu, R. (1982). Secreción de exoenzimas en *Saccharomyces cerevisiae*. Efecto de n-alcoholes, cerulenina y concanavalina A en la secreción de invertasa y α-galactosidasa. Estudios dedicados a Juan Peset Aleixandre, pp. 661-684. Universitat de València.
19. Martínez J. P., Elorza, M. V., Gozalbo, D. and Sentandreu, R. (1982). Regulation of α-galactosidase synthesis in *Saccharomyces cerevisiae* and effect of cerulenin on the secretion of this enzyme. Biochim. Biophys. Acta **716,** 158-168.
20. Moreno, F., Ochoa, A. G., Gascón, S. and Villanueva, J. R. (1975). Molecular forms of yeast invertase. Eur. J. Biochem. **50,** 571-579.
21. Mormeneo, S. and Sentandreu, R. (1982). Regulation of invertase synthesis by glucose. J. Bacteriol. **152,** 14-18.
22. Muesch, A., Hartmann, E., Rohde, K., Rubartelli, A., Sitia, R. and Rapoport, T. A. (1990). A novel pathway for secretory proteins? Trends Biochem. Sci. **15,** 86-89.
23. Nakata, M., Tomita, T., Izuka, T. and Kanegasaki, S. (1989). Inhibitory effect of antibiotic cerulenin on the respiratory burst in phagocites. II. Inhibition by cerulenin of intracellular calcium mobilization in human neutrophils. J. Antibiot. **42,** 1178-1183.
24. Novick, P., Ferro, S. and Schekman, R. (1981). Order of events of the yeast secretory pathway. Cell **25,** 461-469.
25. Novick, P., Field, C. and Schekman, R. (1980). Identification of 23 complementation groups required for posttranslational events in the yeast secretory pathway. Cell **21,** 205-215.
26. Novick, P. and Schekman, R. (1979). Secretion and cell-surface growth are blocked in a temperature sensitive mutant of *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA **76,** 1858-1862.
27. Novick, P. and Schekman, R. (1983). Export of major cell surface proteins is blocked in yeast secretory mutants. J. Cell Biol. **96,** 451-457.
28. Omura, S. (1976). The antibiotic cerulenin, a novel tool for biochemistry as an inhibitor of fatty acid synthesis. Bacteriol. Rev. **40,** 681-697.
29. Omura, S. (1981). Cerulenin. Meth. Enzymol. **72,** 520-532.
30. Palade, G. E. (1975). Intracellular aspects of the process of protein synthesis. Science **189,** 347-358.
31. Payne, G. S. and Schekman, R. (1985). A test of clathrin function in protein secretion and cell growth. Science **230,** 1009-1014.
32. Payne, G. S., Hasson, T. B., Hasson, M. S. and Schekman, R. (1987). Genetic and biochemical characterization of clathrin-deficient *Saccharomyces cerevisiae*. Mol. Cell. Biol. **7,** 3888-3898.
33. Payne, G. S. and Schekman, R. (1989). Clathrin: a role in the intracellular retention of a Golgi membrane protein. Science **245,** 1358-1365.

34. Pfanner, N., Orci, L., Glick, B. S., Amherdt, M., Arden, S. R., Malhotra, V. and Rothman, J. E. (1989). Fatty acyl-Co-enzyme A is required for budding of transport vesicles from Golgi cisternae. Cell, **59,** 95-102.
35. Pfeffer, S. R. and Rothman, J. E. (1987). Biosynthetic protein transport and sorting by the endoplasmic reticulum and Golgi. Annu. Rev. Biochem. **56,** 829-852.
36. Rudolph, H. K., Antebi, A., Fink, G. R., Buckley, C. M., Dorman, T. E., LeVitre, J., Davidow, L. S., Mao, J. and Moir, D. T. (1989). The yeast secretory pathway is perturbed in mutations in PMR1, a member of a Ca^{+2} ATPase family. Cell **58,** 133-145.
37. Ruiz-Herrera, J., Martínez, J. P., Casanova, M., Gil, M. L. and Sentandreu, R. (1987). Separation of chitosomes and secretory vesicles from the «slime» variant of *Neurospora-crassa.* Arch. Microbiol. **149,** 156-162.
38. Sambrook, J. F. (1990). The involvement of calcium in transport of secretory proteins from the endoplasmic reticulum. Cell **61,** 197-199.
39. Schauer, I., Emr, S., Gross, C. and Schekman, R. (1985). Invertase signal and mature sequence substitutions that delay intercompartmental transport of active enzyme. J. Cell. Biol. **100,** 1664-1675.
40. Schekman, R. (1985). Protein localization and membrane traffic in yeast. Annu. Rev. Cell Biol. **1,** 115-143.
41. Schekman, R. and Novick, P. (1982). The secretory process and cell surface assembly. *In* J. Strathern, E. Jones and J. Broach (eds.), The molecular biology of the yeast *Saccharomyces.* Vol. 2, pp. 361-393. Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y.
42. Smith, R. A., Duncan, M. J. and Moir, D. T. (1985). Heterologous protein secretion from yeast. Science **229,** 1219-1224.
43. Tanner, W. and Lehle, L. (1987). Protein glycosylation in yeast. Biochim. Biophys. Acta **906,** 81-89.
44. Tschopp, J., Esmon, P. C. and Schekman, R. (1984). Defective plasma membrane assembly in yeast secretory mutants. J. Bacteriol. **160,** 966-970.
45. Walworth N. C. and Novick, P. J. (1987). Purification and characterization of constitutive secretory vesicles from yeast. J. Cell. Biol. **105,** 163-174.

MICROBIOLOGIA SEM **8** (1992), 9-13

Studies on the distribution of the *Paramecium aurelia* species complex of Poland

Zofia Komala and Ewa Przyboś*

Institute of Animal Systematics and Evolution, Polish Academy of Sciences, 31-016 Kraków, Slawkowska 17, Poland.
(Received April 10, 1990/May 3, 1991)

Summary

Species of the *Paramecium aurelia* complex occurred in the studied regions of the Carpathians and the Sudetes Mts with varying frequency. *P. novaurelia* appeared most often, *P. biaurelia* occurred less frequently but was also very characteristic, both species prevailed over *P. primaurelia. P. tetraurelia* and *P. triaurelia* were very rare.

Key words: Species relationship, geographical distribution, collection methods.

Resumen

Las especies del complejo *Paramecium aurelia* se encuentran en las regiones estudiadas de los Cárpatos y de los Sudetes con una frecuencia variable. *P. novaurelia* es la especie más frecuente, *P. biaurelia* lo es menos, siendo muy característica de las regiones investigadas.

Estas 2 especies prevalecen sobre *P. primaurelia,* mientras que *P. tetraurelia* y *P. triaurelia* se han encontrado muy raramente.

Introduction

From the 15 species of the *Paramecium aurelia* complex known in the world (1, 11) 5 of 8 found in Europe (3, 7) are known in Poland. These are *P. primaurelia, P. biaurelia, P. triaurelia, P. tetraurelia,* and *P. novaurelia.*

Investigations on the distribution of these species along the Carpathians/Carpathian Mts - West Carpathians, i.e. the Tatras, Pieniny, Sacz Beskids, Low Beskids, and the East Carpathians, i.e. Bieszczady, as well as the Carpathian Plateau, i.e. Western and Middle Beskid Plateaus/have been conducted for several years (4, 5).

Lately, the authors extended the studies to the Sudetes Mts (Middle and Western) (6, 8).

Material and methods

The collection of species from nature and establishing of clones for systematic determination in laboratory conditions were carried out according to the routine method (4, 5).

(*) Corresponding author.

TABLE 1
OCCURRENCE OF SPECIES OF THE *PARAMECIUM AURELIA* COMPLEX IN THE CARPATHIANS

Name of the region	Area in km^2	Number of designated clones, studied habitats, and ratio value*				Total number of designated clones and studied habitats in the region
		Paramecium primaurelia	*Paramecium biaurelia*	*Paramecium tetraurelia*	*Paramecium novaurelia*	
Carpathian Mts	5314	76 (14)**	169 (21)	57 (10)	201 (18)	503 (42)
Carpathian Plateau	6545	78 (13)	41 (9)	1 (1)	152 (25)	272 (39)
Total	11859	154 (27) 0.33*	210 (30) 0.37*	58 (11) 0.136*	353 (43) 0.53*	775 (81)

* Ratio value = $\frac{\text{No of habitats for a defined species}}{\text{Total no of habitats of the area}}$

** In brackets number of studied habitats.

The routine procedure of collecting and establishing clones from nature is a follows: samples of water with plankton and plant remnants are taken from the surface of littoral waters. From each sampling point 10 samples (tubes) are taken with about 30 ml of water. As soon as possible, 10 individuals are separately isolated from each sample and clones established. To the samples in which no paramecia are found, a little medium is added and after a week the samples are again examined. Should paramecia be present, only one individual from a sample is isolated and established as a clone.

The identification of species was made by mating investigated clones with mating types of the

TABLE 2
OCCURRENCE OF SPECIES OF THE *PARAMECIUM AURELIA* COMPLEX IN THE SUDETES Mts

Name of the region	Area in km^2	Number of designated clones, studied habitats, and ratio value*					Total number of designated clones and studied habitats in the region
		Paramecium primaurelia	*Paramecium biaurelia*	*Paramecium triaurelia*	*Paramecium tetraurelia*	*Paramecium novaurelia*	
Middle Sudetes	2184	33 (6)**	91 (14)	—	5 (1)	140 (19)	269 (35)
Western Sudetes	1252	20 (1)	43 (9)	13 (1)	—	20 (5)	96 (12)
Total	3436	53 (7) 0.149*	134 (23) 0.48*	13 (1) 0.02*	5 (1) 0.02*	160 (24) 0.51*	365 (47)

* Ratio value = $\frac{\text{No of habitats for a defined species}}{\text{Total no of habitats of the area}}$

** In brackets number of studied habitats.

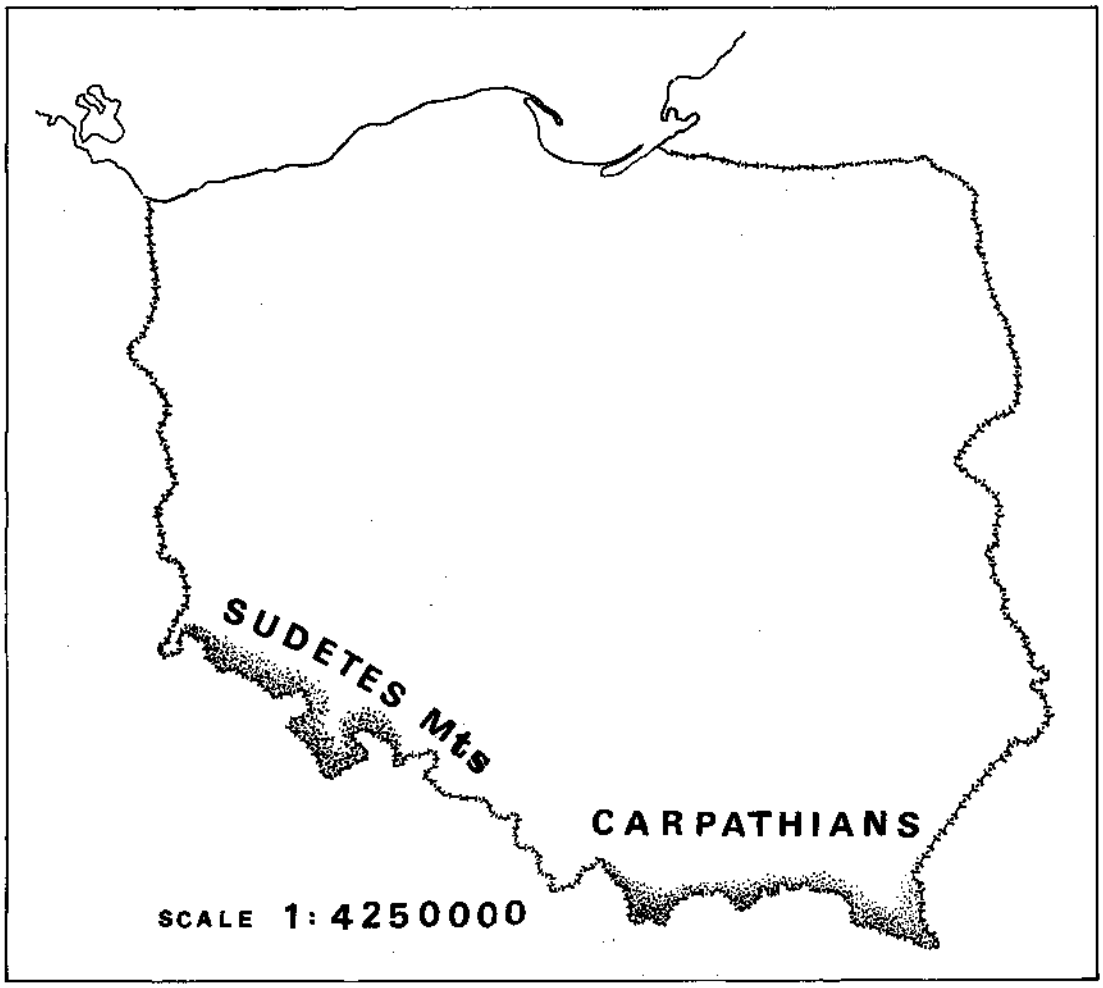


Fig. 1. A sketch-map of Poland showing the Carpathians and the Sudetes Mts.

standard strains of known species according to Sonneborn (10). The standard strains used were as follows: strain 90 (standard of *P. primaurelia*), strain Rieff, Scotland (standard of *P. biaurelia*), strain 324 (standard of *P. triaurelia*), a strain from Sydney, Australia (standard of *P. tetraurelia*), strain 510 (standard of *P. novaurelia*).

Paramecia were cultured in a lettuce medium inoculated with *Enterobacter aerogenes*.

For estimation of the occurrence of species the number of established clones from nature, the number of investigated habitats, and the season (autumn) of collection were taken into consideration.

Results and discussion

The investigations have shown that species of the *Paramecium aurelia* complex occurred with varying frequency, while dominance sometimes differed in particular regions (Tables 1 and 2, Fig. 1, 2).

Fig. 2. Distribution of species of the *Paramecium aurelia* complex along the Carpathians and the Sudetes Mts. Number 1 refers to *P. primaurelia*, number 2 to *P. biaurelia*, number 3 to *P. triaurelia*, number 4 to *P. tetraurelia*, and number 9 to *P. novaurelia*.

It was found that in the Carpathians (Table 1) *P. novaurelia* dominates (353 clones[+] 43 habitats[++]) over *P. biaurelia* (210 c/30 h) and *P. primaurelia* (154 c/27 h). For a precise estimation of the species frequency the following ratio was also stated: number of habitats for a defined species to the total number of habitats of the area, which is 0.53 for *P. novaurelia*, 0.37 for *P. biaurelia*, and 0.33 for *P. primaurelia*. In this region *P. tetraurelia* appeared only in 11 habitats (58 clones), the ratio being 0.136 (4, 5).

In the Sudetes Mts (Table 2) *P. novaurelia* and *P. biaurelia* dominate over *P. primaurelia*, the values for the number of clones and habitats are 160 c/24 h, 134 c/23 h, and 53 c/7 h, respectively. The ratio value for these species being 0.51, 0.48, and 0.149. *P. tetraurelia* (5 c/1 h) and *P. triaurelia* (13 c/1 h) appeared only in one habitat each, the ratio values were 0.02 for both species (6,8).

Considering the whole investigated territory of the Carpathians and the Sudetes Mts, it was found that *P. novaurelia* appeared most often (513 c/67 h, the ratio value - 0.523), *P. biaurelia* occurred less frequently (344 c/53 h, the ratio value - 0.41) but was nevertheless very characteristic. *P. primaurelia* was not so frequent (207 c/34 h, the ratio value - 0.26). However, in the Sudetes Mts *P. primaurelia* appeared rather rarely in comparison with the Carpathians. In turn, *P. tetraurelia* (63 c/12 h, the ratio value - 0.09) and *P. triaurelia* (13 c/1 h, the ratio value - 0.008) were very rare in the whole investigated territory. *P. triaurelia* was not found in the Carpathians, and in the Sudetes Mts only in one habitat.

The authors suggested that the above data, based on uniform collection and identification methods reflect at least approximately the relationship existing in nature between the described species at the particular time and location.

It should be also stressed that the authors succeeded in finding a species not yet known in Poland, i.e. *P. triaurelia* (6), and the new species for Europe - *P. sexaurelia* in Spain (7). This is in

+ clones-c, ++ habitats - h.

agreement to Sonneborn's (9) suggestion that it would not be surprising to find new species elsewhere should a search be made on a similar scale as in the USA.

The observed variation in the frequency of settlement of these species, probably, mainly depends on the adaptive ability of a given species to the specific fluctuating biocenotic conditions, with its tendency to invade new habitats and even to dominate over other species.

Hairston (2) is of the opinion that each species (syngen) of *Paramecium aurelia* complex has a characteristic distribution in time and space. According to him many, but not all of these distributions can be explained on the basis of different sensitivities to temperature differences, but this explanation is not sufficient in the case of *P. biaurelia* and *P. triaurelia.* The restricted space distribution of these two species Hairston explains by inhibition influence of *P. biaurelia* on *P. triaurelia.*

References

1. Aufderheide, K. J., Daggett, P.-M. and Nerad, T. A. (1983). *Paramecium sonneborni* n. sp., a new member of the *Paramecium aurelia* species complex. J. Protozool. **30,** 128-131.
2. Hairston, N. G. (1958). Observations on the ecology of *Paramecium,* with comments on the species problem. Evolution **12,** 440-454.
3. Komala, Z. and Przyboś, E. (1970). The new habitats of *Paramecium aurelia* syngens in Poland. Folia Biol. (Kraków) **18,** 287-293.
4. Komala, Z. and Przyboś, E. (1984). Distribution of the *Paramecium aurelia* species complex (Protozoa, Ciliophora) in the Carpathian chain of Poland. Zool. Scripta. **13,** 161-163.
5. Komala Z. and Przyboś, E. (1985). The occurrence of species of the *Paramecium aurelia* complex on the Dynów Plateau with some remarks on the method. Folia Biol. (Kraków) **33,** 107-116.
6. Komala, Z. and Przyboś, E. (1990). Studies on the distribution of the *Paramecium aurelia* species complex. Folia Biol. (Kraków) **38,** 35-42.
7. Przyboś, E. (1988). Genetic analysis of *Paramecium sexaurelia* (Ciliophora, Protista) in Spain. Genome **30** (suppl. 1), 386.
8. Przyboś, E. and Komala, Z. (1988). Species of the *Paramecium aurelia* complex (Protozoa, Ciliophora) in the Middle Sudetes of Poland. Zool. Scripta **17,** 325-327.
9. Sonneborn, T. M. (1957). Breeding systems, reproductive methods, and species problem in Protozoa. In: E. Mayr (ed.) The Species Problem, pp. 155-324. AAAS, Washington.
10. Sonneborn, T. M. (1970). Methods in *Paramecium* research. In: D. M. Prescott (ed.) Methods in Cell Physiology, vol. IV, pp. 242-339. Academic Press, New York.
11. Sonneborn, T. M. (1975). The *Paramecium aurelia* complex of fourteen sibling species. Trans. Amer. Microbiol. Soc. **94,** 155-178.

MICROBIOLOGIA SEM **8** (1992), 14-20

Effect of aeration rate on the alcoholic fermentation of whey by *Kluyveromyces fragilis*

H. Varela*, M. D. Ferrari, L. Loperena and C. Lareo

Dep. Bioingeniería. Instituto de Ingeniería. Química. Facultad de Ingeniería. J. Herrera y Reissig 565. Casilla de correo 30. Montevideo, Uruguay.

(Received November 5, 1990/May 29, 1991)

Summary

In this paper, the influence of aeration rate on the alcoholic batch fermentation of whey by *Kluyveromyces fragilis* NRRL Y-2415 was investigated.

Assays in 1.5-L fermentor using concentrated whey permeate containing 100 g/L of lactose were carried out at different oxygen supply rate ($K_La C^*$) from 0 to 82 mmol/Lh. Optimum response was obtained at 14 mmol/Lh: ethanol production rate reached was 3.4 g/Lh yielding 0.46 g of product per gram of initial lactose. An increase of $K_La C^*$ from 0 to 14 mmol/Lh improved the ethanol production: maximum specific ethanol production rate (q_{pm}) increased 3.3 times from 0.3 to 1.0 g/gh. For higher aeration levels, ethanol production diminished and biomass formation was stimulated.

The declination of q_{pm} and the increase of μ_m at higher aeration level lead to conclude the importance of a controlled oxygen supply in order to obtain the required balance between yeast biosynthetic needs and ethanol production.

Key words: Kluyveromyces fragilis, *whey, ethanol, fermentation, aeration.*

Resumen

En el presente trabajo se investigó la influencia de la aireación sobre la fermentación etanólica discontinua de suero de leche utilizando *Kluyveromyces fragilis* NRRL Y-2415.

Se efectuaron ensayos en fermentador de 1,5 L con sueros permeados concentrados de 100 g/L de lactosa a diferentes velocidades de suministro de oxígeno ($K_La C^*$) desde 0 a 82 mmol/Lh. La respuesta óptima se obtuvo a 14 mmol/Lh: se alcanzó una velocidad de producción de etanol de 3,4 g/Lh con un rendimiento de 0,46 g de producto por gramo de lactosa inicial. Un aumento de $K_La C^*$ de 0 a 14 mmol/Lh mejoró la producción de etanol: la máxima velocidad específica de producción de etanol (q_{pm}) se incrementó 3,3 veces de 0,3 a 1,0 g/gh. Para niveles de aireación mayores la producción de etanol disminuyó y se estimuló la formación de biomasa.

La declinación de q_{pm} y el aumento de μ_m para los niveles mayores de aireación ensayados permite concluir la importancia de un suministro controlado de oxígeno con el fin de obtener el balance requerido entre las necesidades biosintéticas de la levadura y la producción de etanol.

(*) Corresponding author.

Introduction

In the last ten years, the annual milk consumption for the elaboration of dairy products in Uruguay increased about 70 % from 274 to 474 thousands of m^3 (3). It has been produced an increase of liquid whey, an effluent generated principally in cheese and casein manufacture. Ethanol production from lactose in the whey permits to reduce its BOD (biological oxygen demand) value and appraise it as raw material.

Previous experiments showed that whey permeates can be fermentated to ethanol by *Kluyveromyces fragilis* NRRL Y-2415. As the ethanol concentration reached, 20 g/L, can be no economically attractive, experiments using whey permeates concentrated by reverse osmosis were conducted. The ethanol concentration increased to 39 g/L. However, fermentation rate was low (Varela, H. *et al.* 1989. Res. 2nd Cuban and International Seminar on Biotechnology, S10-044; Varela, H. *et al.* 1989. Res. 2nd Cuban and International Seminar on Biotechnology, S10-043).

Many yeasts are unable to synthesize sterols and unsaturated fatty acids in the absence of oxygen. If the concentration of such compounds are not sufficient in the medium, yeast can find limitations for its growth under anaerobic conditions (1). G. Moulin *et al.* (7) reported that celullar multiplication was possible in whey permeates without additives and under anaerobiosis. This fact permited them to suggest that the medium had enough quantities of such compounds for supporting the yeast growth during fermentation. Lately, L. Zertuche *et al.* (9) found that air flow supply during continuous fermentation with a strain of *K. fragilis* of concentrated whey permeate supplemented with 5 g/L of yeast extract increased the ethanol production rate. On the contrary, P. Vienne *et al.* (8) found that aeration had a negative effect on the ethanol production in continuous culture with *K. fragilis,* but they did not descart the possibility that an optimum might exist.

Taking these researches as a basis, in the present paper oxygen supply effect on different process parameters of concentrated whey fermentation without nutrient addition, was studied in order to increase ethanol productivity.

Materials and methods

Microorganism

Kluyveromyces fragilis NRRL Y-2415 *(Kluyveromyces marxianus* var. *marxianus)* was used. It was grown at 32° C and maintained at 5° C on malt agar slants (DIFCO, USA).

TABLE 1
EXPERIMENTAL CONDITIONS AND RESULTS

F mL/min	N rpm	$K_La C^*$ mmol/Lh	S_o g/L	N_o 10^7 cells/mL	X_o g/L	P_o g/L	t_f h	P g/L	Q_p g/Lh	Y_p g/g	Y_x g/g	q_{pm} g/gh	q_{sm} g/gh	μ_m h^{-1}	X_S %
0	200	0	97	8	2.6	2.2	74	38.7	0.5	0.40	0.00	0.3	0.5	0.00	74
150	200	10	99	9	2.7	1.5	39	45.0	1.1	0.45	0.01	0.6	1.3	0.01	97
60	300	14	97	16	3.4	2.4	13	44.8	3.4	0.46	0.03	1.0	2.2	0.19	94
450	300	28	101	11	2.9	1.5	16	39.8	2.5	0.39	0.06	0.6	1.3	0.18	94
450	400	44	104	10	2.8	1.8	19	27.8	1.5	0.27	0.08	0.4	1.1	0.20	94
900	500	82	105	8	2.6	2.0	18	8.0	0.5	0.08	0.12	0.1	0.6	0.28	80

Note: t_f inform for $K_La C^*$ 0 was the value at which fermentation was stopped.

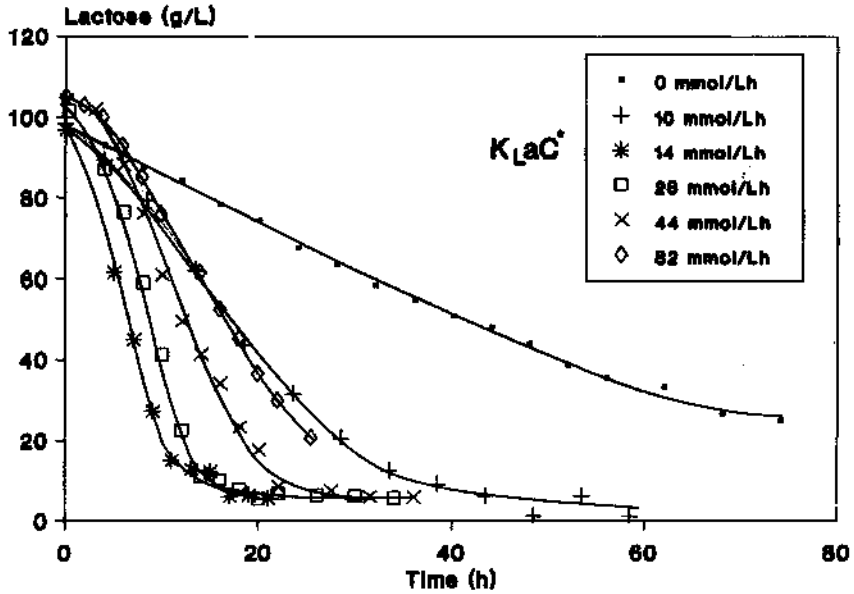


Fig. 1. Effect of $K_La C^*$ on lactose consume.

Fermentation medium

Cheese whey permeate (CONAPROLE, Uruguay) was used. It was concentrated by reverse osmosis using a DDS unit (De Danske Sukkerfadrikker, Denmark) equipped with membranes having a nominal molecular weight cut-off value of 350 daltons. Final lactose concentration was adjusted to 100 g/L by dilution with distilled water and pH was brought to 4.5 with HCl. Medium was pasteurized at 90° C for 20 min.

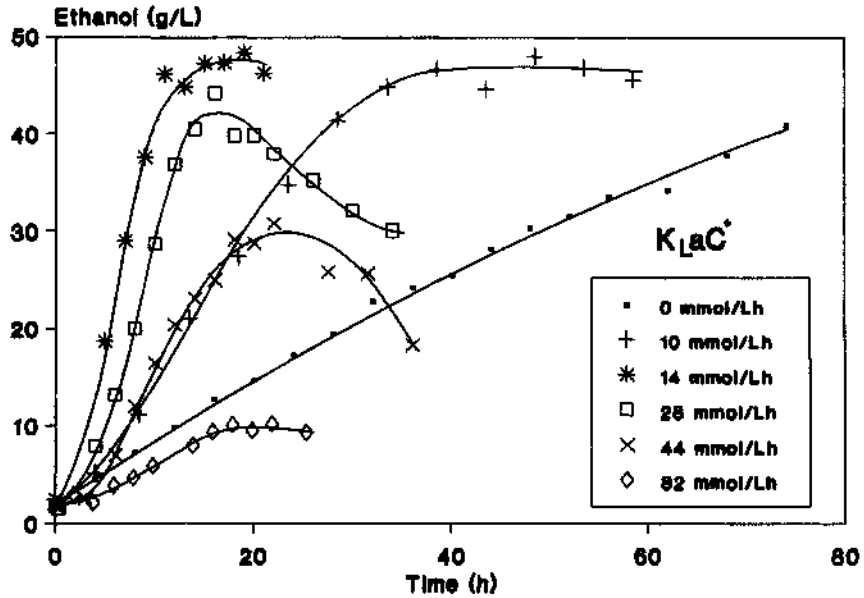


Fig. 2. Effect of $K_La C^*$ on ethanol production.

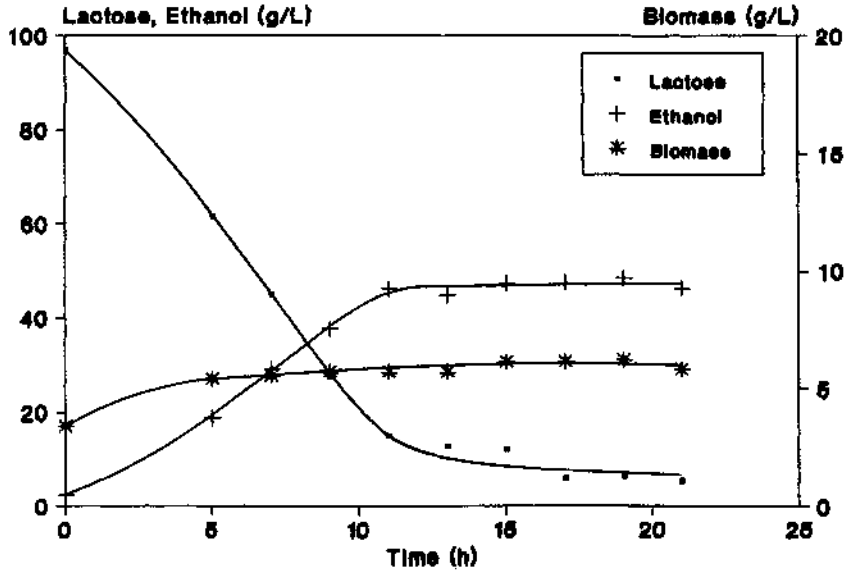


Fig. 3a. Fermentation profiles at 14 mmol/Lh, optimum aeration rates.

Inoculum development

A 48-h-old culture on potato dextrose agar (DIFCO, USA) slant was used. Growth medium for the inoculum was whey permeate adjusted to 40 g/L of lactose, pH 4.5 and supplemented with 6 g/L of yeast extract (DIFCO, USA). Propagation was carried out in two steps in flasks (10 % filling) incubated in 5-cm stroke reciprocant shaker at 32° C and 120 rpm, for 12 hours. Final cell concentration reached was (5-8) $\times 10^8$ cells/mL. The amount of cell necessary for obtaining an initial concentration of 1×10^8 cells/mL were harvested by centrifugation (1230 *g*, 40 min), resuspended in fermentation medium and transfered to the fermentor.

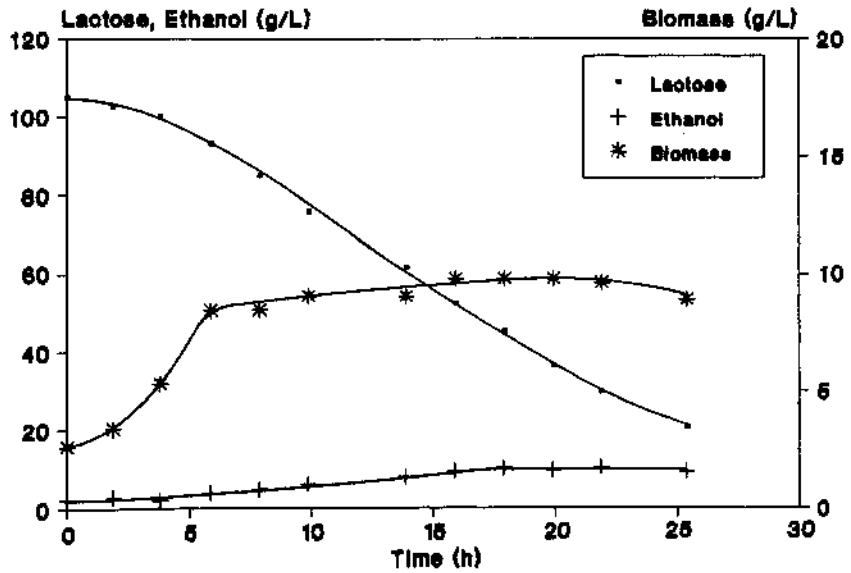


Fig. 3b. Fermentation profiles at 82 mmol/Lh.

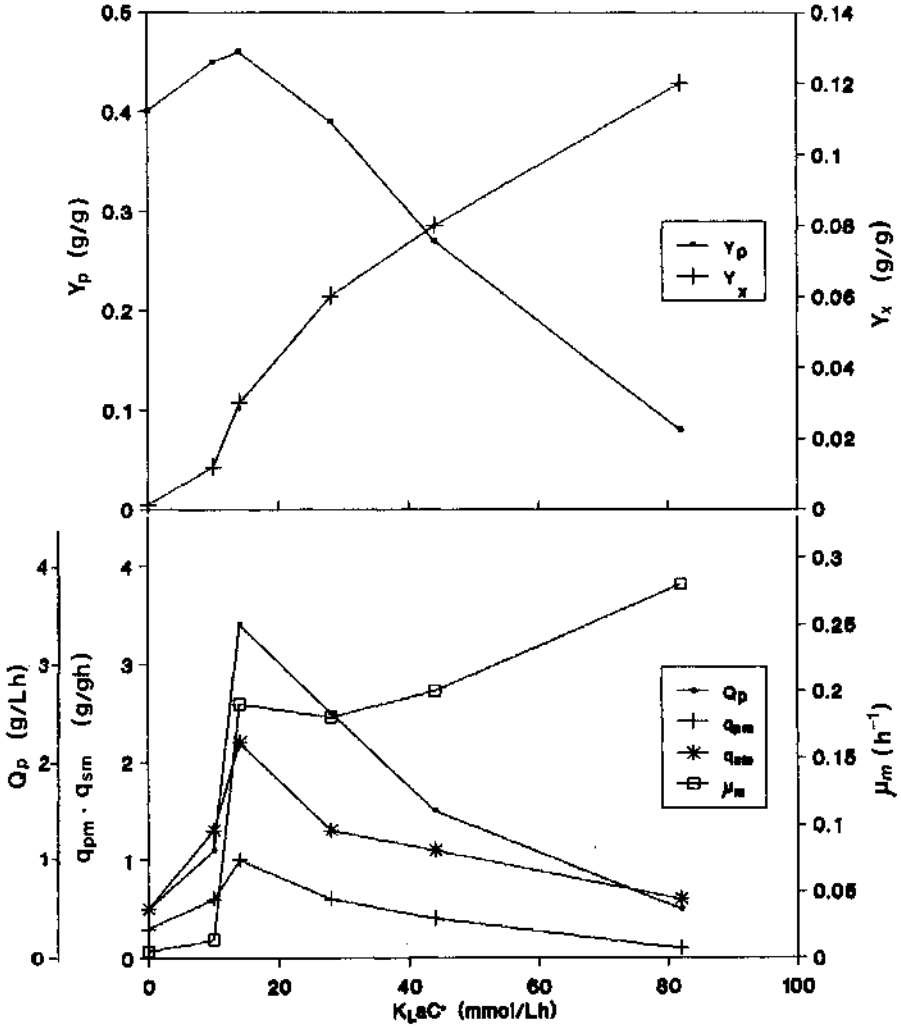


Fig. 4. $K_La C^*$ effect on fermentation parameters.

Fermentation assays

Assays were performed in a fermentor (EYELA, model M-100, Tokyo Rikakikai, Japan) with a working volume of 1.5 L at 32° C. Agitation speed and air flow rate were varied according to the required aeration rate. Polypropylene glycol 2027 (BDH, England) was used as an antifoam agent. A synopsis of the experimental conditions used is given in Table 1.

Analytical methods

Lactose was determined by Chloramine T standard method (4). Ethanol was measured by gas chromatography using Pye-Unicam 304 chromatographer equipped with FI detector and glass column (180 cm × 4 mm) packed with FFAP 10 % on Chromosorb 80/100 mesh (SUPELCO, USA). n-Butanol was the internal standard and nitrogen was used as the carrier gas. Cell concentration and viable fraction were assayed by microscopic counting using a Neubauer chamber and Methylene Blue aqueous solution as a diluent (5). Biomass concentration was measured turbidimetrically at 570 nm using a Spectronic 21 spectrophotometer (Bausch & Lomb, USA). The empirical correla-

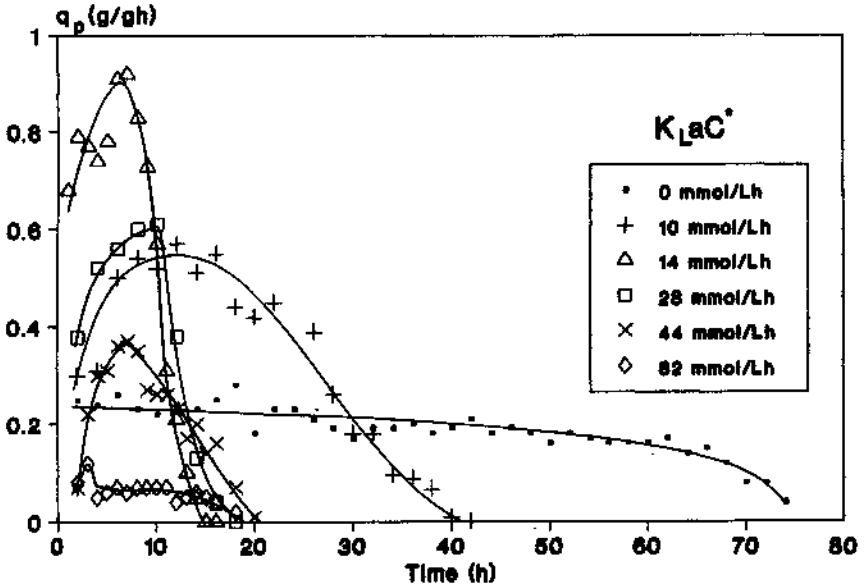


Fig. 5. K_LaC^* effect on specific production rate (q_p).

tion determinated was: one unit of optical density is equivalent to 0.98 g of biomass dried at 70° C. Oxygen supply rate was determinated by sulfite oxidation method (2).

Results and Discussion

Figure 1 and 2 respectively illustrate lactose and ethanol concentration profile for different aeration conditions. Figure 1 shows an increase in lactose consumption rate when $K_{La}C^*$ was risen from 0 to 14 mmol/Lh. However for higher K_LaC^* this rate decreased. Concerning to Figure 2, it can be concluded that fermentation time (t_f: minimum time required for obtaining ethanol production rate close to 0) had a drastic diminution from more than 74 to 13 hours when K_LaC^* changed from 0 to 14 mmol/Lh. Moreover, the maximum ethanol concentration reached was approximately the same, showing an increase on the fermentation rate. Nevertheless, for K_LaC^* values higher than 14 mmol/Lh a decrease in the ethanol production rate and a strong diminution of the maximum ethanol concentration were shown, making evident a shift in the lactose degradation pathway from a fermentative via to an oxidative via. Figures 3a and 3b show completely different fermentation behaviour. Figure 3a presents fermentation profiles for the optimum aeration rate found. In this fermentation, the t_f is coincident with time required to obtain minimum lactose concentration. Figure 3b presents fermentation profiles for the highest aeration rate tested and shows that t_f is much more less than the time required for obtaining the minimum lactose concentration. In addition, the biomass concentration reached in this fermentation was higher than the one obtained in the preceding experience.

The fermentation parameter values obtained for the different K_LaC^* tested are resumed in the Table 1. Figure 4 shows the influence of K_LaC^* on the fermentation parameters. Q_p, Y_p, q_{sm} and q_{pm} were maximum at the optimum K_LaC^* value of 14 mmol/Lh. The variation of K_LaC^* from 0 to 14 mmol/Lh resulted in an increase of 6.8 times of Q_p from 0.5 to 3.4 g/Lh, ethanol yield (Y_p) improved about 15 % from 0.40 to 0.46 g/g and biomass yield (Y_x) rose from 0 to 0.03 g/g. An additional oxygen supply resulted in a drastic decrease of the fermentation performance. Aeration rate higher than 14 mmol/Lh produced a fast diminution of Q_p, Y_p and q_{pm} followed by a continuous ascent of Y_x. On the other hand, μ_m profile had a substantive change, from 10 mmol/Lh to higher

K_LaC^* values. A clear predominance of respiratory metabolism was observed for K_LaC^* values higher than 14 mmol/Lh: the q_{pm}/μ_m ratio was 0.35 g/g for 82 mmol/Lh, while such ratio was 5.3 g/g for the optimum aeration condition (14 mmol/Lh). At high aeration levels, specific lactose uptake rate (q_{pm}) decreased. Under these conditions glucose degradation rate is much lesser than under limited oxygen supply in order to compensate the higher ATP yield through oxidative respiration (Pasteur effect) (6).

Curves of q_p vs time at different aeration rates in Figure 5 show (except experience 1 without aeration) an ascendant phase that makes evident the activation grade reached in the fermentation and a descending phase that indicates an inhibition or limitation phenomenon. Maximum q_p and the time at which occurred illustrate the yeast behaviour in the course of the fermentation. An increase of K_LaC^* from 0 to 14 mmol/Lh enhanced the ethanol production: q_{pm} increased from 0.3 to 1.0 g/gh, making evident the high activation grade reached. The declination of q_{pm} at higher aeration rates leads to conclude the importance of a controlled oxygen supply in order to obtain the balance required between cell growth and ethanol production.

Nomenclature

F	air flow rate, mL/min.
K_LaC^*	oxygen supply rate, mmol/Lh.
N	agitation speed, rpm.
N_o	initial cellular concentration, cells/mL.
P	ethanol concentration reached at t_f corrected by initial concentration, g/L.
P_o	initial ethanol concentration, g/L.
Q_p	ethanol productivity, g/Lh.
q_p	specific ethanol production rate, g ethanol/g biomass h.
q_{pm}	maximum specific ethanol production rate, g ethanol/g biomass h.
q_{sm}	maximum specific lactose uptake rate, g lactose/g biomass h.
S_o	initial lactose concentration, g/L.
t_f	fermentation time, h.
X_o	initial biomass concentration, g/L.
x_s	lactose conversion, %.
Y_p	grams of ethanol produced at t_f per gram of initial lactose, g ethanol/g lactose.
Y_x	grams of biomass produced at t_f per gram of initial lactose, g biomass/g lactose.
μ_m	maximum specific growth rate, h^{-1}.

References

1. Casey, G., Magnus, C. and Ingledew (1984). High-Gravity Brewing: Effects of Nutrition on Yeast Composition, Fermentative Ability, and Alcohol Production. Appl. Env. Microbiol. **48,** 639-646.
2. Cooper, C. M., Fernstrom, G. A. and Miller, S. A. (1944). Performance of agitated gas-liquid contactors. Ind. Eng. Chem. **36,** 504-509.
3. Dirección de Investigaciones Económicas Agropecuarias (1990). Ministerio de Ganadería, Agricultura y Pesca, Uruguay.
4. International Dairy Federation (1964). International Standard FIL-IDF 28:1964.
5. Lee, S. S., Robinson, F. M. and Wang, H. Y. (1981). Rapid determination of yeast viability. Biotechn. Bioeng. Symposium **11,** 641-649.
6. Lehninger, A. L. (1985). Bioquímica, 2.ª ed. Ediciones Barcelona S. A., Barcelona, pp. 547.
7. Moulin, G., Laham-Guillaume, M. et Galzi, P. (1980). Etude de la production d'alcohol sur lactosérum deprotéiné. Industries Alimentaire et Agricoles **97,** 471-474.
8. Vienne, P. and von Stockar, U. (1985). Metabolic, physiological and kinetic aspects of the alcoholic fermentation of whey permeate by *Kluyveromyces fragilis* NRRL Y-665 and *Kluyveromyces lactis* NCYC 571. Enzyme Microb. Technol. **7,** 287-294.
9. Zertuche, L. and Zall, R. R. (1985). Optimizing alcohol production from whey using computer technology. Biotech. Bioeng. **27,** 547-554.

MICROBIOLOGIA SEM **8** (1992), 21-31

Methanosarcina mazei JC2, a new methanogenic strain isolated from lake sediments, that does not use H_2/CO_2

Jordi J. Cairó [1]*, Manuel Clarens [1], Jean Pierre Touzel [2], Montserrat Bardulet [1] and Josep M. París [1]

[1] *Universitat Autònoma de Barcelona. Departament d'Enginyeria Química. 08193 Bellaterra (Barcelona), Spain.*

[2] *Institut National de la Recherche Agronomique. Station de Technologie Alimentaire, BP 39, F-59651 Villeneuve d'Ascq Cedex, France.*

(Received May 30/August 12, 1991)

Summary

A new mesophilic methanogenic strain, which produced methane from acetate, methanol, and methylamines, was isolated from lake sediments obtained from the lake Banyoles, near Girona (Spain). The cells were irregular in shape, from 1 to 3 µm in diameter, aggregated in masses of a few to several hundred units. Colonies were about 1-2 mm and irregularly shaped. Their color was yellow or white. Growth occurred throughout the pH range of 5 to 9 with optimal growth around pH 7. The optimal growth temperature was 37° C. The molar deoxiribonucleic acid base composition was 37.2 % (G + C). Studies of DNA homologies showed that this isolate was a strain of *Methanosarcina mazei,* but it differs from other reported strains, in that was not able to use H_2/CO_2 for growth or methane production.

Key words: archaebacteria, methanogen, Methanosarcina, *DNA-DNA homology.*

Resumen

Se ha aislado una nueva cepa mesófila metanogénica que produce metano a partir de acetato, metanol y metilaminas, a partir de sedimentos del lago de Banyoles, cerca de Girona (España). Las células presentaban una morfología irregular, de 1 a 3 µm de diámetro, agregadas en masas de unas pocas a cientos de unidades. Las colonias presentaban un tamaño de 1 a 2 mm, con bordes irregulares. El color era amarillo o blanco. El crecimiento tenía lugar en un margen de pH de 5 a 9 con un óptimo cerca de 7. La temperatura óptima de crecimiento era de 37° C. La composición molar de bases del ADN (% G + C) era del 37,2 %. Los estudios de homología del ADN mostraron que el aislado era una cepa de la especie *Methanosarcina mazei,* pero diferente de las descritas, dada su incapacidad de utilizar H_2/CO_2 para el crecimiento o la producción de metano.

(*) Corresponding author.

Introduction

Methane-producing bacteria are very interesting because they are included in a distinct phylogenetic group of prokaryotes, named Archaebacteria, which also comprise the sulfur-metabolizing thermophilic archaebacteria and the extremely halophilic archaebacteria (21). Methanogenic bacteria habit a variety of anaerobic environments that include rumen of hervibores, sewage sludge digesters, wetwood root of certain trees, and aquatic sediments (11). On the other hand, the study of biological methanogenesis has attracted wide interest because of the role it plays in anaerobic digestion of organic matter.

Methanosarcinaceae have been described as a distinct group of methanogens characterized by specific morphological features and a broad spectrum of utilized substrates, and are the most cosmopolitan among methanogens. Thus, a considerable diversity exists within the reported strains. In this paper, the isolation and characterization of a new strain of *Methanosarcina mazei,* the unique to date isolated from lake sediments, are reported.

Materials and methods

Source of organism

The JC2 strain was isolated from lake sediments collected from the lake Banyoles, near Girona (Spain), using a Lunz water sampler modified by Ruttner (20). The samples were collected two meters below the interface sludge-water at a depth of 27 meters, from places where previous studies had shown methanogenic activity. Upon arrival in the laboratory, they were anaerobically transferred to N_2-flushed flasks, which were filled with the medium described below, containing carbon and energy sources.

Media and culture conditions

The strict anaerobic methods developed by Hungate (8), and modified by Miller and Wollin (18), were used throughout this study. The medium for the isolation and culture of methanogenic bacteria contained per liter: KH_2PO_4, 0.75 g; K_2HPO_4, 1.45 g; $MgCl_2 \cdot 6H_2O$, 0.2 g; NH_4Cl, 0.9 g; L-cysteine-HCl (sigma), 0.5 g; yeast extract (ADSA), 1 g; trace mineral solution, 10 ml; trypticase (ADSA), 0.5 g; resarzurin (0.2 %), 1 ml; $Na_2S \cdot 9H_20$, 0.5 g; Na_2CO_3, 0.5 g. The trace mineral solution (sulfate-free) contained (in g/l): nitrilotriacetic acid, 1.5 (nitrilotriacetic acid was dissolved in 1000 ml distilled water and pH brought to 6.5 with KOH concentrated solution): $MgCl_2 \cdot 6H_2O$, 3; $MnCl_2 \cdot 4H_2O$, 0.5; NaCl, 1; $FeCl_2 \cdot 4H_2O$, 0.1; $CoCl_2 \cdot 6H_2O$, 0.1; $CaCl_2 \cdot 2H_2O$, 0.1; $CuCl_2 \cdot 2H_2O$, . 0.01; $ZnCl_2$, 0.1; $AlCl_3$, 0.01; H_3BO_3, 0.01; $Na_2MoO_4 \cdot 2H_2O$, 0.01 and $NiCl_2 \cdot 6H_2O$, 0.02.

Solid medium was prepared by the addition of 16 g/l of agar. pH was adjusted to 7.1 with a concentrated solution of KOH. The medium was boiled under nitrogen or nitrogen/CO_2 (80:20) gas mixtures, dispensed in serum vials or roll-tubes and autoclaved (20 min., 120° C). Each tube or vial was flushed with H_2/CO_2 (80:20) gas mixture when needed. The other carbon and energy sources (sodium acetate, 3 g/l; methanol, 0.8 g/l; mono-, di-, trimethylamine, 0.5 g/l of each one; and formic acid, 0.92 g/l) were added before adjusting pH and autoclaving. In medium for physiological assays, yeast extract and trypticase were omitted and replaced by 10 ml of a vitamin solution, containing (in mg/l): biotin, 2; pyridoxal-HCl, 0.1; thyamine-HCl, 5; and nicotinic acid, 5.

A combination of four antibiotics was used in the isolation assays in order to avoid eubacterial growth. The final concentrations into the media were: penicillin, 666 µg/ml; vancomycin, 150 µg/ml;

kanamycin, 150 µg/ml; and streptomycin, 50 µg/ml. The sterile and anaerobic solution of antibiotics was added aseptically to the medium after autoclaving. All the carbon sources, antibiotic solutions, and the reducing solution were added using hypodermic needles and syringes previously sterilized and flushed with N_2 gas. Cells were cultivated in serum vials (100 ml) or roll-tubes (Ø 18 mm, length 15 cm) closed with butyl rubber stoppers and aluminium caps. Unless otherwise indicated, incubation was performed at 37° C, in the dark. All the experiments were done in duplicate.

Metabolic activity determinations and effect of temperature and pH

Determinations of optimal pH and temperature were carried out in appropriately modified culture media using different buffering systems (citrate-phosphate and Tris) and methanol as carbon and energy source. Cultures were incubated at several temperatures (20, 30, 37, 45° C) and pH (5, 6, 7, 8, 9). Maximum specific rate of methanogenesis was determined from methane production curves. It was calculated from the maximum slope of the curve obtained by plotting the logarithm of methane *versus* time.

Microscopy

Light microscopic observation of the cultures was carried out in an OLYMPUS BHT-2 microscope equipped with a phase contrast system BH2-PC and an epi-illuminant ultraviolet system BH2-RFL.

Samples for scanning electron microscopy were prepared according to Anderson (3). They were dehydrated using the critical point technique and sputter-coated with gold for observation. The scanning electron microscope used was an ISI Super III-A.

Transmission electron microscopy was done as described by Glauert and Glauert (6). Cells were fixed with glutaraldehyde (2 %), and buffered with cacodylate solution to pH 7.2 at 4° C for 2 h. Postfixation was done with osmium tetroxide (1 %) in sodium acetate 0.5 M for 24 h. Samples were dehydrated, stained with uranyl acetate, embedded in epoxy resin, sectioned on a LKB ultratome III 8802A, and post-stained with lead citrate. Samples were examined in a Hitachi Hu-12A transmission electron microscope.

DNA extraction

About 5 g of frozen cell paste were disrupted by passage through a French pressure cell at 138,000 kPa. DNA was purified from the lysate with the hydroxylapatite procedure according to Johnson (9).

DNA base composition

The thermal denaturation procedure described by Marmur and Doty (17) was used to determine the guanine plus cytosine content. DNA was dissolved in saline citrate solution (0.15 M NaCl plus 0.015 M trisodium citrate 2-hydrate, pH brought to 7 with HCl 0.1 N). Absorbance at 260 nm and cell temperature were digitally recorded every 1 min intervals with a Kontron SP800 spectrophotometer equipped with a Huber PD415 temperature programmer. For reference, *Escherichia coli* ATCC 10536 DNA (50 mol % G + C) was used.

TABLE I
CHARACTERISTICS OF THE SEDIMENT STUDIED

Parameter*	
TS ($kg \cdot m^{-3}$)	47.9
VS ($kg \cdot m^{-3}$)	13.8
VS/TS (%)	28.9
CODt ($kg \cdot m^{-3}$)	17.3
pH	6.9
acetic acid ($mg \cdot l^{-1}$)	—**

* TS, Total solids; VS, Volatile solids; COD_t, total COD; COD_s, soluble COD.
** Not detected.

DNA homologies

DNA homologies were determined according to Touzel *et al.* (27). All of the DNA samples were sonicated at 0° C for 1 min with a Braun Labsonic 1510 sonicator at 10-W energy setting and a 4-mm probe was used. The sonication procedure was repeated four times after 1 min resting periods. After nick translation, labelled DNA was separated from excess nucleotide by chromatography on Sephadex G-50 on a spun column as described by Maniatis *et al.* (16). DNA of *Methanosarcina barkeri* MS and *Methanosarcina mazei* MC3 were used as probes.

Fig. 1. Scanning electron micrograph of the isolated *Methanosarcina*. Dashed line represents his value in the below figure.

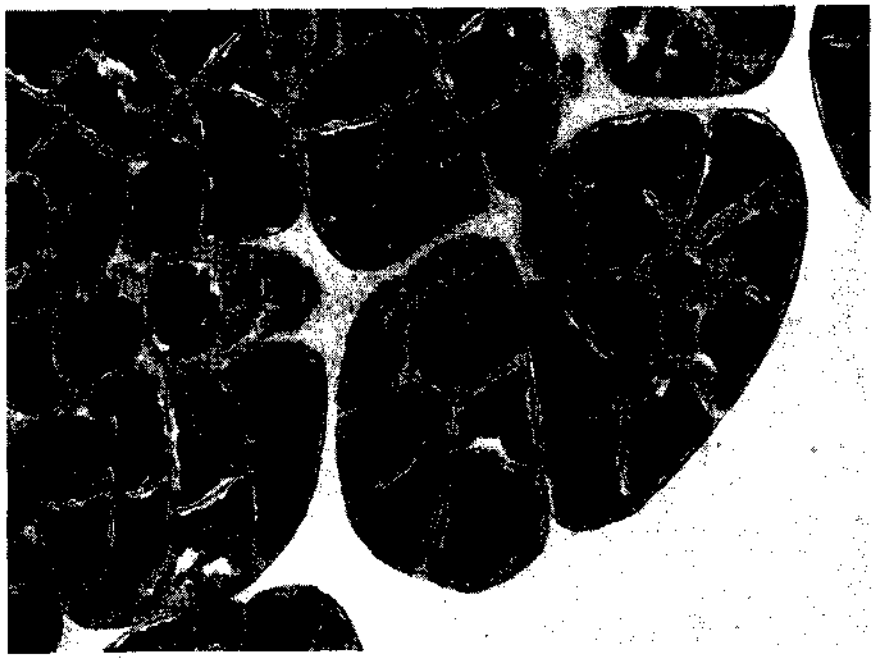


Fig. 2. Transmission electron micrograph of the isolate. Long bar represents 1 µm.

Elimination of S1 nuclease-resistant duplexes was performed by chromatography on a hydroxylapatite column in a Pasteur pipette. DNA samples were denatured by heating for 10 min in a boiling bath. Phosphate concentration was brought to about 0.1 M, and the sample was deposited on the top of the column. Fractions eluted by phosphate buffer (0.12 M, pH 6.8) were monitored for radioactivity and pooled. The pooled fractions were freeze-dried and dissolved in 0.1 ml of water. Phosphate buffer was changed to STE (Salt-Tris-EDTA) buffer (16) by spun-column chromatography on Sephadex G-50. The reassociation reaction mixtures contained 5 µl (0.01 to 0.025 µg, 6000 to 12,000 cpm) of labelled DNA, 100 µl (40 µg) of unlabelled DNA, 35 µl of water, and 280 µl of buffer containing 600 mM NaCl, 15 mM ethylenediamine tetraacetate (pH 8), 15 mM tris (hydroxymethyl) aminomethane buffer (pH 8), 0.0225 % SDS, and 75 % formamide. DNA mixtures were denatured by heating in boiling water for 10 min. Hybridizations were conducted for 18 h at 35° C. The single-stranded DNAs were eliminated by incubating the preparation for 1 h at 30° C in the presence of a suitable dilution of S1 nuclease, and the hybrids were precipitated with an equal volume of 10 % trichloroacetic acid, collected on Whatman GF/F fibreglass disks, washed five times

TABLE 2
CARBON AND ENERGY SOURCES USED BY THE ISOLATE

Carbon source	Growth	Methane production
Acetate	+	+
Formate	–	–
Methanol	+	+
Methylamines	+	+
H_2/CO_2	–	–

+ observed; – not observed

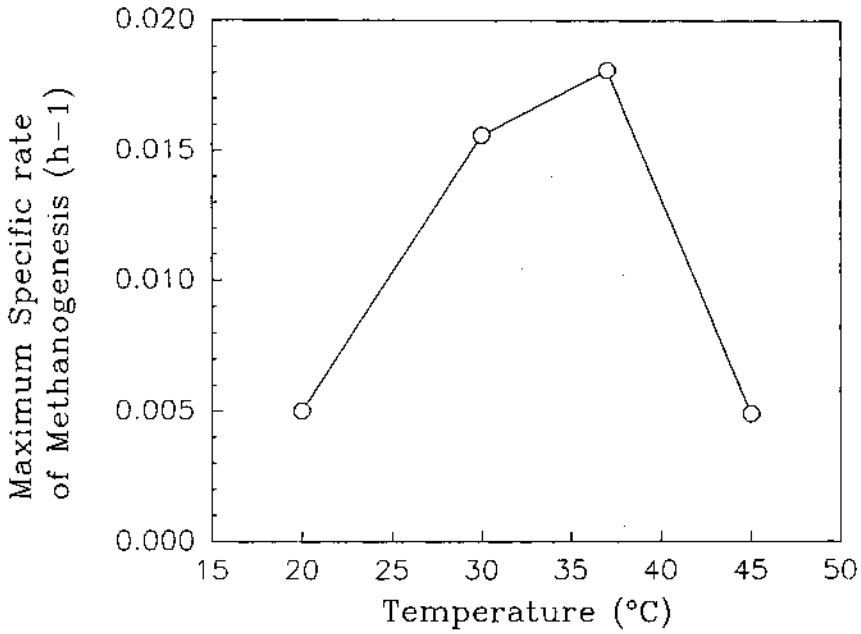


Fig. 3. Temperature range for growth on methanol of the isolated *Methanosarcina*.

with ice-cold trichloroacetic acid and then with ethanol, dried, and counted in the presence of 10 ml of Insta-gel scintillation mixture.

Other analysis

Methane and carbon dioxide contents of the biogas produced were determined with a Hewlett-Packard 5890 gas chromatograph, equipped with a thermal conductivity detector and a Porapak Q column (Ø 1/8", length 3 m) with helium as carrier (flow rate 20 ml/min). Injector, column, and detector temperatures were 110, 30 and 130° C, respectively.

Chemical Oxygen Demand (total, COD_t, and soluble, COD_s), Total Solids (TS) and Volatil Solids (VS) were determined according to Standard Methods (2).

Chemicals

All the chemicals used were of the highest purity available (analytical grade).

Results

General characteristics of the sediments

The physical and chemical characteristics of the sediments samples were determined (Table 1). The TS concentration suggested that the sediment was not a very concentrated sludge. This might be due to the fact that the sediments are fluidized in the deep of the lake. However, the percentage of VS shows that the sediments is not rich in biomass, and the major components is an inorganic matrix. This is confirmed by the very low value of COD_t.

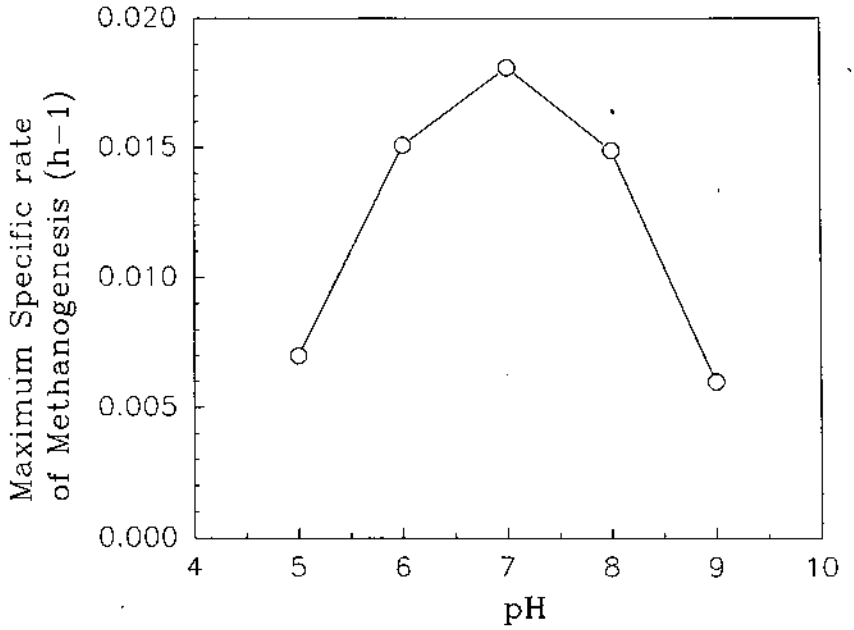


Fig. 4. Range of pH for growth on methanol of the isolated *Methanosarcina*.

Isolation

Five successive transfers of anaerobic sediment samples were done at 2-week intervals into enrichment medium. For isolation, the last enrichment culture was vigorously shaken and then serially diluted to 10^{-8} and purified in roll-tubes. Methane-producing colonies with high tendency to aggregate appeared after 2 weeks. They were picked and allowed to grow in the same liquid medium, and afterwards the procedure of mechanical disruption and serial dilution was repeated once more. Finally, the purity of the isolate was checked under light microscope. Only fluorescent aggregates typical of *Methanosarcina* were observed in the medium.

Morphology and ultrastructure

On solid medium, colonies were granular, irregular, yellow or white, from 0.5 to several mm in diameter. In liquid medium, aggregates presented the same morphology and their size depended of the shaking speed. The cells stained gram negative. Under epi-illuminant microscopy, aggregates exhibited the characteristic fluorescence of methanogens when irradiated at 420 nm.

Scanning electron microscopy revealed the typical appearance of the well known *Methanosarcina* species (Fig. 1), agglomerated in globular cysts from 1 to 3 μm that formed masses of several cysts. Transmission electron microscopy (Fig. 2) showed that the aggregates were made up of irregular cells held together by cell wall. The cells exhibited an electron dense cytoplasm. The round black bodies in the cytoplasm could be polyphosphate-like inclusions as evidenced by Scherer and Bochem (22) and Aldrich *et al.* (1). Cell shape varied between 1 to 3 μm. The typical three-layer membrane delimited cytoplasm. It appeared to be a S-layer as described by Sleytr (23). Spores, motility or flagels were never detected. A high number of cells in division were observed. The individual cells were highly irregular because the planes of division were not perpendicular. In some ca-

TABLE 3
MAXIMUM METABOLIC ACTIVITIES FOR EACH SUBSTRATE

Substrate	Concentration	Maximum metabolic activity ($hours^{-1}$)
Methanol	0.8 g/l	0.0181
Acetate	3 g/l	0.0062
Methylamines	1.5 g/l	0.0143
H_2/CO_2	80%/20%	–

ses, it was possible to observe vesicules transversely and longitudinally cut. Cell morphology did not change with growth and the agregates never desappeared. Cocci, single or in pairs, were not observed with the medium used.

Substrates for growth and methane formation

The carbon and energy sources for growth and methane production tested are listed in Table 2. The isolate grew well on methanol, mono-, di and trimethylamine, and acetate as the sole carbon and energy sources. No growth occurred on H_2/CO_2 or formate. Trypticase, yeast extract and vitamins were not required.

Optimal growth conditions

Growth was observed at temperature ranging between 20 to 45° C, with an optimum at 37° C (Fig. 3). No growth was observed at 45° C. The isolate grew in a pH range from 5 to 9, with an optimal pH at 6.8-7.0 (Fig. 4).

Maximum specific rate of methanogenesis was determined as described before. The results are shown in Table 3. It can be observed that methanol and methylamines were the best substrates for methanogenesis and growth, both at the same level. Although acetate was used by the isolate, its level was two fold lower.

DNA studies

The DNA base composition was 37.2 % G + C (Table 4). *Escherichia coli* ATCC 10536 was used as reference strain.

The percentages of DNA-DNA hybridization of the strain JC2 were 65.68 for *Methanosarcina barkeri* strain MS and 103.68 for *Methanosarcina mazei* strain MC3.

TABLE 4
RESULTS OBTAINED IN THE DETERMINATION OF THE % G + C OF THE ISOLATE

	E. coli ATCC 10536	*E. coli**	*Methanosarcina* strain JC2
Tm	89.88 ± 0.29	91.0	84.64 ± 0.31
% G + C	49.97 ± 0.72	50.0	37.20 ± 0.73

* From Marmur and Doty, 1962.

TABLE 5
SPECIES AND STRAINS OF THE GENUS *METHANOSARCINA* PREVIOUSLY DESCRIBED

Strain	Reference	Carbon and energy sources	Temp.	% G + C	Source
Methanosarcina barkeri					
MS	1,4	H_2, acetate, CH_3OH, methylamines	M	38.8	Sewage sludge
UBS	1,4	H_2, acetate, CH_3OH, methylamines	M	43.5	Anaerobic digestor
227	15	H_2, acetate, CH_3OH, methylamines	M	38.8	Anaerobic digestor
Fusaro	7	H_2, acetate, CH_3OH, methylamines	M	ND	Lake sediment
Methanosarcina acetivorans					
C2A	24	Acetate, CH_3OH, methylamines	M	42.0	Marine sediment
Methanosarcina mazei					
S6	14	H_2, acetate, CH_3OH, methylamines	M	42.0	Anaerobic digestor
LYC	13	H_2, acetate, CH_3OH, methylamines	M	ND	Alkaline swamp sediment
MC3	24	H_2, acetate, CH_3OH, methylamines	M	38.9	Anaerobic digestor
Methanosarcina vacuolata					
Z761	28	H_2, acetate, CH_3OH, methylamines	M	36.3	Anaerobic digestor
Methanosarcina thermophila					
TM1	29	Acetate, CH_3OH, methylamines	T	42.0	Anaerobic digestor
CHTI55	26	Acetate, CH_3OH, methylamines	T	39.3	Anaerobic digestor
MP	19	Acetate, CH_3OH, methylamines	T	38.8	Water hyacinths, ground termites

M = Mesophilic. T = Thermophilic. ND = Not determined.

Discussion

Strain JC2 and *Methanosarcina barkeri* Fusaro (7) are the only methanogenic microorganisms of the genus *Methanosarcina* which have been isolated from lake sediment. The strain JC2 is mesophilic and presents the typical morphology of the *Methanosarcina* genus (i.e. cell aggregates). In Table 5 are listed the previously described *Methanosarcina* strains. Strain JC2 belongs to one of the mesophilic species: *Methanosarcina barkeri, Methanosarcina acetivorans, Methanosarcina mazei* or *Methanosarcina vacuolata.*

The *Methanosarcina vacuolata* species can be rejected because our isolate does not present neither the typical high-vacuolated cytoplasm nor the ability to use H_2/CO_2. Strain JC2 is distinct to the *Methanosarcina acetivorans* strain C2A considering the higher value of % G + C of the latter (41.1 mol %). Likely, *Methanosarcina barkeri* strain UBS and *Methanosarcina mazei* strains S-6 and LYC are different according to the % G + C values. Finally, the study of DNA-DNA hybridization allowed us to conclude that our isolate is a *Methanosarcina mazei* strain.

Strain JC2 can use acetate, methanol, mono-, di- and trimethylamines as sole carbon and energy source but not H_2/CO_2 substrate. So, the fluorescence emited by the strain JC2 when irradiated at 420 nm could be explained by the requirement of cofactor F_{420} in other reactions than the reduction of Methenyl-H_4MPT to Methylene-H_4MPT (H_4MPT = tetrahydromethanopterin) invol-

ved in the conversion of CO_2 to CH_4. Reactions known to be F_{420}-dependent are the reduction of $NADP^+$ to NADPH (12) and the reduction of the heterodisulfide HTP-S-S-CoM to HTP-SH (7-mercaptoheptanoylthreonine phosphate) and CoM-SH (coenzyme M) involved in the conversion of CH_3-S-CoM to CH_4 (5).

Although methanol and methylamines are common substrates for all the *Methanosarcina mazei* strains described (10), acetate and H_2/CO_2 utilization by *Methanosarcina mazei* species are not well clarified yet. Strain LYC uses efficiently H_2/CO_2 but not acetate (13) while strains S-6 and MC3 use efficiently acetate but poorly H_2/CO_2 (14, 25). So, it appears that either acetate or H_2/CO_2 may be used efficiently by the *Methanosarcina mazei* strains. The incapacity of strain JC2 to use H_2/CO_2 corroborates this feature and it would appear that two physiologically distinct groups exist in the *Methanosarcina mazei;* species acetate using strains and H_2/CO_2 using strains. On the other hand, the strict incapacity of strain JC2 to use H_2/CO_2 could suggest that is a new strain of the species *Methanosarcina mazei.*

Acknowledgements

This work was partially supported by grants No. EN3B-0050-E(B) from EEC and IER (Spanish Ministry of Industry and Energy), No. RG078787 from NATO. We thank R. Tello for her assistance. Thanks are also due to María Francisca Roviralta Foundation for providing general laboratory equipment.

References

1. Aldrich, H. C., Robinson, R. W. and Williams, D. S. (1986). Ultrastructure of *Methanosarcina mazei.* System. Appl. Microbiol. **7,** 314-319.
2. American Public Health Association (1980). Standard methods for the examination of water and wastewater. 15th ed. Washington D. C., AHPA-AWWA-WPCF.
3. Anderson, T. F. (1951). Techniques for the preservation of three dimensional structures in preparing specimens for the electron microscopy. Vol. XIII, pp. 130-139. Trans. N. Y. Acad. Sci. New York.
4. Bryant, M. P. and Boone, D. R. (1987). Emended description of strain MS (DSM 800), the type strain of *Methanosarcina barkeri.* Int. J. System. Bacteriol. **37,** 169-170.
5. Deppenmeier, U., Blaut, M.; Mahlmann, A. and Gottschalk, G. (1990). Membrane-bound $F_{420}H_2$-dependent heterodisulfide reductase in methanogenic bacterium strain Göl and *Methanolobus tindarius.* FEBS Lett. **261,** 199-203.
6. Glauert, A. M. and Glauert, R. H. (1958). Araldite as an embedding medium for electron microscopy. J. Biophys. Biochem. Citol. **4,** 409-414.
7. Hippe, H.; Caspari, D., Fiebig, K. and Gottschalk, G. (1979). Utilization of trimethylamine and other N-methyl compounds for growth and methane formation by *Methanosarcina barkeri.* Proc. Nat. Acad. Sci. USA. **76,** 494-498.
8. Hungate, R. E. (1969). A roll tube method for cultivation of strict anaerobes. In: J. R. Norris and D. W. Ribbons (eds.), Methods in Microbiology, vol. 3B. pp. 117-132. Academic Press Inc. New York.
9. Johnson, J. L. (1981). Genetic characterization. In: P. Gerhardt, R. G. E., Murray, R., Costilow, E. W., Nester, W. A., Wood, N. R. Krieg and G. Briggs Phillips (eds.). Manual of Methods for General Bacteriology, pp. 450-472. American Society for Microbiology, Washington, D.C.
10. Jarrell, K. F. and Kalmokoff, M. (1988). Nutritional requirements of the methanogenic archaebacteria. Can. J. Microbiol. **34,** 557-576.
11. Jones, W. J., Nagle, D. P. and Whitman, W. B. (1987). Methanogens and the diversity of archaebacteria. Microbiol. Rev. **51,** 135-177.
12. Keltjens, J. F. and van der Drift, C. (1986). Electron transfer reactions in methanogens. FEMS Microbiol. Rev. **39,** 259-303.
13. Liu, Y., Boone, D. R., Sleat, R. and Mah, R. A. (1985). *Methanosarcina mazei* LYC, a new methanogenic isolate which produces disaggregating enzyme. Appl. Environ. Microbiol. **49,** 608-613.
14. Mah, R. A. (1980). Isolation and characterization of *Methanococcus mazei.* Curr. Microbiol. **3,** 321-326.
15. Mah, R. A., Smith, M. R. and Baresi, L. (1978). Studies on acetate-fermenting strain of *Methanosarcina.* Appl. Environ. Microbiol. **35,** 1174-1184.

16. Maniatis, T., Fristsch, E. F. and Sanbrook, J. (1982). Molecular cloning, a laboratory manual. Cold Spring Harbor Laboratory. Cold Spring Harbor, New York.
17. Marmur, J. and Doty, P. (1962). Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. J. Mol. Biol. **5,** 109-118.
18. Miller, T. L. and Wolin, M. J. (1974). A serum bottle modification of the Hungate technique for cultivating obligate anaerobes. Appl. Environ. Microbiol. **27,** 985-987.
19. Ollivier, B., Lombardo, A. and García, J. L. (1984). Isolation and characterization of a new thermophilic *Methanosarcina* strain (strain MP). Ann. Microbiol. **135,** 187-198.
20. Ruttner, F. (1970). Fundamentals of Limnology. University of Toronto Press, Toronto.
21. Sanz, J. L. and Amils, R. (1988). Archaebacteria: their phylogenetic relationship with the eubacterial and the eucaryotic kingdom. Microbiologia SEM **4,** 5-27.
22. Scherer, P. A. and Bochem, H. P. (1983). Ultrastructural investigation of 12 *Methanosarcinaceae* and related species grown on methanol for occurrence of polyphosphate-like bodies. Can. J. Microbiol. **29,** 1190-1199.
23. Sleytr, U. B. (1978). Regular arrays of macromolecules on bacterial cell walls: Structure, assembly, and function. Internat. Rev. Cytol. **53,** 1-64.
24. Sowers, K. S., Baron, S. F. and Ferry, J. G. (1984). *Methanosarcina acetivorans* sp. nov., an acetotrophic methane-producing bacterium isolated from marine sediments. Appl. Environ. Microbiol. **47,** 971-978.
25. Touzel, J. P. and Albagnac, G. (1983). Isolation and characterization of *Methanococcus mazei* MC3. FEMS Microbiol. Lett. **16,** 241-245.
26. Touzel, J. P., Petroff, D. and Albagnac, G. (1985). Isolation and characterization of a new thermophilic *Methanosarcina,* the strain CHTI 55. System. Appl. Microbiol. **6,** 66-71.
27. Touzel, J. P., Prensier, G., Roustan, H. L., Thomas, I., Dubourgier, H. C. and Albagnac, G. (1988). Description of a new strain of *Methanothrix soehngenii* and rejection of *Methanothrix concilii* as synonym of *Methanothrix soehngenii.* Int. J. System. Bacteriol. **38,** 30-36.
28. Zhilina, T. N. and Zavarzin, G. A. (1987). *Methanosarcina vacuolata* sp. nov., a vacuolated Methanosarcina. Int. J. System. Bacteriol. **37,** 281-283.
29. Zinder, S. H., Sowers, K. R. and Ferry, J. G. (1985). *Methanosarcina thermophila* sp. nov., a thermophilic, acetotrophic, methane-producing bacterium. Int. J. System. Bacteriol. **35,** 522-523.

MICROBIOLOGIA SEM **8** (1992), 32-39

Densidad y actividad de microorganismos del ciclo del carbono bajo el dosel de *Myrica gale* L.

J. M. Pozuelo González[1]*, F. J. Gutiérrez Mañero[1], F. Llinares Pinel[1] y F. Bermúdéz de Castro[2]

[1] *Laboratorio de Biología. Colegio Universitario S. Pablo (CEU). Monteprincipe, Boadilla del Monte. 28660 Madrid.*
[2] *Departamento de Ecología. Facultad de Biología. Universidad Complutense. 28040 Madrid.*
(Recibido febrero 7/diciembre 17, 1991)

Summary

Plants, especially actinorhizal, regulate edaphic microflora through various ways, modifying thus nutrients recycling. *Myrica gale* effect on microorganisms in the carbon cycle is studied in this work by comparing soil samples collected under the canopy in summer and control samples. The results indicate that under *M. gale* C-organic and N-total concentration and anaerobic cellulolytic, hemicellulolytic and amilolytic density increase, and pH, C/N ratio and aerobic cellulolytic microorganisms density decrease. Microbial activity in soil is also modified.

Key words: carbon, most probable number, microbial activity, canopy, Myrica gale.

Resumen

Las plantas, especialmente las actinorrizas, regulan la microflora edáfica por varias vías, modificando así el reciclado de nutrientes. En este trabajo se estudia el efecto de *Myrica gale* sobre microorganismos del ciclo del carbono, comparando muestras de suelo recogidas en verano bajo el dosel y muestras control. Los resultados indican que bajo *M. gale* aumentan las concentraciones de C-orgánico y N-total y las densidades de microorganismos celulolíticos anaerobios, hemicelulolíticos y amilolíticos y disminuyen el pH, la relación C/N y la densidad de celulolíticos aerobios. También se modifica la actividad microbiana del suelo.

Introducción

La materia orgánica es muy valiosa para el desarrollo de muchas propiedades del suelo que afectan al crecimiento de las plantas. La recuperación del suelo, la actividad microbiana y la productividad de un ecosistema terrestre están directamente relacionadas con la calidad y cantidad de materia orgánica del suelo (25). En la degradación de la materia orgánica intervienen microorganismos que realizan las transformaciones necesarias para el reciclado de los nutrientes. Dichas

(*) A quien debe dirigirse la correspondencia.

transformaciones se integran en ciclos biogeoquímicos. Uno de los más importantes es el ciclo del carbono, en cuyas etapas edáficas intervienen microorganismos celulolíticos, hemicelulolíticos, ligninolíticos, pectinolíticos, amilolíticos y quitinolíticos, entre otros.

La mineralización de la materia orgánica se ve afectada por factores bióticos y abióticos. Alexander (1) considera como principales factores abióticos: composición química de la materia orgánica y temperatura, humedad, pH y concentración de oxígeno del suelo. En cuanto a los factores bióticos cabe destacar la vegetación, que regula la microflora edáfica (17) a través de 4 vías principales: formando microclimas por efecto dosel; aportando materia orgánica por lecho de hojarasca, lavado del dosel y exudados radicales; aireando y estructurando el suelo por medio de las raíces, y cambiando la composición iónica del suelo por acción del aparato radical (26). Este control sobre el sustrato es más intenso en las plantas actinorrizas debido a que sus tejidos son muy ricos en compuestos nitrogenados (23), excretan una amplia gama de aminoácidos (13) y factores de crecimiento (3, 18, 22) y regulan eficazmente el pH del suelo (6, 19). Además se ha observado experimentalmente que enriquecen el suelo en materia orgánica (8, 9) y modifican diversos grupos de microorganismos edáficos (2, 4, 12).

En este trabajo pretendemos estudiar las modificaciones de la microflora edáfica del ciclo del carbono bajo el dosel de *Myrica gale* L., arbusto dioico de hoja caduca que vive en suelos ácidos al borde de arroyos, en turberas y en tremedales. Es una especie actinorriza que desarrolla nódulos radicales en simbiosis con *Frankia brunchorstii* (14). Aparece siempre nodulada en su hábitat natural y cede al suelo cantidades de nitrógeno que oscilan, según los lugares, entre 9 y 34 kg ha^{-1} $año^{-1}$ (20).

Materiales y métodos

Zona de muestreo

El muestreo se realizó en el Valle de la Viuda, situado en el centro de la provincia de Ciudad Real, en el término municipal de Piedrabuena, entre las cotas 640 y 700 metros. Por la parte inferior discurre transversalmente la carretera que une Piedrabuena con Navalpino. El fondo del valle estaba cubierto por un brezal de *Erica tetralix* L. que se extendía a ambos lados del arroyo del Valle de la Viuda, en cuyas orillas crecía abundantemente *M. gale* entremezclada con el brezo. En 1981 se explanó la parte baja del valle para plantar *Pinus pinaster* L. y *Populus x canadiensis* Moench (I-214 clónico) arrasando totalmente la vegetación. Pronto *M. gale* rebrotó y libre de competidores se extendió por una mancha turbosa, a mitad del valle, formando el sotobosque de la chopera de repoblación. Los ejemplares de *M. gale* presentaban en la época de muestreo nódulos abundantes y tenían una actividad reductora de acetileno (ARA) media de 50,88 nMC_2H_4 h^{-1} g^{-1} (peso seco).

Muestreos

Se muestreó en verano bajo el dosel de ejemplares de *M. gale* y a más de 3 metros de los mismos, lejos de su posible influencia. En ambas zonas se recogieron 3 muestras independientes constituidas en cada una de ellas por una mezcla homogeneizada de 10 porciones de suelo. El matorral de *M. gale* estaba situado en la parte baja del valle, cerca de la carretera citada, al borde del arroyo que surca el fondo del valle (coordenadas UTM 30SUJ814293).

Las muestras de suelo se recogieron siguiendo las técnicas habituales para análisis microbiológicos, en los 15 primeros centímetros de suelo, después de eliminar la capa superficial de hojarasca.

TABLA 1
CONDICIONES DE CULTIVO DE LOS GRUPOS FUNCIONALES ANALIZADOS

Grupo funcional	Número de réplicas	Inóculo (ml)	Diluciones	Temperatura (° C)
Celulolíticos aerobios	3	1	10^{-1} - 10^{-6}	28
Celulolíticos anaerobios	3	1	10^{-1} - 10^{-6}	28
Hemicelulolíticos	3	0,5	10^{-1} - 10^{-8}	28
Amilolíticos	3	1	10^{-1} - 10^{-8}	28

Cultivos microbianos

Para el análisis de cada grupo funcional se emplearon los medios de cultivo y técnicas descritas por Pochon y Tardieux (16). A los medios de cultivo, inoculados con las suspensiones-diluciones de suelo, se les añadió 1 ml/l de solución de oligoelementos (12). Los grupos funcionales estudiados y las condiciones de cultivo se resumen en la Tabla 1.

Medida del pH, carbono orgánico y nitrógeno total de los suelos

El pH se midió con un pHmetro Crison digit 501, provisto de un electrodo combinado de vidrio modelo Radiometer GK 2401C, en una mezcla de 20 g de suelo y 20 ml de agua destilada, previamente agitada durante 20 minutos.

El carbono orgánico se valoró por el método de Walkley y Black (27), modificado por García (11).

La determinación del nitrógeno total se realizó mediante la digestión de muestras según el método de Kjeldahl. El amonio liberado se determinó con un electrodo específico Orion 95-10-00 conectado a un mv/pHmetro Crison digit 501.

Tratamiento de la información

El número más probable de microorganismos por gramo de suelo (NMP) se evaluó por el método de McCrady (15). Para ello se calculó el número característico en función de los tubos positivos que aparecieron en cada prueba. La actividad biológica de cada grupo funcional se observó trazando la curva correspondiente en función del tiempo y del índice de dilución media límite (ID), ID = N/n, donde N representa el número total de tubos positivos y dudosos y n el número de réplicas por dilución.

Para comparar el NMP se utilizó un análisis de la varianza bidireccional con réplicas y 2 factores de variación, zonas y grupos fisiológicos. Cuando las diferencias fueron significativas se contrastaron las medidas con el test LSD (24). Los valores fisicoquímicos se comparan por el test «t de Student».

Resultados

Los valores de pH, carbono orgánico, nitrógeno total y relación carbono/nitrógeno aparecen en la Tabla 2. Los pH son ácidos en todos los suelos analizados, aunque la acidez es significativamente mayor bajo *M. gale.*

TABLA 2

RESULTADOS DE LOS ANALISIS FISICOQUIMICOS Y MICROBIOLOGICOS DE LOS SUELOS RECOGIDOS EN EL VALLE DE LA VIUDA

	Dosel (1)	Control (2)	Incremento respecto control (%)
N_2 total (mg/g)	3,42 ± 0,42 (a)	2,28 ± 0,01 (b)	+50,00
C orgánico (%)	3,78 ± 0,38 (a)	3,12 ± 0,11 (a)	+21,15
pH	5,33 ± 0,03 (a)	5,90 ± 0,06 (b)	–9,66
C/N	11,10 ± 0,24 (a)	13,68 ± 0,53 (b)	–18,86
Microorganismos celulíticos aerobios ($\times 10^2$)	1,93 ± 0,93 (a)	1,95 ± 0,90 (a)	–0,86
Microorganismos celulíticos anaerobios (×10)	5,17 ± 2,34 (a)	3,50 ± 0,58 (a)	+47,63
Microorganismos hemicelulolíticos ($\times 10^5$)	3,98 ± 2,77 (a)	1,22 ± 1,59 (b)	+225,00
Microorganismos amilolíticos ($\times 10^5$)	2,17 ± 1,67 (a)	1,47 ± 3,03 (b)	+46,89

Se indica la media ± el error estándar de 3 muestras.

Si la letra que figura entre paréntesis es distinta, existen diferencias entre ambos suelos, significativas al nivel $p < 0,01$.

(1) Suelos recogidos bajo el dosel de *M. gale.*

(2) Suelos recogidos a más de 3 m de *M. gale.*

Las concentraciones de carbono orgánico más elevadas se encuentran bajo el dosel de las plantas, 21,15 % más que en los suelos control. Sin embargo, dicha variación no resultó significativa. En cuanto a las concentraciones de nitrógeno total, se aprecian valores significativamente mayores bajo *M. gale.* Debido a que en este caso las diferencias son muy acusadas, la relación carbono/nitrógeno es menor bajo las plantas, 18,86 % con respecto al control, y dicha disminución es significativa.

El NMP medio de microorganismos celulolíticos aerobios varía entre $1,93 \times 10^2$ y $1,95 \times 10^2$ microorganismos por gramo de suelo (Tabla 2) y el valor más bajo corresponde a las muestras recogidas bajo la planta. La actividad biológica (Fig. 1) oscila entre 1,44 y 1,83 unidades del ID durante el primer período de lectura. Entre los 7 y 15 días de incubación se aprecia en ambos casos un incremento notable de la actividad que se mantiene menor bajo *M. gale.*

Hay menos microorganismos celulolíticos anaerobios que aerobios y la densidad media varía entre $5,17 \times 10$ y $3,5 \times 10$ microorganismos por gramo de suelo. En contra de lo observado en el grupo anterior, la densidad mayor se obtiene bajo *M. gale.* En ambos suelos se alcanza la misma actividad a los 7 días de incubación y luego el mayor incremento de actividad se produce en las muestras recogidas bajo *M. gale.*

Los microorganismos hemicelulolíticos experimentan mayor incremento bajo el dosel (225 %), pero su actividad biológica es muy parecida a la del control, como se observa en las curvas de la Figura 2.

En los microorganismos amilolíticos se advierte un incremento del 46,89 % en la muestra recogida a pie de planta, muy parecido al de los celulolíticos anaerobios. Las diferencias de actividad biológica entre el suelo del dosel y control son muy escasas.

Al considerar globalmente todos los grupos estudiados, se aprecia que la densidad microbiana es 1,79 veces superior bajo el dosel de *M. gale* con respecto al control. Esta diferencia es muy significativa con $p = 0,0006$ y $F = 15,14$. También las diferencias entre los distintos grupos fisiológicos fueron significativas ($p = 0,0006$ y $F = 18,29$), siendo el NMP de microorganismos hemicelulo-

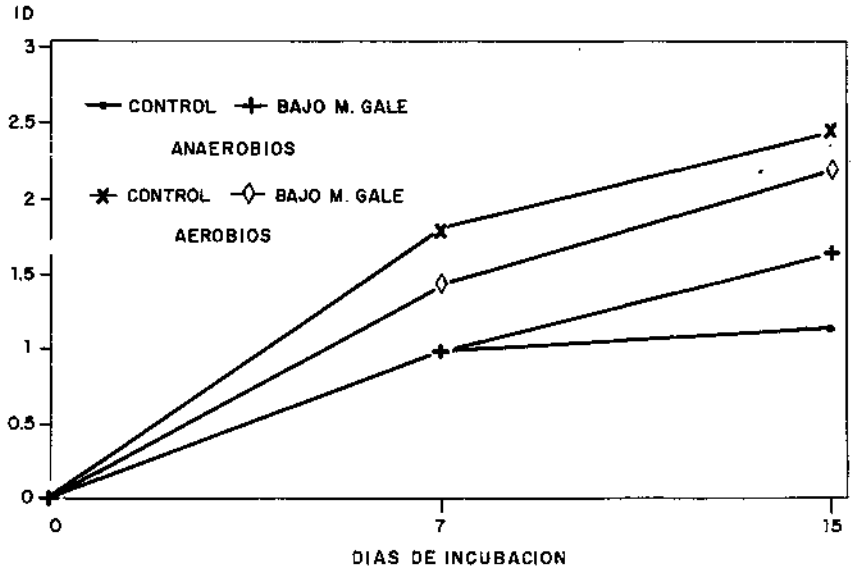


Fig. 1. Curvas de actividad de los microorganismos celulolíticos.

líticos y amilolíticos significativamente mayor que el de celulolíticos ($p < 0,01$, LSD = $14,41 \times 10^3$), entre los microorganismos celulolíticos aerobios y anaerobios no se observan diferencias significativas. Las diferencias altas se manifiestan también en la actividad que es prácticamente el doble en amilolíticos y hemicelulolíticos con respecto a los celulolíticos (Figs. 1 y 2). La interacción entre las densidades de los grupos fisiológicos y las zonas de muestreo resultó significativa ($p = 0,003$ y $F = 7,35$) debido al fuerte incremento del NMP de microorganismos hemicelulíticos y amilolíticos bajo la planta (Tabla 2).

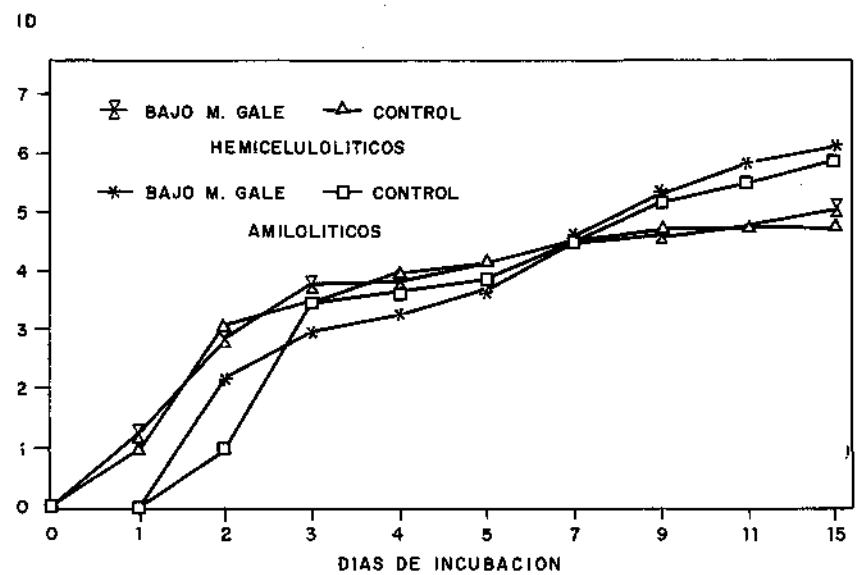


Fig. 2. Curvas de actividad de los microorganismos hemicelulolíticos y amilolíticos.

Discusión

A causa de los innumerables factores capaces de modificar la actividad y densidad de los microorganismos estudiados, las técnicas empleadas se refieren a capacidades funcionales. En este caso interesa considerar el efecto *in situ* de *M. gale.* Por ello se debe considerar que la materia orgánica se degrada como resultado de la actividad combinada de varias poblaciones microbianas en virtud de una acción cometabólica, por lo que los métodos seguidos deben poner de relieve interacciones de sinergia y antagonismo, como se logra al cultivar en tubos la comunidad microbiana variando su densidad (7).

Como el ARA es una medida indirecta de la cantidad de nitrógeno fijada y éste, a su vez, determina el vigor de la planta y la concentración de formas nitrogenadas en los órganos, existe una relación entre esta actividad y las condiciones de desarrollo de la comunidad microbiana edáfica. Por ello se debe suponer que la interacción entre el suelo y la planta en verano es menos estrecha e inespecífica que en otras estaciones más favorables al crecimiento vegetal y microbiano, en las que no se dan situaciones de estreses hídrico y térmico, como se ha demostrado al estudiar las fluctuaciones de otros grupos de microorganismos edáficos en el mismo lugar donde hemos realizado este trabajo (12).

Las concentraciones de nitrógeno total concuerdan con el hecho numerosas veces comprobado de que la cantidad de nitrógeno es superior bajo dosel de una planta diazotrófica. Dicho efecto se justifica por la concentración elevada de compuestos nitrogenados en las hojas de las plantas diazotróficas, como consecuencia de la capacidad fijadora de nitrógeno de los nódulos radicales (23), así como por la liberación de productos nitrogenados en la necrosis nodular y los aportes de la hojarasca y del lavado (5). También aparece en este punto la concentración más elevada de materia orgánica, debido al aumento en el aporte de productos orgánicos y de biomasa por el mayor aprovechamiento del sustrato. Los valores de pH comprueban el hecho ya mencionado por otros autores (6, 19) de que las plantas diazotróficas suelen acidificar el suelo, por intercambio iónico entre las raíces y el sustrato (10) y por el contenido elevado en grupos carboxilos y fenólicos, dada la riqueza en lignina de las hojas de *M. gale* (21). Las poblaciones microbianas encuentran así, en torno a *M. gale,* un sustrato más favorable para su desarrollo, fundamentalmente compuestos carbonados fácilmente asimilables, como lo muestran las diferencias de densidad entre los grupos fisiológicos encargados de atacar sustratos de resistencia elevada a la degradación, microorganismos celulolíticos y los que metabolizan sustratos más fácilmente degradables, amilolíticos y hemicelulíticos. Por ello las plantas que se desarrollan próximas a *M. gale* encuentran un medio edáfico más adecuado para su desarrollo y producen una biomasa más rica en compuestos carbonados de asimilación fácil.

Bibliografía

1. Alexander, M. (1980). Introducción a la microbiología del suelo. AGT Ed. S. A. México.
2. Bermúdez de Castro, F. y Gutiérrez Mañero, F. J. (1987). Las plantas actinorrizas y la microflora edáfica del ciclo del nitrógeno. En: J. I. Castelló y J. Terrades (eds.). Bases ecologiques per la gestió ambiental, pp. 101-102. Servei Parcs Naturals. Diputació. Barcelona.
3. Bermúdez de Castro, F. y Rodríguez-Barrueco, C. (1976). Estudio sobre las sustancias con actividad citokinina de los nódulos radiculares fijadores de nitrógeno de *Alnus glutinosa* (L.) Gaernt. Anuario. Centro de Edafología y Biología Aplicada. CSIC Salamanca. **2,** 105-114.
4. Bermúdez de Castro, F., Burguete, I. y Schmitz, M. F. (1984). Variaciones en la densidad de los microorganismos nitrificantes en alisedas. Studia Oecologica. **4,** 109-116.
5. Bollen, W. B. and Lu, K. C. (1968). Nitrogen transformations in soils beneath red alder and conifers. En: J. M. Trappe, J. F. Franklin, R. F. Tarrant and G. M. Hansen (eds.). Biology of Alder, pp. 141-148. USDA, Portland, Oregon.
6. Bormann, B. T. and Debell, D. S. (1981). Nitrogen content and other soil properties related to age of red alder stands. Soil. Sci. Am. J. **45,** 428-432.

7. Bungay, H. R. and Bungay, M. L. (1968). Microbial interactions in continuous culture. Adv. Appl. Microbiol. **10,** 269-290.
8. Carlson, P. J. and Dawson, J. O. (1985). Soil nitrogen changes, early growth and response to soil internal drainage of a plantation of *Alnus jorullensis* in the Colombian highlands. Turrialba. **35,** 141-150.
9. Danière, S., Capellano, A. et Moiroud, A. (1986). Dynamique de l'azote dans un peuplement naturel d'*Alnus incana* (L.) Moench. Oecol. Plant. **7,** 165-175.
10. Franklin, J. F., Dryness, C. T., Moore, D. G. and Tarrant, R. F. (1968). Chemical soil properties under coastal Oregon stands of alder and conifers. En: J. M. Trappe, J. F. Franklin, R. F. Tarrant and G. M. Hansen (eds.). Biology of Alder, pp. 157-178. USDA, Portland, Oregon.
11. García, A. (1981). Experimentos en microbiología del suelo. Compañía Editorial Continental, S. A. México.
12. Gutiérrez Mañero, F. J. y Bermúdez de Castro, F. (1983). Modificaciones de la microflora edáfica del ciclo del nitrógeno bajo *Myrica gale* L. An. Edafol. Agrobiol. **42,** 1233-1244.
13. Leaf, G., Gardner, I. C. and Bond, G. (1959). Observations on the composition and metabolism of the nitrogen-fixing root nodules of *Myrica.* Biochem. J. **72,** 662-667.
14. Lechevalier, M. P. and Lechevalier, H. A. (1989). Genus *Frankia* Brunchorst 1886, 174^{AL}. En: S. T. Williams, M. E. Sharpe and J. G. Holt (eds.). Bergey's Manual of Systematic Bacteriology 4, pp. 2410-2417. Williams and Wilkins Co. Baltimore.
15. McCrady, M. H. (1918). Tables for rapid interpretation of fermentation tube results. Can. J. Pub. Health. **9,** 20.
16. Pochon, J et Tardieux, P. (1962). Techniques de Analyse en microbiologie du sol. Editions de la Tourelle. St. Mandé.
17. Remacle, J. et De Leval, J. (1975). L'application des indices de richesse et d'activité pour la caractérisation microbiologique des sols. Rev. Ecol. Biol. Sol. **12,** 193-199.
18. Rodríguez-Barrueco, C., Miguel, C. and Palmi, L. M. (1979). Cytokinins in root-nodules of the nitrogen-fixing non-legume *Myrica gale* L. Z. Pflanzenphysiol. **95,** 275-278.
19. Schmitz, M. F., Aranda, Y., Esteban, M. L. y Bermúdez de Castro, F. (1990). Nodulación de *Elaeagnus angustifolia* L. en el bosquete de Valdemoro (Madrid), Ecología. **4,** 121-129.
20. Schwintzer, C. R. (1979). Nitrogen fixation by *Myrica gale* root nodules in a Massachusets wetland. Oecologia. **43,** 283-294.
21. Schwintzer, C. R. (1984). Production, decomposition and nitrogen dynamics of *Myrica gale* litter. Plant and Soil. **78,** 245-258.
22. Silver, W. S., Bendana, F. E. and Powell, R. D. (1966). Root nodule symbiosis. II The relation of auxin root geotropism in root and root nodules of non-legumes. Physiol. Plant. **19,** 207-218.
23. Silvester, W. B. (1977). Dinitrogen fixation by plants associations excluding legumes. En: R. W. F. Hardy and A. H. Gibson (eds.). A treatise on dinitrogen fixation. IV, pp. 141-190. John Wiley and Sons. New York.
24. Sokal, R. R. and Rohlf, F. J. (1969). Biometry. W. H. Freeman and Company. New York.
25. Stroo, H. F. and Jencks, E. M. (1982). Enzyme activity and respiration in mine soils. Soil Sci. Soc. Am. J. **46,** 548-553.
26. Turner, D. P. and Franz, E. H. (1985). The influence of western hemlock and western redcedar on microbial numbers, nitrogen mineralization and nitrification. Plant and Soil. **88,** 259-267.
27. Walkley, A. and Black, I. A. (1934). An examination of the Degtjareff method for determining soil organic matter and a proposed modification of the chromic acid titration method. Soil Sci. **37,** 29-38.

MICROBIOLOGIA SEM 8 (1992), 39-43

Single cell protein production from beet pulp by mixed culture

K. M. Ghanem

Botany Department. Faculty of Science, Alex. Univ., Alex., Egypt.
(Received March 24, 1991/January 8, 1992)

Summary

Different mixed cultures of *Trichoderma reesei* and a yeast were cultivated on beet pulp (BP) – containing medium. *T. reesei* and *Kluyveromyces marxianus* offered a combination that gave high SCP yields (51 %) and efficiently converted BP into proteins (39.4 %). The yeast extract in the basal medium could be substituted and the BP level multiplicated from 2 to 4 % upon using mixed culture of *T. reesei* and *K. marxianus*. Under these conditions the protein yields reached to a maximum value of 54 % and highest efficiency of BP conversion into proteins (41.8 %). The obtained protein proved to contain all essential amino acids, which compared favourably with those of the FAO guideline and soy bean oil meal.

Resumen

Thrichoderma reesei y una levadura se cultivaron conjuntamente en un medio que contenía pulpa de remolacha (BP). La combinación de *T. reesei* y *Kluyveromyces marxianus* dio el mejor rendimiento de SCP (51 %) y convirtió eficientemente BP en proteínas (39,4 %). El extracto de levadura se eliminó del medio base y BP se incrementó del 2 al 6 % en cultivos conjuntos de *T. reesei* y *K. marxianus*. Bajo estas condiciones los valores de proteína alcanzaron un valor máximo de 54 % y la más alta conversión de BP en proteínas (41,8 %). Se comprobó que la proteína obtenida contiene todos los aminoácidos esenciales.

Introduction

Most experiments on single cell protein (SCP) production from cellulosic wastes were carried out with monocultures (Peitersen, 1975a). However, particular attention had been given to the utilization of mixed microbial systems which had some advantages for efficient biodegradation and protein production from complex lignocellulosic substrates (De la Torre, 1982). Many workers used varying forms of mixed cultures for increased protein yields (1, 2, 5, 6, 7, 8).

In Egypt, surplus quantities of beet pulp (BP), a by-product of sugar production from sugar beet, are available. The author (Ghanem *et al*, 1991) succeeded in obtaining high protein yield (49.3 %)

using treated BP as the sole carbon source by a monoculture of *Trichoderma reesei.* The present article aims to increase the protein outputs from BP, as high as possible, using mixed cultures.

Materials and methods

Beet pulp (BP)

The crude BP was kindly supplied by the Delta Sugar Company, Kafr El-Sheikh, Egypt. The dried BP was physically treated by milling in Wiley mill and passed through a 60 gauge mesh sieve, followed by chemical pretreatment with 3 % NaOH, to provide the most suitable BP for protein yields by *T. reesei* (Ghanem *et al,* 1991).

Maintenance and cultivation

The pure stock cultures were maintained on glucose-peptone agar slants with transfers at monthly intervals. Cultivation was carried out with a medium, which was found by the author (Ghanem *et al,* 1991) to be the most suitable for SCP by *T. reesei,* of the following composition (g/l): NaOH-pretreated milled BP, 20; $(NH_4)_2SO_4$, 3; KH_2PO_4, 2; $MgSO_4 \cdot 7H_2O$, 0.3; $CaCl_2 \cdot 6H_2O$, 0.3; yeast extract, 1; $FeSO_4 \cdot 5H_2O$, 0.01, and pH = 5.0. The organisms were allowed to grow in 100 ml portions of the medium dispensed in 250 ml Erlenmeyer flasks. The flasks were sterilized by autoclaving at 121° C for 15 min, inoculated with 8 ml spore suspension of 5-day-old cultures of *T. reesei* plus 2 ml of 48-hr-old cultures of the tested yeasts and incubated at 30° C under shaked conditions (200 shakes/min, amplitude 7 cm) for 7 days.

Analyses

The fermentation residue (residual BP + microbial growth of *T. reesei* and the yeast) was separated by centrifugation at 4000 rpm for 20 min, washed with distilled water and dried at 30° C for constant weight, this residue referred to as the dry weight. The dried residue was analysed for its crude protein content (SCP) by the microKjeldahl technique (total nitrogen × 6.25), and amino acid constituents using Backman Amino Acid Analyzer.

Results and discussion

SCP production by mixed culture

Four different species of yeasts in addition to *T. reesei* were tested. The results (Table 1) revealed that the mixed culture of *T. reesei* and *K. marxianus* offered a combination that gave as high as 51 % yield of protein and efficiently converted about 39.4 % of the supplemented BP into SCP. This might be attributed to the availability of some nutrients (amino acids, vitamins, ... etc.), in the fermentation medium, provided either by the living or lyzed dead yeast cells of *K. marxianus* that stimulated the metabolic activities of *T. reesei.* In this respect, it was reported that the protein yields from cellulosic wastes were increased using mixed cultures (1, 2, 8).

As the basal medium has 1 g/l yeast extract, the increased activities of *T. reesei* in mixed culture can be attributed to the continuous consumption of the released reducing sugars during the fermen-

TABLE 1
DRY WEIGHTS AND SCP PRODUCTION BY THE TESTED MIXED CULTURES

Microbial system	Final pH	Dry wt mg	SCP		Conversion efficiency
			mg	% D. wt	
Monoculture of:					
Trichoderma reesei (control)	5.1	1450	714.9	49.3	35.7
Mixed culture of:					
T. reesei + *Kluyveromyces marxianus*	5.1	1547	788.5	51.0	39.4
T. reesei + *Candida utilis*	4.8	1521	762.0	50.1	38.1
T. reesei + *Saccharomyces cerevisiae*	5.0	1536	758.8	49.4	37.9
T. reesei + *Saccharomyces uvarum*	4.9	1467	724.7	49.9	36.2

* Dry weight = residual BP + microbial growth.

** Conversion efficiency = $\frac{\text{mg protein}}{\text{mg BP in 100 ml medium}} \times 100$.

tation of the cellulosic waste (BP) from the medium by *K. marxianus* yeast resulted in low concentration of reducing sugars (detected as few milligrams/liter) which in turn increase the synthesis and activity of cellulases of *T. reesei*. In this respect, it was stated that the cellulases regulatory mechanism (catabolite repression) can be avoided by using mixed cultures (3).

Effect of yeast extract levels

Different concentrations (0.0-2 g/l) of yeast extract were tested. The results (Table 2) indicated that the omission of yeast extract stimulated protein yields (52%), while its presence favoured a trifle increase in growth yields. These results revealed that *K. marxianus* in mixed culture provides nutrients to the mould, and the catabolite repression of cellulases of *T. reesei* may be avoided. Thus, the yeast extract in the basal medium can be substituted by *K. marxianus*.

Effect of BP level

The basal medium, lacking 1 g/l yeast extract, was modified by adding different levels of NaOH-pretreated milled BP ranging from 10 to 60 g/l. The results (Table 3) revealed that at 4% BP

TABLE 2
DRY WEIGHT AND SCP PRODUCTION BY THE MIXED CULTURE OF *T. REESEI* AND *K. MARXIANUS* AS INFLUENCED BY DIFFERENT YEAST EXTRACT LEVELS

Yeast extract level (g/l)	Dry wt mg	SCP		Conversion efficiency
		mg	% D. wt	
0.0	1515	789.0	52.0	39.5
0.5	1532	786.1	51.3	39.3
1.0	1547	788.5	51.0	39.4
1.5	1551	787.8	50.8	39.4
2.0	1556	785.9	50.5	39.3

TABLE 3
DRY WEIGHT AND SCP PRODUCTION BY MIXED CULTURE OF *T. REESEI* AND *K. MARXIANUS* AS INFLUENCED BY BP LEVELS

BP level g/100 ml	Final pH	Dry wt (mg)	SCP		Conversion efficiency
			mg	% D. wt	
1	5.0	519	260.5	50.2	26.1
2 (basal)	5.1	1517	789.0	52.0	39.5
3	5.1	2295	1212.2	52.8	40.4
4	5.2	3096	1672.1	54.0	41.8
5	5.2	3816	2030.1	53.2	40.6
6	5.2	4465	2340.0	52.4	39.0

maximum protein yiels (54 %) with promising efficiency to convert BP into SCP (41.8 %) were estimated with the mixed culture. The author (Ghanem *et al*, 1991) found that 2 % BP were optimum for SCP by monoculture of *T. reesei.*

Amino acids composition

The good nutritional quality of the tested fungal proteins obtained from the mixed culture had been assessed through the GLC analyses of the amino acids of the fermentation products (unfer-

TABLE 4
AMINO ACID CONTENT (g/100 g PROTEIN) OF A MONOCULTURE OF *T. REESEI* (GHANEM ET AL, IN PRESS) AND A MIXED CULTURE OF *T. REESEI* AND *K. MARXIANUS* AS COMPARED WITH THE FAO REFERENCE AND SOY BEAN OIL MEAL PROTEIN

Amino acid	Monoculture *(T. reesei)*	Mixed culture *(T. ressei + K. marxianus)*	FAO	Soy bean oil meal
Essential:				
Cystine	4.8	4.5	2.0	1.4
Isoleucine	9.6	5.2	4.2	5.7
Leucine	7.1	7.9	4.8	7.7
Lysine	4.2	3.7	4.2	6.5
Methionine	0.9	0.8	2.2	1.4
Phenylalanine	3.8	5.4	2.8	5.1
Threonine	5.0	6.2	2.8	4.0
Tyrosine	4.6	4.4	2.8	2.7
Valine	6.9	8.0	4.2	5.0
Non-essential:				
Alanine	6.5	5.8	–	–
Arginine	4.8	3.3	–	–
Aspartic acid	9.5	10.0	–	–
Glutamic acid	8.5	13.2	–	–
Glycine	5.5	5.3	–	–
Histidine	3.8	3.8	–	–
Proline	4.8	5.7	–	–
Serine	5.3	5.1	–	–

mented BP + microbial growth) obtained under the tested cultural conditions. The results (Table 4) indicate that the tested proteins show a profile that compares favourably with the FAO reference, and the amino acids of soy bean oil meal values or higher, except for methionine and lysine which showed lower values.

As the amino acids profile of mixed culture *(T. reesei + K. marxianus)* compared with that of the monoculture *(T. reesei),* under comparable conditions, reported by the author (Ghanem *et al,* 1991), the values of some amino acids (leucine, phenylalanine, threonine, valine, aspartic, glutamic and proline) are higher in mixed culture than in monoculture, while (cystine, methionine, tyrosine, glycine, histidine and serine) were of comparable values in both samples. This finding is in accordance with the results of some workers (6).

The previous study indicated that introducing mixed culture of *T. reesei* and *K. marxianus* for SCP production from BP is more efficient than using monoculture of *T. reesei,* where the protein yields increased from 49.3 to 54 %, while the conversion efficiency of BP into proteins raised from 35.7 to 41.8 % and the BP level could be multiplicated upon using mixed culture. On the other hand, the yeast extract in the basal medium could be substituted by the presence of *K. marxianus* in the mixed culture.

References

1. Callihan, C. D. and Dunlap, C. E. (1971). U. S. Environmental Protection Agency, Rep. SW-24c. PB. 203-630. Cited in Kristensen, T. P. (1978). European J. Appl. Microbiol. Biotechnol. **5,** 155-163.
2. Callihan, C. D. and Dunlap, C. E. (1973). U. S. Environmental Protection Agency, PB. 223-873. Cited in Kristensen, T. P. (1978). Eyropean J. Appl. Microbiol. Biotechnol. **5,** 155-163.
3. De La Torre, M. (1982). SCP production from cellulosic wastes. Conservation and Recycling. **5** (1), 41-46.
4. Ghanem, K. M., El-Refai, A. H. and El-Gazaerly, M. A. (1991). Protein-enriched feedstuff from beet pulp. World Journal of Microbiology and Biotechnology. **7,** 365-371.
5. Kristensen, T. P. (1978). Continuous single cell protein production from *Cellulomonas* sp. and *Candida utilis* grown in mixture on barley straw. European J. Appl. Microbiol. Biotechnol. **5,** 155-163.
6. Molina, O. E., Gálvez, N. I. P. and Callieri, D. A. (1983). Bacterial protein production from sugar cane bagasse pith. Acta. Cient. Venezolana. **34,** 59-64.
7. Peitersen, N. (1975a). Production of cellulase and protein from barley straw by *Trichoderma viride.* Biotechnol. Bioeng. **17,** 361-374.
8. Peitersen, N. (1975b). Cellulase and protein production from mixed cultures of *Trichoderma viride* and a yeast. Biotechnol. Bioeng. **17,** 1291-1299.

MICROBIOLOGIA SEM 8 (1992), 44-48

The occurrence of *Salmonella* serotypes in marine recreational waters of Valencia, Spain

J. L. Alonso[1]*, M. A. Alonso[2], M. A. Usera[3] and A. Echeita[3]

[1] *Instituto de Hidrología y Medio Natural. Universidad Politécnica. Camino de Vera, s/n. 46071 Valencia. Spain.*
[2] *Servicio de Análisis Clínicos. Hospital General del SVS de Alicante. Spain.*
[3] *Laboratorio de Referencia de Salmonella. Servicio de Bacteriología. Centro Nacional de Microbiología. 28220 Majadahonda (Madrid). Spain.*
(Received October 25, 1991/January 10, 1992)

Summary

Salmonellae serotypes were studied in order to know their prevalence in marine recreational areas of Valencia. Two hundred eight strains were isolated. The strains belonging to serogroups B, C, D, E and G. The serotyping yielded twenty one different serotypes. The most frequent salmonellae serotypes were *S. anatum* and *S. bredeney.* Our results were compared with those reported by other authors in Spain.

Key words: Salmonella serotypes, *sewage, marine waters.*

Resumen

Se han estudiado los serotipos de *Salmonella* para conocer cuáles son los predominantes en las aguas marinas de uso recreativo en Valencia. Se han aislado cepas que pertenecían a los serogrupos B, C, D, E y G. El número de serotipos identificados ha sido 21. Los serotipos más frecuentes han correspondido a *S. anatum* y *S. bredeney.* Los resultados obtenidos en este estudio se han comparado con los reseñados por otros autores en España.

Introduction

Recent epidemiological reports indicate continued increases in the incidence of human salmonellosis (3). *Salmonella* infections are maintained by cycles in which the polluted environment and particularly contaminated surface water, plays a major role (6). The presence of salmonellas in marine recreational waters constitutes a potential public health hazard, because salmonellae are one of the pathogenic microorganisms most frequently found in surface polluted waters.

Serotyping is the most useful epidemiological marker for *Salmonella,* differentiating over 2,000 serotypes according to the Kauffmann-White Scheme (9).

(*) Corresponding author.

TABLE 1
SEROGROUP DISTRIBUTION OF THE TOTAL NUMBER OF STRAINS ISOLATED

Serogroup		Number of strains	Frequency (%)
B		93	44.71
C	C-1	24	11.54
	C-2	29	13.94
D	D-1	4	1.92
E	E-1	41	19.71
	E-3	2	0.96
	E-4	3	1.44
G		8	3.85
Autoagglutinable		4	1.92
Total		208	100.00

In this study we examine *Salmonella* strains isolated from marine recreational areas of Valencia in order to know the serotype prevalence.

Methods

Water samples

Four marine recreational zones of Valencia were chosen for this study (Puebla de Farnals, Port Saplaya, Malvarrosa and Perelló). A total of 46 water samples were obtained. Samples at each zone were provided as follows: on near the sewage discharge area (sites 1) and the second separated by about 200 meters from the first point (sites 2). Water samples were collected at monthly intervals during the period from September 1990 to February 1991.

Isolation of Salmonella

Salmonella analyses were carried out using the technique described by Alonso *et al.* (2). In short, one liter of sample was filtered through 0.45 μm-pore sterile membrane filters and the membranes introduced into flasks containing NR10/43 (100 ml) (1) or NR10(10)/43 (100 ml) (11) enrichment broths. Both enrichment media were incubated at 42° C for 24 h. All flasks of NR10/43 and NR10(10)/43 were streaked onto duplicate plates of Hektoen enteric agar (Difco) and Rambach agar (Technogram), and incubated at 37° C for 24 h.

Biochemical identification

From each plate two colonies were subcultured for purification on Nutrient agar (Difco) and identified using the API Z system (API system, France).

Serological identification

The antigenic formula was established by slide agglutination (5) and microagglutination (13) with both polyvalent and specific sera.

TABLE 2
SEROTYPE DISTRIBUTION OF THE TOTAL NUMBER OF STRAINS ISOLATED

Serotype	Number of strains*		Total number of strains	Frequency (%)
	Sites 1	Sites 2		
S. anatum	14	15	29	13.94
S. bredeney	9	18	27	12.98
S. brandenburg	7	10	17	8.17
S. typhimurium	11	5	16	7.69
S. derby	13	2	15	7.21
S. indiana	9	6	15	7.21
S. ohio	8	5	13	6.25
S. london	9	3	12	5.77
S. muenchen	7	5	12	5.77
S. newport	5	4	9	4.32
S. worthington	5	3	8	3.85
S. virchow	0	6	6	2.88
S. goldcoast	4	1	5	2.40
S. infantis	0	4	4	1.92
S. hadar	2	1	3	1.44
S. senftenberg	0	3	3	1.44
S. agona	3	0	3	1.44
S. harrisonburg	1	1	2	0.96
S. enteriditis	2	0	2	0.96
S. panama	2	0	2	0.96
S. mikawasima	0	1	1	0.48
S. autoagglutinable	2	2	4	1.92
Total	113	95	208	100.00

* Sites 1 near from the sewage discharge area and sites 2 separated by about 200 meters from the first point.

Results

A total of 208 *Salmonella* strains were isolated from marine sewage-polluted recreational waters. Table 1 shows the serogroups identified. *Salmonella* serogroup B was the more frequently isolated (44.71 %). We found 21 different serotypes and the number of isolations corresponding to each serotype is represented in Table 2. The most frequently salmonellae serotypes were *S. anatum* (13.94 %) and *S. bredeney* (12.98 %). In Table 3 are compared the serotypes isolated from marine recreational waters of Valencia, whose percentage is over 1 %, with those of non-human origin reported by other authors (7, 10) in Spain.

Discussion

As it can be seen from the results (Table 2), salmonellae were detected at a distance of approximately 200 meters from the point of discharge. It might appear that the survival rate of salmonellae serotypes in seawater is quite high. These findings agree with those indicated by other authors

TABLE 3

COMPARISON BETWEEN *SALMONELLA* SEROTYPES OF NON-HUMAN ORIGIN ISOLATED IN VALENCIA (IHMN), ZARAGOZA (IMPS) AND GRAND CANARY ISLANDS (DCCCTP)

Serotype	IHMN* (%)	IMPS† (%)	DCCCTP‡ (%)
S. anatum	13.94	1.30	15.54
S. bredeney	12.98	5.00	—**
S. brandenburg	8.17	2.00	—
S. typhimurium	7.69	6.79	0.52
S. derby	7.21	2.14	—
S. indiana	7.21	—	—
S. ohio	6.25	11.61	0.52
S. london	5.77	11.60	—
S. muenchen	5.77	3.75	—
S. newport	4.32	2.14	9.33
S. worthington	3.85	—	—
S. virchow	2.88	6.25	1.04
S. goldcoast	2.40	2.14	9.33
S. infantis	1.92	7.68	12.40
S. hadar	1.44	1.30	—
S. senftenberg	1.44	0.70	—
S. agona	1.44	2.32	8.81
S. autoagglutinable	1.92	—	—

* IHMN: Instituto de Hidrología y Medio Natural, marine polluted waters (This work).

† IMPS: Instituto Municipal de Salud Pública de Zaragoza, sewage origin (7).

‡ DCCCTP: Departamento de Ciencias Clínicas y Centro de Tecnología Pesquera de Gran Canaria, sewage and marine origin (10).

** No data available.

(14). The serotypes *S. virchow, S. senftenberg* and *S. mikawasima* were isolated from sites 2 but they were not detected in sites 1 probably due to overgrowth of accompanying microflora.

Salmonella strains belonging to serogroup B, C and E represent over 90% of the isolates. In Spain the serogroups of non-human origin more frequently detected are B, C and D (4). Most serotypes isolated in our study are important in human salmonellosis in Spain. *S. enteriditis* generally dominates Spanish list as the common serotype isolated from human and non-human sources (4). The WHO *Salmonella* surveillance data for 1979-87 showed that *S. enteriditis* appears to be increasing on at least the continents of North America, South America and Europe (12). In 1987 8 (89%) of 9 European countries reported *S. enteriditis* as their most common *Salmonella* serotype. Our results indicate a low prevalence of *S. enteriditis,* being most frequent *S. typhimurium* in marine polluted waters (Table 2). In sewage of Zaragoza (7) it has also been observed a similar frequency of isolation of *S. typhimurium* (Table 3). In our study the more frequently serotypes isolated were *S. anatum* and *S. bredeney.* In sewage and marine waters of Gran Canary Islands (10) the first place was also occupied by *S. anatum.*

We have detected the presence of *S. mikawasima,* an uncommon serotype in Spain. This serotype increased significantly due to an outbreak in Catalonia, that affected a large number of people in 1986 (4).

Seagull feces can play a role in the spread of salmonella serotypes in marine waters. In seagull feces have been detected *S. typhimurium, S. panama, S. infantis* and *S. brandenburg* (6).

The entrance of a serovar into a food chain may be the origin of its implantation in a country (8). Marine sewage-polluted waters can act as a reservoir of salmonellae serotypes, that can be introduced into salmonellae infection cycles by ingestion of shellfish from these marine areas.

Acknowledgments

This work was supported in part by the Universidad Politécnica de Valencia. We thank R. Díaz and R. Gutiérrez from the *Salmonella* Reference Laboratory (Spain) for expert technical assistance.

References

1. Alcaide, E., Martínez, J. P., Martínez-Germes, P. and Garay, E. (1982). Improved *Salmonella* recovery from moderate to highly polluted waters. J. Appl. Bacteriol. **53,** 143-146.
2. Alonso, J. L., Botella, M. S., Amorós, I. and Rambach, A. (1992). *Salmonella* detection in marine waters using a short standard method. Wat. Res. (in press).
3. D'Aoust, J. Y. (1987). Recent developments in *Salmonella* epidemiology and methodology. Food Lab. Newsletter. **9,** 32-36.
4. Echeita, M. A. and Usera, M. A. (1989). Prevalence of *Salmonella* serotypes isolated in Spain from human and non-human sources (1983-1987). Microbiol. SEM. **5,** 95-103.
5. Ewing, W. H. (1986). Edwards and Ewing's identification of enterobacteriaceae. 4th ed. Elsevier Science Publishing Co., Inc. New York.
6. Kampelmacher, E. H. (1977). The spread and significance of salmonellae in surface waters in The Netherlands. In: A. W. Hoadley and B. J. Dutka (eds.). Bacterial indicators/health hazards associated with water, pp. 148-158. American Society for Testing and Materials ASTM STP 635, Philadelphia.
7. Lafarga, M. A., Castillo, J., Navarro, M. y Gómez-Lus, R. (1991). Serotipos de *Salmonella enterica* en aguas residuales de Zaragoza. Comparación con aislamientos clínicos. 1982-1989. Microbiol. SEM. **7,** 23-34.
8. Le Minor, L. (1984). Genus III. *Salmonella* Lignières 1900. In: N. R. Krieg and J. G. Holt (eds.). Bergey's Manual of Systematic Bacteriology. Vol. I, pp. 427-458. Williams & Wilkins. Baltimore.
9. Le Minor, L. and Popoff, M. Y. (1988). Antigenic formulas of the *Salmonella* serovars 5th revision. WHO Collaborating Centre for Reference and Research on *Salmonella*. Institut Pasteur. Paris.
10. Monzón, C., González Z., López, R. H. y O'Shanahan, L. (1991). Inventario de *Salmonella* aislados en Gran Canaria. Rev. Esp. Microbiol. Clin.. **6,** 140-142.
11. Moriñigo, M. A., Borrego, J. J. and Romero, P. (1986). Comparative study of different methods for detection and enumeration of *Salmonella* spp. in natural waters. J. Appl. Bacteriol. **61,** 169-176.
12. Rodríguez, D. C., Tauxe, R. V. and Rowe, B. (1990). International increase in *Salmonella enteriditis:* A new pandemic? Epidemiol. Infect. **105,** 21-27.
13. Shipp, C. R. and Rowe, B. (1980). A mechanised microtechnique for *Salmonella* serotyping. J. Clin. Pathol. **33,** 595-597.
14. Yoshpe-Purer, Y. and Shuval, H. I. (1972). Salmonellae and bacterial indicator organisms in polluted coastal water and their hygienic significance. In: M. Ruivo (ed.). Marine pollution and sea life, pp. 574-580. Fishing News (Books) Ltd.