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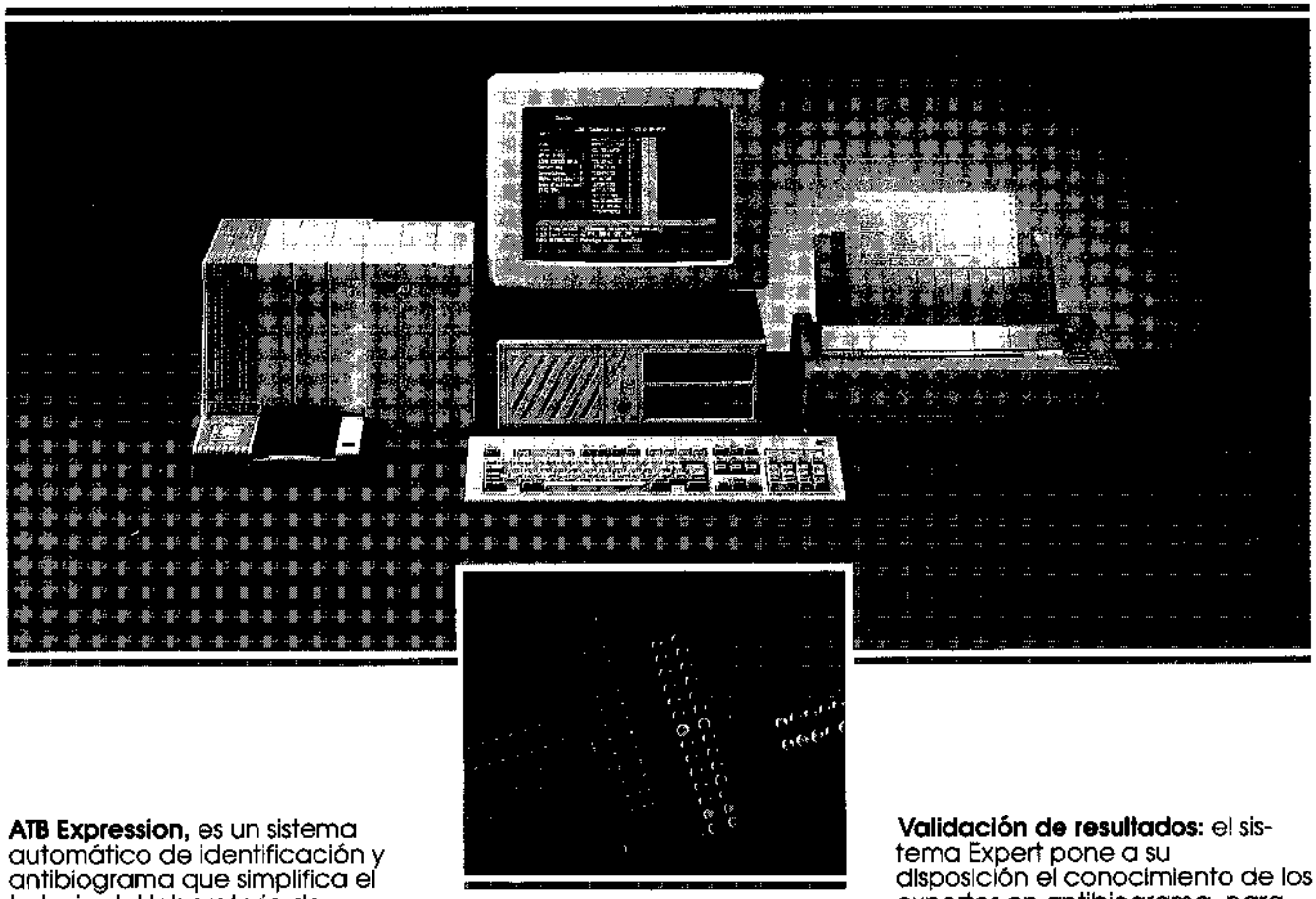

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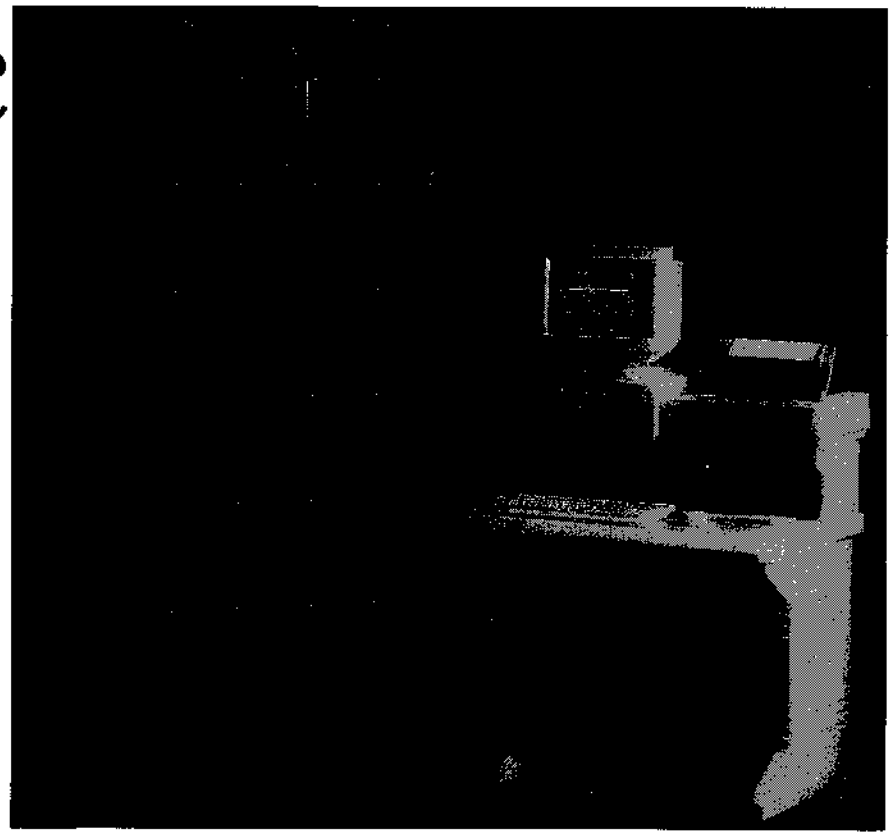

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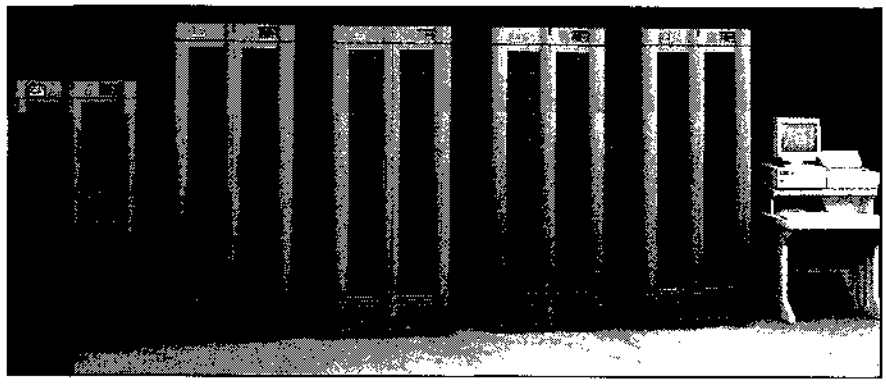

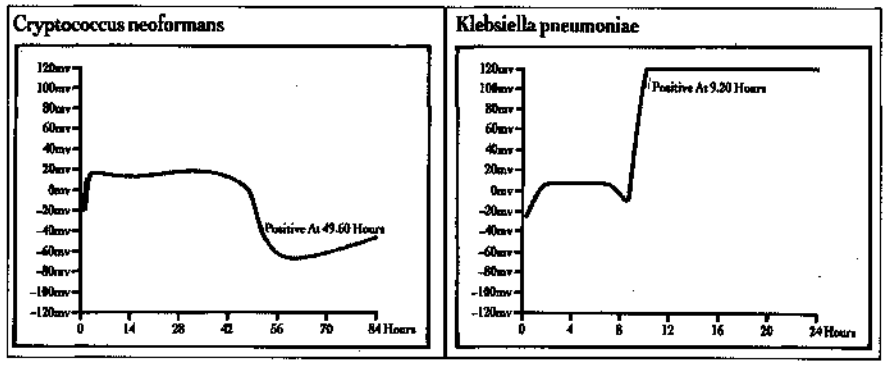

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MICROBIOLOGIA SEM 9 (1993), 77-89 **MINIREVIEW**

Thermophilic enzymes and their biotechnological potential

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Summary

The ability of many microorganisms to grow at high temperatures has held a particular fascination for microbiologists and biochemists since a long time. As any of their cellular components, their proteins are inherently more stable to heat than those of conventional organisms. This thermal stability is not due to any specific characteristic, but results a consequence of various changes which contribute to the whole stability of the protein in an additive manner. These enzymes are not only more thermostable, but also more resistant to chemical agents than their mesophilic homologous, what makes them extremely interesting for industrial processes. Despite this, most of the enzymes used at present in industrial processes have been isolated from mesophiles due to the limited knowledge and difficulties to grow thermophiles in high scale. The objective of this review is to consider briefly the importance of the thermostability in order to apply enzymes in the industry, and to overview the most recent advances in the identification of new thermophilic organisms and enzymes. Furthermore, the recent development of genetic model systems for moderate and extreme thermophiles are referred.

Key words: Enzymes, thermostability.

Resumen

La capacidad de muchos microorganismos para crecer a elevadas temperaturas ha fascinado a microbiólogos y bioquímicos durante mucho tiempo. Al igual que ocurre con el resto de los componentes celulares, las enzimas de éstos son inherentemente más termoestables que las procedentes de organismos mesófilos. Esta estabilidad térmica no es debida a ninguna característica específica, sino que ocurre como consecuencia de diversos cambios que contribuyen a la estabilidad de forma aditiva. Además de ser más termoestables, estas enzimas son también más resistentes a diversos agentes químicos que sus homólogas mesófilas, lo que las hace interesantes para su aplicación en procesos industriales. A pesar de ello, muchas de las enzimas utilizadas actualmente por la industria proceden de microorganismos mesófilos, debido al desconocimiento existente acerca del aislamiento y crecimiento de microorganismos termófilos a gran escala. El objetivo de esta revisión es discutir brevemente la importancia de la termoestabilidad para la aplicación de las enzimas en la industria y resumir los más recientes avances en la identificación de nuevos organismos termófilos y sus enzimas. Además, se comentan los más recientes sistemas genéticos modelo desarrollados en bacterias termófilas moderadas y extremas.

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Introduction

One of the most amazing properties of the microorganisms is their ability to adapt to extreme environments, in which factors such as pH, temperature, pressure or salt concentration overtake higher values from those regarded as standard for most living organisms (7).

Among all these factors, temperature is the one which has the greatest influence on the functionality of biological molecules and structures. In fact, most of the organisms we presently know can solely grow within a narrow temperature range, usually between 5 to 45 °C, which for this reason is considered as the «normal» temperature range. However, the existence of stable geothermic environments has allowed the selection, or the persistence, of microorganisms that not only resist, but also require higher temperatures for living. These organisms are called «thermophiles». Depending on their growth temperatures range, thermophilic organisms have been arbitrarily divided into three classes (8): «Moderate thermophiles», with optimal growth temperature between 45-65 °C; «Extreme thermophiles» which grow between 65-90 °C; and «Hyperthermophiles», that grow optimally at temperatures over 90 °C.

As it could be expected, the taxonomic diversity of the organisms decreases as the temperature rises (8). Thus, most eukaryotes are mesophiles, but very few of them, restricted to scarce Fungi and Algae, could be included within the «moderate thermophiles». Something similar happens with most prokaryotes, although in this case there are many groups with strains able to grow optimally over 45 °C. Following this reduction of the diversity, only a few groups of prokaryotes could be classified as «extreme thermophiles», being the photosynthetic ones completely excluded from this group, with the exception of *Chloroflexus spp.* (7). Even more, within the "hyperphermophilus", essentially prokaryotes of the Archaea Domain have been described being isolates from the genus *Thermotoga* and *Aquifex* which are included within Bacteria, the exceptions to this rule (20).

The various bacterial metabolism models known in mesophiles also suffer the stringency imposed by high temperatures. Photothrophs are the first to disappear when temperatures rise to 70 °C (limit for *Cloroflexus*). Then, aerobic heterotrophs disappear, with a border line around 85 °C (some *Thermus* isolates), probably due to the low solubility of oxygen at high temperatures. At the end, the most resistant metabolism seems to be that from chemolitotrophic anaerobes, being the limits for Bacteria and Archaea around 95 and 115 °C, respectively.

The question about the upper temperature limit for life has been frequently quoted (5, 7, 8, 10). In 1967 Thomas Brock noted «Bacterial are able to grow...at any temperature at which there is liquid water, even in pools which are above the boiling point.» (5). To confirm this, several attempts were done to isolate hyperthermophilic organisms able to grow over the life temperature limits previously found. Such attemps allowed the identification of submarine thermal environments in which the gradient temperatures available ranged up to 350 °C (10). A number of isolates were obtained from these environments that, as predicted by T. Brock, had an optimal growth temperature above 100 °C (8, 19, 55). However, the fact that no organisms living at temperatures higher than 115 °C (the Tmax of growth for *Pyrodictium occultum*) have been described so far (55, 56), and that the half life of universal biological molecules, like ATP, are extremely short at temperatures around 130-150 °C, makes improbable the isolation of microorganisms more thermophilic than the ones already mentioned. These most probably represent the actual temperature limit for life.

Molecular basis of thermophily

Although thermophilic microorganisms have in their cytoplasm high concentration of thermostabilizing substances such as poliamines (spermine, spermidine, thermine), the essential rule remains that the thermostability lies on the intrinsic properties of each cellular component (8). In this sense, it has

TABLE 1
MAIN ADVANTAGES OF THERMOSTABLE ENZYMES IN INDUSTRIAL PROCESS

Property	Advantages
Thermostability	The half life of the enzymes increases. The purification of the enzymes is easier...
Resistance against various chemical agents	They can tolerate hard conditions including important amounts of organic solvents, diverse pH level frequently necessaries during industrial process.
High optimal temperature	Low activity at room temperature. It does not requiere active cooling in fermentation. High diffusion rates of substrates and products.
Solubility	At high temperatures the concentrations of substrates can be increased, with the exception of gases.
Viscosity	Decreases. Mixing and pumping can be also increased.
Microbial contamination	The probability of contamination decreases as the temperature rises. Contaminant enzymes are inactivated at high temperature.

been known since a long time that thermophilic membranes are built up by lipids containing long, saturated, and methylated forms of fatty acids, which allow the physiological fluidity degree within the temperature growth range of the organism (7). Also, classically a high G+C content in the DNA from thermophiles has been described as a main element for adaptation to thermophily, although the existence of mesophiles with this property (i.e. *Streptomyces spp.*), and the recent description of hyperthermophiles with low G+C content (19) makes the relevance of this factor doubtful.

The role of proteins in the adaptation to thermophily is extremely important. It is very well known that single mutations in the amino acids sequence of a protein could give thermosensitive forms of it, which in many cases makes the organisms unable to grow at the same («restrictive») temperature as its parental strain. However, the converse effect is much less spectacular, since a stabilizing punctual mutation rarely represents more than 3-5 °C in the unfolding critical point of a protein (18). In fact, all current evidence indicates that the enhanced stability of thermophilic enzymes can not be attributed to a single common determinant (i.e. any «special» interaction), but is the result of several amino acids changes with additive effects on the stability with respect to the structure of homologous mesophilic proteins (36).

Industrial applications of thermostable enzymes

Besides cost, thermal sensitivity has been the main obstacle to the extensive use of enzymes in industry (27). For this reason, the most attractive characteristic of the thermophilic microorganisms from a biotechnological point of view is their ability to produce enzymes able to catalyze industrially relevant process at higher temperature than the corresponding enzymes from mesophiles.

Thus, the ability of thermophilic enzymes to work at high temperatures implies many advantages for their applications in industrial reactors (or fermenters). All of these advantages, resumed in Table 1, are directly derived from the exceptional stability of these proteins: i) At high temperature, the viscosity of culture broth decreases, increasing the mass transfer and making easy the mixing and pumping of the substrates. This advantage is extremely important in some of the present applications of the enzymes, as for the hydrolysis of polysaccharides (2, 3, 27, 41). ii) Sterility requirements are not as strin-

TABLE 2.
MAIN PROBLEMS FOR THE APPLICATION OF THERMOPHILIC ENZYMES IN THE INDUSTRY

Property	Main problem observed
Thermal sensitivity	There are may substrates, products or enzyme cofactors unstable at high temperatures.
Solubility of gases	Decrease. The diffusion of gases limits some reactions.
Enzyme stability	The inactivation of the enzyme results extremely difficult.
Equipment Stress	All the materials are damaged in a short time, unless especially designed.

gent as when mesophilic enzymes are used due to the low probability of contamination by thermophilic saprofites. iii) Besides, these enzymes have little activity at low temperature, what allows to stop the reaction just by cooling, and iv) Are more resistant than mesophilic counterparts to denaturing agents (organic solvents, high and low pH) often present in the reactions (18). v) Finally, as many high scale fermentation processes generate heat, the common and expensive cooling of reactors could be avoided if thermophiles or their enzymes were used.

However, despite the many economically important advantages of thermophilic enzymes, there are also disadvantages for specific applications (Table 2) that can be resumed as follows: i) Some cofactors frequently used in the pharmaceutical industry are damaged by heating. ii) The solubility for gases such as oxygen decreases. iii) The irreversible inactivation of enzymes is difficult, and, finally, iv) the materials of the reactors are easily damaged, unless they are especially designed for this work conditions.

Despite the many advantages that thermophilic enzymes have for their routinely use at industrial level the biotechnological applications of thermophiles have been very limited until now. The reasons for this contradiction are many, some of them related with the cost and risk that any new method implies, and others related to the scarce number of thermophilic strains available from official collections (ATCC, DSM) which could be used to screen for specific thermostable enzymes (8, 27). Nevertheless, during the recent years several thermophilic enzymes have been commercialized (27), being the most important from an economical point of view proteases used for the production of detergents.

What could thermostable enzymes be used for? Table 3 shows the actual biotechnological applications of thermophilic bacteria and their enzymes, which in the following section are further commented.

1. Starch hydrolysis

Industrial starch hydrolysis is usually developed in two main steps. Initially, starch is solubilized at 110 °C. After this, it is digested to maltodextrins with an α-amylase commonly obtained from *Bacillus subtilis* and *Bacillus amyloliquefaciens*. Although, the last is moderatelly thermostable these enzymes have to be added twice, before and after the solubilization step. Recently, a more thermostable α-amylase from *Bacillus licheniformis* has been isolated to overcome partially the problem (41), but others still more thermostable are being investigated even at the genetic level (Table 4).

After the treatment with α-amylase, the maltodextrins generated are digested by pullulanase or glucoamylase action to oligosaccharides and glucose. The pullulanase commonly used is purified from *Klebsiella aerogenes*, but its instability over 50 °C implies the rapid cooling of the maltodextrin solution. Furthermore, the cooling also produces an important increase in the viscosity of the reaction mixture with the concomitant yield losses.

TABLE 3.
MAIN APPLICATIONS OF THERMOSTABLE ENZYMES AT PRESENT.

Enzyme	T (°C)*	Application	Origin**
Enz. Acting on Carbohydrates			
α-amylase	60-90	Starch hydrolysis	*Bacillus licheniformis*
pullulanase	50-60	Starch hydrolysis	*Klebsiella aerogenes,*
xylose isomerase	50-55	Sweetening of corn syrups	*Actinoplanes missouriensis*
Cellulase	55-65	Hydrolysis of cellulose, ethanol production, paper bleaching	*Clostridium thermocellum*
Proteases			
Neutral protease	40-80	Food processing	*Bacillus stearothermophilus*
Alkaline protease	40-80	Detergents	*Bacillus licheniformis*
Molecular Biology			
Taq polymerase	45-95	DNA amplification (PCR)	*Thermus spp.*
Vent DNA polymerase	50-98	DNA amplification (PCR)	*Thermococcus litoralis*
Pfu DNA polymerase	50-98	DNA amplification (PCR)	*Pyrococcus furiosus*
Tth polymerase	45-95	Reverse transcription of RNA	*Thermus thermophilus HB8*
RNA polymerase	65-75	RNA synthesis	*Thermus spp.*
Restriction Enzymes	65-75	DNA specific digestion	*Thermus spp. Bacillus, Sulfolobus*
Anaerobic treatment of residual waters	50-60	Organic compounds elimination	Methanogenic bacteria *Metanobacterium, Metanosarcina*

* Range temperature at which the enzyme is used.
** Microorganisms from which the enzyme has been obtained.

Two groups are trying to clone and overexpress in *Bacillus* (2, 23) a pullulanase from the hyperthermophilic Archaea *Pyrococcus woesei* which is so thermostable that can be heated at 110 °C for hours without apparent losses of activity (2). Other pullulanases have been detected in isolates from *Thermus spp.*, *Clostridium spp.*, and *Thermoanaerobacter spp.* It is expectable that their use in this process will increase the final yield of this industry in the near future.

2. Cellulose

The production of sugars from vegetal wastes is one of the main future goals for the food industry. One of the obligate conditions in this process is the necessity for higher temperatures to increase the solubility and the accessibility of the extremely viscous substrate. The enzymes most commonly used come from the mesophilic fungi *Trichoderma sp.* However, there exists cellulase complexes from the anaerobic moderate thermophile *Clostridium thermocellum* (11, 53) that seem to be a good alternative, because it is quite stable at 60 °C. Furthermore, this strain is also able to ferment cellulose directly to ethanol (45) which represents a promising goal for the future.

Another important industrial application of this cellulase or even of other more thermostable ones is their use in paper bleaching for recycling, for which process even higher temperatures are still necessary (90-95 °C). For these last processes the use of other enzymes like xylanases, pullulanases and β-glucosidases seem to be adequate alternatives for it.

TABLE 4.
THE SOURCE OF THERMOSTABLE ENZYMES

Enzyme	Genus from which it has been isolated
Dehydrogenases	
Alanine DH	*Thermus*
Alcohol DH	*Thermoanaerobacter, Sulfolobus; Bacillus*; Thermoanaeromicrobium; Fervidobacterium*
CO-DH	*Carboxydothermus*
Gliceraldehyde 3 P DH	*Thermus*; Methanothermus; Thermotoga*; Bacillus*; Thermoproteus; Pyrococcus**
Glu DH	*Pyrococcus; Sulfolobus; Thermococcus*
Glucose DH	*Sulfolobus*
Isocitrate DH	*Thermus*; Bacillus*
Isopropilmalate DH	*Thermus*;*
Lactate DH	*Thermus; Thermotoga; Bacillus**
Leu DH	*Rhodothermus*; Bacillus**
Malate DH	*Thermus*; Sulfolobus; Thermoplasma; Methanothermus; Chlorobium; Chloroflexus; Bacillus*
NADH DH	*Thermus; Bacillus; Sulfolobus*
Other oxidoreductases	
Cytochromes	*Thermus*; Hydrogenobacter*
Hydrogenase	*Calderobacterium; Methanococcus; Pyrococcus*; Desulfurolobus; Bacillus*
Malic enzyme	*Sulfolobus*
Peroxidases	*Thermus; Thermomicrobium; Thermoleophilum*
Hydrolases	
Acetyl esterase	*Thermoanaerobacter*
Alkaline phosphatase	*Thermus; Flavobacterium*
α-amylase	*Bacillus*; Pyrococcus*; Thermus*; Rhodothermus; Thermococcus; Thermotoga*
β-amylase	*Clostridium**
Aminoacylase	*Bacillus*
Aminopeptidase	*Thermus*; Sulfolobus*
Arginase	*Thermus; Bacillus; Sulfolobus*
L-Asparaginase	*Thermus*
D-Asparaginase	*Thermus*
ATPase	*Thermus; Sulfolobus*
Carboxyesterase	*Bacillus; Archaeglobus*
Carboxypeptidases	*Thermus*
Cellulases	*Clostridium*; Dictyoglomus; Caldocellum**
Endo-β-glucanase	*Thermoanaerobacter**
Esterases	*Sulfolobus; Bacillus; Unidentified submarine isolates*
α-Galactosidase	*Thermus**
β-Galactosidase	*Sulfolobus*; Thermus*; Thermoanaerobacter; Thermococcus; Desulfurococcus*
α-Glucosidases	*Bacillus; Thermococcus*
β-Glucosidases	*Caldocellum; Thermus; Thermococcus; Thermotoga; Pyrococcus; Clostridium*
α-Glucuronidase	*Thermoanaerobacter*
Lipases	*Bacillus*; Pyrobaculum; Thermus*
Proteases	*Bacillus*; Sulfolobus*; Thermus*; Desulfurococcus; Thermococcus*
Pullulanase	*Thermus; Thermoanaerobium*; Clostridium; Pyrococcus*; Thermococcus; Bacillus*

Pyrophosphatase	*Thermus; Bacillus; Sulfolobus*
Ribonucleases	*Thermus**
Restriction Endo	*Thermus*; Sulfolobus; Bacillus; Rhodothermus; Thermotrix*
Transglucosylase	*Sulfolobus; Desulfurococcus; Thermotoga; Thermococcus*
Xylanase	*Bacillus; Thermotoga; Thermonospora; Rhodothermus*; Caldocellum**
β-Xylosidase	*Caldocellum**
Transferases	
Ala-Amino Tase	*Bacillus; Thermus, Pyrococcus*
Asp Amino Tase	*Sulfolobus*; Bacillus; Thermus, Pyrococcus*
Asptranscarbamylase	*Thermotoga, Thermus*;* Unidentified hyperthermophilic strain
DNA polymerase	*Thermus*; Thermotoga; Sulfolobus; Bacillus; Pyrococcus*; Clostridium; Thermoactinomyces; Scotothermus; Methanobacterium*
5'Methyl Thioadenosine Phosphorilase	*Bacillus; Thermotoga; Sulfolobus; Pyrococcus*
Phe-Amino Tase	*Thermus; Bacillus*
Phosphofructo Kinase	*Thermus; Bacillus; Flavobacterium*
Propylamino Transferase	*Sulfolobus*
Pyruvate kinase	*Thermus; Thermoplasma*
RNA polymerase	*Thermus; Sulfolobus; Thermoproteus; Desulfurococcus; Methanobacterium; Methanothermus; Methanococcus*
Tetrahydromethanopterin Met Tase	*Methanobacterium*
tRNA methyl Tase	*Thermus*
Lyases	
Aldolase	*Thermus*
Citrate Syn	*Sulfolobus; Thermoplasma**
Fumarate Hydratase	*Bacillus*
Threonine deanimase	*Thermus*
Urease	*Unidentified isolates from Yellowstone*
Ligases	
Aminoacyl tRNA Syn (Phe, Gle, Val, Ser, Thr)	*Thermus; Bacillus**
CarbamoilPSyn	*Thermus**
DNA ligase	*Thermus*; Rhodothermus*; Scotothermus**
Glutamine Syn	*Bacillus; Pyrococcus*
Trp Syn	*Thermus**
Isomerases	
Protein disulphide isomerase	*Sulfolobus*
Reverse gyrase	*Sulfolobus*
Triose3P Isomerase	*Methanothermus*
Xylose (Glucose)isomerase	*Thermus*; Themotoga; Bacillus*

The asterisk (*) indicates those genera fron which the corresponding gene has been cloned.
The data shown in this table are taken essentially from references (3, 9, 27, 41) and from the abstracts book from the 1992 FEMS Symposium «Thermophiles: Science and Technology», held in August 1992 in Iceland.
The enzyme abbreviations used are:
DH: Dehydrogenases.
Tase: Transferases.
Syn: Synthetases.
Endo: Endonucleases.

3. Xylose-Isomerase

Another important application of thermophilic enzymes in the food industry is the production of sweet corn syrups by increasing their fructose content. Fructose –the sweetest natural sugar– is usually obtained from glucose by a very expensive chemical process. However, the discovery that xylose (glucose)-isomerase catalyzes this reaction allowed the development of reactors with the enzyme immobilized to solid substrate (Aluminium oxide) (22). The continued addition of corn syrup to the reactor allows the obtention of a sweeter syrup with approximately 52% of fructose and 48% of glucose. As corn syrup is quite viscous, the conversion reaction must be done at least at 60-65 °C, limiting the half life of the mesophilic enzyme. Even more, it has been calculated that the fructose yield could be increased to 55-60% by rising the temperature of the process in 10-15 °C. Taking in mind that the market of this product represents about $8 billions per year (48), such increase in the yield results extremely interesting. To allow this, thermostable mutants of this enzyme have been obtained by site directed mutagenesis (47, 48), although the recent identification of homologous activities on isolates from *Thermus* (stable in solution at 70 °C during 1 month!), *Thermotoga* and thermophilic *Bacillus*, will probably substitute the preceding enzymes (12, 13, 60).

4. Proteolytic enzymes

Proteases represent by far the most world sold enzymes, as they constitute about 2/3 in volumen from the whole enzyme market. The reason is their «classical» use as components of dairy and industrial detergents (27, 41). The most commonly used protease is the alkaline protease from *Bacillus licheniformis*, although its limited thermostability makes desirable its substitution by others from more thermophilic microorganisms (14, 54). However, during the last years several new proteases of elevate thermostability have been identified and even cloned (57).

There is also interest for the identification of thermophilic proteases with high specificity of cleavage in order to be applied in research biotechnology. In this sense, the recent description of aminopeptidases and a procesive carboxypeptidase from *Thermus* which could be used for the direct sequencing of proteins are very good examples (33).

5. Molecular biology

The application of thermophilic enzymes in recently developed methods for molecular biology, such as the spectacular example of the PCR, has supposed a revolution on the genetic engineering only comparable with the appearance of restriction endonuclease. All the DNA or RNA manipulations that were difficult because of secondary structures are now overcame with the use of RNA and DNA polymerase from thermophilic microorganisms (32, 40, 50). In fact, DNA polymerases from hyperthermophiles have been cloned recently (35).

6. Synthesis of special compounds

Although actually in the theoretical field, thermophiles are potential sources of new compounds and applications in various fields:

Vitamin A production: Carotenoids have a considerable utility as colouring in the food industry and as precursors of vitamin A (61) to which α and β-carotenoids can be converted with an stequiometry of 1:1 and 2:1, respectively. Until now, the best known microorganism from which carotenoids could be purified was *Blakeslea trispora*, but it needs a very complex medium to grow. An alternative

microorganism without this exigency is *Thermus aquaticus*, in which about 80% of the total content of the lipids are carotenoids.

Amino acids production and detection: Aminoacil dehydrogenases from *Bacillus sphaericus* have been used to synthetize L-leucine and L-alanine. Recently it has been discovered that homologous enzymes purified from *Bacillus stearothermophilus* and expressed in *E. coli* (42) are more active. Besides, these enzymes could be used in amino acids detection kits after a thermal inactivation step of the rest enzymes of the sample.

New antibiotics: Most of the antibiotics already produced are secondary metabolites from *Actinomycetes* (mainly *Streptomyces*) that have been identified in screening programs of pharmaceutical companies. However, most (if not all) of the screening programs have been directed to isolate mesophilic organism with optimal growth temperature about 30 °C while it is now clear that there are several «new» thermophilic strains in thermal environments. In a few cases it has been shown how members of the thermophilic Actinomycetes (*Thermoactinomycetes* mainly) are able to syntetase products with antibiotic activity like thermomicine, thermothiocine, or thermorubin (61). Although few products have been carefully analyzed, they seem to be exclusive of these thermophilic strains, and may represent in the future the reservoir for new antibiotics families.

7. Waste treatment

In the anaerobic treatment of residual water, the insoluble organic material is transformed in methane, which is insoluble and escapes from the fermentative process. This treatment is carried out essentially by bacterial consortia in which the methanogens may represent the limiting factor in the development of thermophilic reactors, although it has been described high yield reactors working at 50-60 °C (62). The principal advantages of these treatments are the increase in the reaction rate, the decrease of the substrate viscosity, and the elimination from the water of mesophilic pathogens (8).

The source of thermostable enzymes

The general feeling exists between the «thermophiles hunters» that most of the bacteria living in natural hot springs are still unknown due to the isolation methods used so far (8, 9, 27). This means that new isolation method most probably will be recompensed with the identification of new bacteria carrying new enzymes. A demonstration for this has been the recent private and official international programs that have allowed the identification and in many cases purification and analysis of potentially interesting enzymes from thermophiles (i.e. European Community Programs). As a consequence of these programs, not only many thermophilic enzymes, but also many new species have been isolated. In Table 4, we have tried to collect data about the thermophilic enzymes analyzed and the bacterial genus from which they have been obtained. It is important to note here that many of the isolates listed in the table still have not a validated name from a taxonomic point of view, although their physiology, and in some cases their genetics suggest them to be new genus.

Although the most important, thermostability is not the unique characteristic needed for the effective industrial use of a specific enzyme. It is also necessary a cheap high scale production of the enzyme. This is one of the main problems when the enzyme comes from thermophiles due to the special growth conditions that most of them could requiere , especially the hyperthermophiles. The last are especially fastidious to grow because conditions such as anaerobiosis (Methanogens), or acidity (Thermoacidophiles), have to be added to high temperatures, making their high yield production extremely difficult, although not impossible (2). To circumvent such problems the election system is the cloning

and expression of the gene responsible for the synthesis of the enzyme in a mesophilic host which should be easy to grow (ie: *E. coli* or *Bacillus subtilis*).

Different enzyme genes from thermophilic microorganisms have already been cloned in *E. coli*, such as those indicated with an asterisk in Table 4 (1, 4, 21, 31, 37, 46, 52). The production of the corresponding enzyme in these mesophiles has been attempted in general with diverse success. Nevertheless, differences in promoter structure or in codon usage between *E. coli* and the producer strain have decreased sometimes the expected enzyme yield. With the aim to overcome these problems, as well as those derived from the scarce knowledge about the genetics and molecular biology of thermophiles, our group (16, 17, 28, 29, 30) and others (34, 36, 49) have focussed their research project on the development of genetic tools and technics to clone, express and overproduce the thermophilic enzymes in phylogenetically related thermophilic hosts.

Two models have been successfully studied with this purpose: *Bacillus stearothermophilus* (34) and *Thermus thermophilus* (30). Both are thermophilic, although covering different temperature ranges. While *B. stearothermophilus* grows between 45 and 70 °C, *Thermus thermophilus* does between 55 and 85 °C. This allows a very important application of thermophilic genetics: The use of both systems in a sequential way to increase the stability of mesophilic enzymes, through a «step by step» direct genetic selection system. This has been described for a kanamycin nucleotidil transferase (34) and for a chloramphenicol acetyl transferase genes (58).

In addition to its extreme thermophilic character the selection of *Thermus thermophilus* as the future thermophilic genetic engineering model system was based in a series of advantages:

1. This genus, described by Brock in 1969, includes a high variety of strains (thousands of isolates) from moderate to extreme thermophiles. In fact, different strains have been isolated from widely distributed geothermal areas along the world (8), like Japan (43, 51), Iceland (44), Russia (15), New Zealand (3), USA (6, 39), etc... Among them, there exists a considerable phenotypic diversity, which by numeric taxonomy allowed their grouping in 6 to 7 clusters. Such diversity is especially represented by the many enzymes of industrial potential relevance detected in different *Thermus* strains (Table 4), which would be expressed in *T. thermophilus* from their own genetic signals.

2. It can grow fast (generation times about 35 min) in liquid medium under aerobic conditions, yielding a high number of cells per ml. It can also grow in plates as isolated colonies, what makes possible the selection of mutants. Therefore, only minor changes are necessary with respect to the classical plating techniques: The agar (1.5-2%) plates should be incubated at 70 °C into a water saturated atmosphere.

3. This strain can be transformed with external DNA with a reasonable efficiency without special treatments to make «competent cells» (25, 29).

4. Most *Thermus* strains carry natural cryptic plasmids that could be used for the development of cloning vectors as it has been demonstrated recently (38, 49, 59). In additon, there are also strains like *Thermus thermophilus* HB27, free of them (25, 26), what avoids compatibility problems.

5. The development by our group of a thermostable kanamycin resistance cassette actually allowed not only the construction of positively selectable plasmids, but also the possibility to isolate insertional mutants in a directed way, which will help to the improvement of the genetic analysis in *Thermus* (29). Furthermore, the recent development by our group of a secretion vector (30) will allow us to produce thermostable enzymes directly in the culture medium of this strain.

Concluding remarks

There exists a high diversity of thermophilic enzymes whose application in industrial processes could improve them extraordinarily. Once identified the enzyme of interest from a thermophile, it its

necessary to produce it in a cheap way, namely by cloning and expressing in a suitable host. Although the mesophilic host systems make the cloning process easier, the yield of the expression could be increased if a more natural host were available. In this sense, new moderate and extreme thermophiles host systems are being developed, although scarce expression examples are actually available. However, the improvement of the systems during the coming years are expected to overcome the actual problems. Furthermore, when no homologous thermophilic counterpart could be found, the genetic systems for thermophiles will also be useful to thermostabilize any mesophilic enzyme simple by selecting sequentially at higher temperatures.

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The proteolytic system of lactic acid bacteria

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Summary

Lactic acid bacteria are widely used throughout the world, empirically or deliberately, in the manufacturing of several food and feed stuffs, including milk products (such as cheese, butter, yoghurt, buttermilk, etc.), fermented vegetables (pickles, olives and sauerkraut), sausages, sourdough bread and silage, due to their ability to convert sugars into lactic acid.

Of these, dairy products are of outstanding economic importance. Starter cultures used in the dairy industry are mixtures of carefully selected lactic acid bacteria which are added to the milk to fulfil the desired fermentation. Dairy starter cultures must reach high densities in milk in order to produce lactic acid at the required rates for manufacturing. Under these conditions, amino acids supply becomes limitant due to their scarce concentration in milk and to the auxotrophies shown by many starter bacteria. This implies the necessity of a proteolytic system, able to degrade the most abundant protein in milk, casein, into assimilable amino acids and peptides. Casein degradation and utilization require the concerted action of proteinases, peptidases and amino acid and peptide uptake systems. This whole set of enzymes constitutes the proteolytic system.

In this article an overview of the recent biochemical and genetic data on the proteolytic system of lactic acid bacteria will be presented.

Key words: Proteolytic system, proteinases, peptidases, amino acid and peptide uptake systems, lactococci, lactobacilli, lactic acid bacteria.

Resumen

Las bacterias lácticas se utilizan en todo el mundo, de forma empírica o deliberada, en la elaboración y fabricación de diversos productos para la alimentación humana o animal, incluyendo productos lácteos (como quesos, mantequilla, yogurt, leches ácidas, etc.), vegetales fermentados (encurtidos, aceitunas, etc.), embutidos, panes ácidos y ensilados, debido fundamentalmente a su habilidad para convertir los azúcares presentes en la materia prima en ácido láctico.

De estos productos los más importantes, desde el punto de vista económico, son los productos lácteos. Los cultivos iniciadores o fermentos utilizados en la industria láctea son mezclas de bacterias lácticas cuidadosamente seleccionadas que se añaden a la leche para llevar a cabo la fermentación deseada. Los cultivos iniciadores deben de alcanzar en leche altas densidades celulares para producir ácido láctico a la velocidad deseada para cada tipo de fabricación. En estas condiciones, el suministro de aminoácidos se convierte en limitante debido a su baja concentración en leche y al grado de auxotrofía que muestran las bacterias presentes en los cultivos iniciadores. Esto implica que las bacterias de los ini-

ciadores han de poseer sistemas proteolíticos capaces de degradar la proteína más abundante en leche, la caseína, a péptidos asimilables y/o aminoácidos libres. El proceso de degradación y utilización de caseína necesita de la acción concertada de proteinasas, peptidasas y sistemas de transporte de aminoácidos y péptidos. Este conjunto de enzimas forma lo que denominamos el sistema proteolítico.

En este artículo se hace una revisión de los datos más recientes sobre la bioquímica y la genética del sistema proteolítico de las bacterias lácticas.

Introduction

Lactic acid bacteria are those producing lactic acid as the major end product from the carbohydrates fermentation. The term includes several unrelated bacteria, but the most typical are grouped in the families *Streptococcaceae* and *Lactobacillaceae*, being the most important genera *Lactococcus, Leuconostoc* and *Pediococcus*, in the first family, and *Lactobacillus* in the second.

Starter cultures mainly consist in various species of lactic acid bacteria which are carefully selected and deliberately added to many fermentation processes to initiate and carry through the desired fermentation. The economic value of the processes in which lactic acid bacteria are involved is broadly accepted. Moreover, the modern manufacturing production techniques, which consider of outstanding importance standardization and product quality, envisage starter cultures as absolutely indispensable.

Dairy products are the most abundant and economically important of the different fermented products. Milk can be transformed into well over a thousand products as a consequence of controlled manufacturing environments, which includes the microorganisms contributing to flavour and texture of the finished products (112).

The primary technological requirement of most starter cultures is the production of lactic acid and volatile flavoured compounds. Furthermore, bacterial growth contributes to proteolysis and lipolysis of the raw material and, at the same time, in the inhibition of pathogenic and spoilage microorganisms. In this way, starter cultures are essential in the configuration of the organoleptical, rheological, shelf life and safety properties of fermented products.

To carry over all these functions, starter cultures must reach high densities in milk. For an optimal growth in milk, lactic acid bacteria depends on lactose, as a carbon source, and on the presence of small peptides and free amino acids, as a nitrogen source. Many of these amino acids are either stimulatory or essential for growth (73, 109). In raw milk, the concentration of free amino acids and small assimilable peptides, however, is only enough to support lactic acid bacteria growth to cell densities corresponding to 25% of those found in fully-grown milk cultures (87, 133, 134). Bovine milk contains 3.0-3.5% (w/w) of protein, about 80% of it consists of α_{s1}-, α_{s2}-, β-and k-casein. Their abundance, as well as their conformation, make caseins the major organic nitrogen source for lactic acid bacteria to grow in milk.

This review is devoted to the latest biochemical and genetic studies on the proteolytic system of lactic acid bacteria, a critical factor in the applied industrial use of these microorganisms. The reader's attention is also drawn to the recent excellent reviews dealing with several aspects of this subject (65, 67, 68, 70, 75, 106, 123, 131, 133, 134).

The proteolytic system of lactic acid bacteria

The majority of the biochemical and genetic studies on the proteolytic system of lactic acid bacteria have historically been done in strains of *Lactococcus*. However, interest in the proteolytic systems

of lactic acid bacteria other than lactococci has been increasing over the last few years due to their potential applications in the food industries.

The general scheme of casein degradation by lactic acid bacteria will include: (i) extracellular proteinase(s), (ii) extracellular peptidases, (iii) amino acids and peptide transport systems, and (iv) intracellular peptidases. All these enzymes together form the proteolytic system of lactic acid bacteria.

Proteinases

The cell envelope proteinase of lactococci

The cell envelope-attached caseinolytic proteinase is the first enzyme in the pathway of casein degradation. Due to the technological importance of casein breakdown, numerous studies on the lactococcal proteinase have been carried out in the last few years, and its biochemical, immunological and genetical properties have been determined (67, 70, 75).

First biochemical studies on lactococcal proteinases showed that the enzymes from several *Lactococcus lactis* strains were (i) high molecular weight proteins (molecular sizes ranging from 80-145 kDa), (ii) having a pH optimum close to 6.0, (iii) Ca^{++}-ions, in low concentration, either activated or stabilized activity, and (iv) were inhibited by phenyl methylsulphonyl-fluoride (PMSF) of diisopropyl-fluorophosphate (DFP), both specific inhibitors of serine-type proteinases (134).

The proteolytic activity was cell associated in all the cases, except in *L. lactis* subsp. *cremoris* ML1, and could be partially or wholly released from the cell by incubation in a Ca^{2+}-free buffer (70, 132) or by lysozyme treatment (20).

In whole cells of several *L. lactis* subsp. *cremoris* strains two acidic activities could be distinguished (referred to as P_I and P_{III}), differing in temperature optima (30 ºC for P_{III} and 4 ºC for P_I) (35). Based on the different action of purified proteinases on bovine caseins two enzyme specificities could be detected: an HP-type (from *L. lactis* subsp. *cremoris* HP) (36,140) and an AM1-type (from *L. lactis* subsp. *cremorsis* AM1) (39, 142). The HP-type, which acts primarily on β-casein, corresponded to the P_I-type of activity and was found to be the unique type in the cell envelope of *L. lactis* subsp. *cremoris* HP, FR, AC1, C13, Wg2, and H2 (45, 92, 141). The AM1 –type which acts on α_{s1}– and β-casein, and shows different electrophoretic pattern of β-casein digestion from that obtained with the P_I-type, corresponded to the P_{III}-type and was found in *L. lactis* subsp. *cremoris* SK11 and AM1 (45, 92, 107, 141). *L. lactis* subsp. *cremoris* FD27 and E8 (141) and *L. lactis* subsp. *lactis* NCDO 763 (94) and UC 317 (76) all have intermediate patterns between the P_I- and P_{III}- types. Both proteinase types act, in a lesser extent, on α_{s2}- and k-casein (10, 94, 141). Maximal growth of *L. lactis*, in chemically defined media, is supported by a combination of β-casein and a low concentration of k-casein (37).

The analysis of the cleavage sites in β-casein by P_I- (10, 45, 91, 92, 93, 108, 141) and P_{III}- type proteinases (45, 92, 108, 142) indicated that the specificity of both proteinases towards β-casein is rather broad, and no particular amino acid residues or consensus sequence could be identified. However, glutamine and serine residues are found most frequently at the cleavage sites. Characteristically, they lie in regions of the β-casein molecule having a high hydrophobicity, high proline content, and low charge (93).

Polyclonal antibodies against purified proteinases of a number of *L. lactis* subsp. *cremoris* strains identified four proteins (A, A', B and C) as part of the proteolytic system of these strains. Proteins A and B have been shown to be proteolytically active. Component A is present in all strains tested and is always accompanied by at least one of the other three proteins. Proteins A and A' share common antigenic determinants, as do B and C (50). These studies were lately confirmed using monoclonal antibodies (69).

Biochemical and immunological data led to the conclusion of an apparent complexity of the cell wall-attached proteinase system in different strains of *L. lactis*, until genetic studies of proteinases and the corresponding genes were undertaken.

Some 15 years ago, it became clear that lactococci harboured several plasmids in which genes coding for some technologically important metabolic traits were present (21, 83). Plasmid location often makes these activities unstable, which consequently may be lost. In most lactococci strains the production of proteinase was associated to plasmids (65, 82, 100). Proteinase negative variants (Prt^-) show a reduced acid production and are unable to grow in milk up to the high cell densities necessary for good fermentation processes.

The cloning and sequencing of the genetic determinant of the proteinase from *L. lactis* subsp. *lactis* Wg2 was a milestone in the elucidation of the complex lactococcal proteinase system (63). In this strain, the loss of a 26 kbp plasmid was accompanied by loss of the proteolytic ability (102). A fragment of this plasmid was inserted in a lactococcal cloning vector and transferred to a proteinase-deficient *L. lactis* subsp. *lactis* strain. The strain thus obtained expressed a functional proteolytic activity and was able to grow normally in milk. With immunological techniques, it was shown that the cloned genetic determinant specified proteins A and B of the proteolytic system of Wg2 (63).

By now, a number of proteinase genes from different lactococcal strains have been cloned and sequenced [among others, *L. lactis* subsp. *cremoris* Wg2 (64), *L. lactis* subsp. *cremoris* SK11 (145), and *L. lactis* subsp. *lactis* NCDO 763 (61)]. Comparison of the nucleotide sequences showed that the genes were highly conserved between strains, with an overall homology of 98% on the nucleotide and amino acid levels. The Wg2 and the SK11 proteinases differ in only 44 amino acids (from a total of 1902) (67). The most prominent difference between the two proteinases is a duplication of 60 amino acids near the C-terminus of the SK11 enzyme. However, these small differences result in different caseinolytic activities (P_I-type in Wg2 and P_{III}-type in SK11) (67). The Wg2 and the *L. lactis* subsp. *lactis* NCDO 763 proteinases seem to be much more similar and only differ in 18 amino acids.

Only one gene has been observed in the strains having a specificity pattern intermediate between the P_I- and the P_{III}-types (61, 67, 76). The nucleotide sequence was indeed intermediate between the sequences of the P_{III}-strain SK11 and the P_I-strain Wg2 proteinase genes (61, 76). Recently, hybrid P_I/P_{III} proteinase genes have been constructed to investigate which of the amino acid substitutions contributed to the differences in specificity. Two regions involved in cleavage specificity were identified. One of them, in the N-terminal portion, comprises seven amino acid differences. In the other region, located in the C-terminal region, at least two amino acid residues are involved. Interestingly, some of the hybrid proteins had a specificity of casein degradation different from either of the wild-type enzymes (146).

Protein homology comparisons indicated three regions in the lactococcal proteinase which have extensive homology with serin proteinases of the subtilisin family, recently referred to as subtilases (117); especially, around the amino acids surrounding the active centre (viz. Asp 32, His 64, and Ser 221 of the subtilisin molecule) (61, 64, 67, 145). The homologous sequences are separated by stretches of amino acids not present in subtilases. As subtilisins, the lactococcal proteinase is synthesized as a pre-pro-enzyme. At the N-terminus a typical signal peptide-like sequence of 33 amino acids is present and the pro-region consits of 154 amino acids in all the cloned proteins (61, 64, 145).

A clear difference between subtilisin and the lactococcal proteinases is their location. While subtilisin is fully secreted into the medium, the proteinase activity of lactococci is associated with the cells (19, 35, 44, 50, 51, 69, 132). At the extreme C-terminal of the lactococcal proteinase a region was identified which shows homology with membrane attachment sites of proteins of Gram-positive bacteria (61, 64, 145). Removal of the C-terminus domain from the proteinases results in the release of the proteins from the cells (66).

A second open reading frame (ORF) is present immediately upstream of the proteinase gene which is faced in the opposite direction. This ORF has been found in all the proteinase gene regions sequenced so far and is completely conserved (46, 61, 144). Deletion analysis has shown that the ORF is essential for proteolytic activity (46, 144). The two genes were consequently renamed as *prtP* and *prtM*, respectively. Cells carrying an intact proteinase gene but lacking *prtM* synthesize and correctly locate the proteinase but they are proteolytically negative (46, 144). *PptM* is a membrane-located lipoprotein which is, in a way not yet understood, involved in the activation of the proteinase (45).

The available genetic data, gathered in recent years, have allowed a reinterpretation of the former biochemical and immunological data and a working model for the lactococcal proteinase has been elaborated. The first important conclusion is that the extracellular serin proteinases of lactococci are all very similar enzymes which differ only by a few amino acids. The small amino acid differences divided the proteinases in two principal classes according to their specificity (P_I-type and P_{III}-type). The second new concept is that the proteinases are attached to the cells via a membrane anchor which is present in the C-terminal end of the molecules. Removal of this sequence results in the release of the proteinases. The third aspect of the model indicates that a closely linked gene, *prtM*, is essential to obtain a functional proteinase. In this respect, the lactococcal proteinases differ from subtilases which perform an autocatalytic activation.

To reconcile the biochemical and genetic work with genetic data, a sequential proteinase degradation model was postulated. The lactococcal proteinase is synthesized as a pre-pro-protein. The size of the proteinase of *L. lactis* subsp. *cremoris* Wg2, as deduced from its nucleotide sequence, is 200 kDa. Removal of the signal peptide sequence and the pro-region would result in a mature enzyme with a molecular size of 187 kDa. However, the size of the purified proteinases from various strains range between 80 to 165 kDa, and all these enzymes start at the N-terminus with Asp. 188. Apparently, large C-terminal deletions can occur without abolishing proteinase activity or specificity. In the proposed model Ca^{2+}-ions are thought to play an important role in proteinase degradations as they can reduce the proteolytic activity at concentrations higher than 10 mM or enhance this activity at lower concentrations. The preferred method of protein isolation, the «release» of the enzyme from the cells in a Ca^{2+}-free buffer, is considered to result in autocatalytic degradation (71). The protein degradation model would explain the occurrence of four immunologically distinct proteins.

Proteinases in lactic acid bacteria other than lactococci

Very little data are available on the presence of proteinases in species of lactic acid bacteria other than lactococci, and most of the work refers to the presence and quantitative assessment of enzyme activity. Proteinases have been detected in *Lactobacillus helveticus, Lb. delbrueckii, Lb. paracasei* (formerly, *Lb. casei*), *Lb. acidophilus* and *Lb. plantarum*.

The enzymes from *Lb. acidophilus* (cited in, 67) and *Lb. helveticus* (151) have been isolated and characterized, and their biochemical properties resemble those of the lactococcal counterpart. Both proteinases can be liberated from the whole cells by washing in Ca^{2+}-free buffer. The molecular size of these enzymes was estimated to be in the range of that of the lactococcal proteinases (approximately 140 Kda). Furthermore, both enzymes were severely inhibited by PMSF and DFP, indicating that they are serin-type proteinases. The low molecular mass peptides released from α- and β-caseins by the proteinase of *Lb. helveticus* have been identified (151). Some of the cleavage sites are the same as those hydrolysed by the lactococcal proteinase but some are different. However, there appear to be different specificity types among the proteinases isolated from different strains of this species (59).

The most intensively studied proteinase systems among lactobacilli is the one of *Lb. paracasei* subsp. *paracasei*, which shows many features similar to that of the lactococci. The enzyme is cell en-

velope-associated and has been shown to be released by washing the cells in Ca^{2+}-free buffer or by lysozyme treatment (16, 33, 58, 62, 97). However, the gene has been located in the chromosome. It encodes a polypeptide of 1902 amino acids, and the inferred protein shows extensive homology to the *L. lactis* subsp. *cremoris* Wg2 proteinase. Similar to the situation in *Lactococcus*, a maturation gene was found upstream of that of the proteinase (49). As with *Lib. helveticus*, there is contradictory evidence on the specificity of the proteinases from different strains of this species (58, 62).

The proteinase from *Lb. plantarum* has been found to have similar properties to the *Lb. paracasei* enzyme (33), although again there is conflicting evidence on its specificity (16, 33, 58). In *Lb. delbrueckii* subsp. *bulgaricus* a multiplicity of proteinase forms have been reported and further work is required to solve this apparent complexity (41, 72).

Streptococcus thermophilus is generally regarded as being less proteolytic than lactococci or lactobacilli. However, the proteinases from the scarce proteinase positive strains share a number of properties with those from lactococci, including cell-envelope location, pH and temperature optima, activators and inhibitors (53, 115, 116). The most relevant difference is that no release of proteinase activity from the cells occurs in Ca^{2+}-free buffer. Both α- and β-caseins are hydrolysed by the *S. thermophilus* proteinases, but β-casein is the preferred substrate (116).

Regarding other lactic acid bacteria, all strains of *Micrococcus* analysed so far have been found to be proteinase positive, degrading both β-and, to a lesser extent, α_{s1}-casein (8, 43, 105). Proteinases from *Micrococcus* have been classified as metalloproteinases. *Micrococcus* GF has been found to possess two extracellular proteinases differing in molecular size, pH optimum and sensitivity to EDTA inhibition (43). *Enterococcus faecalis* appears to possess a 35 kDa metalloproteinase with high homology to the *Bacillus* neutral proteinase (78, 125). Caseinolytic activity has been detected in bifidobacteria (34), and in crude extracts from various strains of *Leuconostoc mesenteroides, Lc. lactis* (31), and pediococci of dairy origin (9).

Peptidases

The specific peptide transport system of lactic acid bacteria was soon recognized to present a size restriction for peptides between 4 and 6 amino acid residues (74, 87, 110, 113). These peptides are much smaller than the majority of those generated from caseins by the proteinases (10, 91, 93, 108, 141, 142). Thus, further hydrolysis is necessary to convert the relatively large peptides produced by the proteinases in to smaller assimilable products. This second step in the casein breakdown is taken over by several peptidases.

First reports about peptidases in lactic acid bacteria described general information on various peptidase activities in cell-free extracts (1, 23, 32, 48, 54, 95). The use of cell-free extracts, even when they are fractionated, may result in the failure to resolve distinct peptidase activities. Today, with the improvement of separation techniques, the detailed biochemical characterization always follows to an initial isolation and purification of single peptidases.

Many different peptidases have now been purified in lactic acid bacteria, including general aminopeptidases [aminopeptidase N (2, 3, 12, 24, 40, 57, 79, 89, 126, 136], aminopeptidase C (99, 148), aminopeptidase A (38, 101)], di-peptidases (52, 139, 147), tri-peptidases (7, 15), prolyl iminopeptidases (6), prolidases (13, 55), X-prolyl dipeptidyl aminopeptidase (4, 11, 14, 30, 56, 60, 77, 84, 88, 152), and endopeptidases (127, 149, 150). All these peptidase types have been found in lactococci. The general picture of the peptidase complement in lactic acid bacteria is far from clear; even less is known of the peptidases involved in the casein oligopeptide breakdown. An even more complex and confusing picture exists in lactic acid bacteria other than lactococci, where there seems to be a large number of different peptidases present in any one strain, which, of course, might reflect the complexity of the pep-

tidase system in these bacteria (31, 34, 48, 75, 106, 133, 134). Part of this complexity, however, is caused by the variety of substrates used to characterize the enzymes by different authors, which makes it difficult to compare peptidases from different strains. Carboxypeptidase activities have only been described in strains of *Lb. plantarum* and *Lb. casei* (1). Much more work need to be done on the properties of peptidases, specificity, distribution and cellular location before their role in the proteolytic pathway can be clearly assesed.

The cellular location of peptidases has been a matter of discussion in recent years since extracellular enzymes are necessary to continue the action of the proteinase. Despite many attempts, none of the above-mentioned peptidases has been unequivocally assigned to an extracellular location. In a recent study the location of five different peptidases (aminopeptidase N and C, an X-prolyl dipeptidyl aminopeptidase, an endopeptidase and a tripeptidase) was detected by polyclonal antibodies. All five peptidases were detected in protoplast extracts even after proteinase K treatment (128). These results are consistent with an intracellular location. More recently, a cell wall-bound peptidase has been purified from a *L. lactis* subsp. *cremoris* strain. The peptidase, a trimer composed of identical subunits of 24 kDa, is able to hydrolyze di- and tripeptides, with a preference for those containing leucine (114).

In lactococci, the majority of the peptidase activity is largely based upon the action of the general aminopeptidases, X-prolyl dipeptidyl aminopeptidase and glutamyl aminopeptidases.

X-prolyl dipeptidyl aminopeptidases

As the proline content of casein is high (16,7% for β-casein), the casein-derived products generated by the proteinase are rich in proline (93, 140, 142). Most peptidases are not able to release N-terminal or penultimate proline residues. To degrade these proline-rich oligopeptides, lactic acid bacteria depend on proline-specific peptidases. Aminopeptidase P, proline iminopeptidase, iminodipeptidase (prolinase), imidodipeptidase (prolidase), and X-prolyl dipeptidyl aminopeptidase, have all been detected in the various genera of lactic acid bacteria (48, 54, 95).

The X-prolyl dipeptidyl aminopeptidases [or PepX, as the new nomenclarure proposed in (131)] hydrolyze specifically X-Pro-Y-... containing peptides by the release of the N-terminal X-Pro dipeptide. This peptidase has been detected in a vast array of lactic acid bacteria including strains of lactococci and lactobacilli (17, 32, 48, 54, 95, 133, 134). The enzyme has been isolated and characterized from several species (4, 11, 14, 30, 56, 60, 77, 84, 88, 152). The native enzymes are dimers with identical subunits of 80-90 kDa. Interestingly, the X-Pro dipeptides produced by PepX are high affinity substrates for the essential di-tripeptidase transport system of lactococci (118, 120). Furthermore, the intracellular prolidase of *L. lactis* is highly specific for X-Pro dipeptides. The prolidase has been recently purified from a *L. lactis* subsp. *cremoris* strain and presents a molecular size of 43 kDa. The prolidase is inhibited by specific metalloproteinase inhibitors (55).

The first peptidase genes characterized at the nucleotide level have been those coding for X-prolyl dipeptidyl aminopeptidase from two strains of *Lactococcus*. The PepX gene of *L. lactis* subsp. *lactis* P8-2-47 was isolated by screening an *Escherichia coli* plasmid bank of total DNA of P8-2-47 for PepX activity using a chromogenic substrate. Expression of the gene in this heterologous host and in *Bacillus subtilis* proved that the structural gene for PepX had been cloned (80). In an independent study, *pepX* of *L. lactis* subsp. *lactis* NCDO 763 was cloned by constructing a total DNA bank in an isogenic PepX mutant. Subsequently, this bank was screened using an enzymatic plate assay and a chromogenic substrate (98). Both genes have been sequenced and contain 763 codons. The amino acid comparison indicated that they were almost identical (80, 98). The size of the protein deduced from the inferred amino acid sequence (88 kDa) corresponded well with that of the purified enzyme (approximately 90 kDa) (60, 152). The N-terminus of the predicted protein does not contain a signal peptide-like sequence. Ins-

tead, the N-terminal amino acid sequence of the purified PepX of *cremoris* P8-2-47 was identical to the N-terminal stretch of amino acids deduced from its gene (80). These results indicate that PepX is not processed at the N-terminus and that, most probably, the enzyme is localized into the citoplasmatic fraction, which is in agreement with the results obtained with polyclonal antibodies.

To determine the precise «in vivo» function of PepX, mutants obtained with methyl methane sulphonate and by gene replacement were grown in milk and in a defined synthetic medium with casein as the only nitrogen source. Both kind of mutants grew equally well in both media and their rate of acid production did not significantly differ from that of the wild type strains. However, in the absence of the enzyme there is a marked difference in the resulting peptide profile from the degradation of metenkephalin (81). These results therefore suggest that although PepX is not essential for the growth of lactococci in milk, altered expression of the gene might have a dramatic impact on the peptide composition of fermented milk products.

General aminopeptidases

General aminopeptidases are defined as enzymes capable of N-terminal hydrolyzation of several di-, tri- and tetrapeptides in which no special amino acids (proline, glutamic acid, etc.) are involved; they thus present a broad substrate profile. Several aminopeptidases have been purified and characterized from various strains of lactococci (2, 24, 38, 40, 99, 101, 126) and lactobacilli (3, 12, 57, 89, 148). The best known of them are aminopeptidase N (PepN) and aminopeptidase C (PepC) which have been recently characterized at the nucleotide level.

PepN, isolated from *L. lactis* subsp. *cremoris* Wg2 (126) and HP (40), is a 95 kDa metallopeptidase which can hydrolyse Ala_3 to Ala_6 peptides. In general, PepN hydrolyzes peptides containing aliphatic or negative residues, but not peptides which contain proline at the second position. Casein-derived peptides which can be regarded as bitter have also been shown to be hydrolysed by PepN (130). The gene encoding PepN has been isolated and characterized (2, 124, 129). It consists of an ORF coding for a protein of 846 amino acids with a molecular weight of 95 kDa. The amino acid sequence of PepN was found to be homologous to the corresponding human, rat and mouse enzymes. No signal peptide-like sequence was identified at the N-terminus of the enzyme and only the initial methionine was absent in the mature enzyme.

PepC is an aminopeptidase, with a broad substrate specificity, purified from *L. lactis* subsp. *lactis* AM2. This enzyme hydrolyzes naphtylamide substituted amino acids as well as di- and tripeptides. The native enzyme is an hexamer composed of identical subunits, and its peptidase activity is inhibited by inhibitors of the cysteine proteinase family (99). The gene encoding PepC predicts an ORF of 436 amino acids. The deduced amino acid sequence of the protein showed homology to the active site of cysteine proteinases like papain and others. No signal peptide-like sequence could be identified (18).

Glutamyl aminopeptidases

Aminopeptidases with specificity for peptides containing glutamyl and aspartyl residues at the N-terminal position (designated as GAP) are technologically interesting since peptides with glutamyl and aspartyl residues confer desirable flavours. GAP's have been purified from *L. lactis* subsp. *cremoris* HP (38) and *L. lactis* subsp. *lactis* NCDO 712 (101) and are made of trimers and multimers of 41-43 kDa subunits, respectively. Several clones specifying GAP's have been obtained by polymerase chain reaction amplification (M. J. Gasson, personal communication).

Endopeptidases

Endopeptidases are likely to play a major role in the degradation of peptides generated from casein by the proteinases. The purification and characterization of two endopeptidases from *L. lactis* subsp. *cremoris* H61, LEPI and LEPII, have been reported (149, 150). Recently, another endopeptidase (LEPIII) has been isolated and purified from *L. lactis* subsp. *cremoris* Wg2 (127). All three endopeptidases are inhibited by EDTA, indicating that they probably belong to the metallopeptidase family, but they have different substrate specificities. The molecular sizes of the enzymes are 98, 40 and 70 kDa for LEPI, LEPII and LEPIII, respectively. LEPI and LEPIII appear to function as monomers, while LEPII is active as a dimer. Other endopeptidases, designated intracellular proteinases, have been purified from *L. lactis* subsp. *lactis* NCDO 712 (96) and *L. lactis* subsp. *lactis* bv. *diacetylactis* (22), in contrast to the LEP endopeptidases the two latter hydrolysed whole casein molecules.

The gene specifying LEPIII, *pepO*, has recently been cloned and sequenced (85). It is probably the last gene of an operon encoding the binding-protein-dependent oligopeptide transport system of *L. lactis*. The ORF found can encode a protein of 627 amino acids, with a calculated molecular weight of 71.5 kDa. The N-terminal amino acid sequence deduced from the nucleotide sequence was identical to that determined from the purified endopeptidase, except for the initial methionine, which is absent in the mature enzyme. The inferred amino acid sequence of PepO showed similarity to the mammalian enkephalinase. By means of gene disruption, a PepO negative mutant was constructed. Growth and acid production of the mutant in milk were not affected, indicating that the endopeptidase is not essential for growth of *L. lactis* in milk. Although PepO is not essential, it was observed that the endopeptidase is able to release specific peptides from the α_{s1}-CN(f1-23) casein fragment generated by the action of chymosin (85).

Other peptidases

Recently, the cloning of several other peptidases has been reported. Among these, we should mention the gene for an aminopeptidase from *Lb. casei* subsp. *rhamnosus* of 87 kDa, that has been cloned in *E. coli* (111), an iminopeptidase gene from *Lb. delbrueckii* subsp. *bulgaricus* (5), a general aminopeptidase gene from *Lb. helveticus* (42) and the genes coding for a dipeptidase and a tripeptidase isolated from *L. lactis* subsp. *cremoris* Wg2 (86). All these genes are currently being analysed and it would be too premature to speculate on the functional role of these enzymes in the casein breakdown pathway.

It is also remarkable the work of R. Plapp's Group in Germany working with a *Lb delbrueckii* subsp. *lactis* strain, from which several peptidases and other genes of the proteolytic system of that strain have been cloned. These included the lactococcal counterparts of PepX and PepN; a prolinase, PepP, a leucyl aminopeptidase, PepL, and the special peptidase which hydrolyses carnosine, PepD, (R. Plapp, personal communication).

Amino acid and peptide transport systems

As was stated before, most lactic acid bacteria can utilize amino acids and peptides to satisfy their nitrogen requirements (73, 74, 87, 109, 110, 113). In lactic acid bacteria primary (in which energy comes from ATP) and secondary (in which energy is provided by an electrochemical gradient) amino acid and peptide transport systems have been found (68, 110, 118, 119, 121, 122). In the last few years fundamental knowledge on the biochemical and bionergetic properties of amino acid and peptide transport systems has been obtained (68). Lactococci, at least, possess separate transport systems for amino

acids, di- and tripeptides and oligopeptides. A brief summary of the recent data will be presented in this section.

Amino acid transport

In lactococci three different transport systems have been described apart from passive diffusion (68). (i) Most of the amino acids are taken up by secondary transport systems in which uptake is coupled to the proton-motive force (pmf). The transport of methionine, leucine, isoleucine,valine, serine, threonine, alanine, glycine and lysine is pmf-driven. The uptake of histidine, cysteine, tyrosine and phenylalanine might also be coupled to the pmf (25, 27, 28). (ii) The uptake of glutamate, glutamine, asparagine and possibly aspartate is catalysed by primary transport systems, and it is driven by ATP (or another energy-rich phosphate bound) (103). (iii) Arginine is taken up in exchange for ornithine with 1:1 stoichiometry (26, 104, 135). Translocation of arginine via this system does not require energy, the driving force is supplied by the concentration gradient of the two amino acids.

Peptide transport

Different peptides with up to 6 amino acid residues can be incorporated to meet the several amino acid deficiencies of lactococci (74, 87, 110). Since high extracellular concentration of the amino acids constituting the peptides do not inhibit uptake (74, 110, 138), these must enter by uptake systems independent from those used by amino acids. Peptide transport has been shown not to be directly coupled to peptide hydrolysis (118). At present, two independent transport systems have been demostrated in lactococci: a di- and tripeptide transporter and an oligopeptide uptake system.

Di- and tripeptide transport occurs by a secondary transport system which is driven by the pmf and has a broad specificity (118). By selecting variants resistant to the toxic chlorated dipeptide L-alanyl-β-chloro-alanine, spontaneous negative mutants of the di- tripeptide transport system have been isolated (119). These mutants had full proteinase and peptidase activities and the uptake of amino acids and tetra-,penta- and hexapeptides was unaffected. The mutants neither grew in milk nor in media containing casein as the sole nitrogen source, indicating that, during casein degradation, one or more essential amino acids become available only as di- or tripeptide. *L. lactis* was believed not to take up proline in a free form (119), however, very recently a low-affinity proline uptake system (K_m >5 mM) has been described in this bacterium (90), but in milk-growing bacteria this amino acid, most likely, enters the cell in a peptide-bound configuration. It has also been shown that this di-, tripeptide transporter has a relatively high affinity for proline-containing peptides (119, 120).

The genetic determinat of this di-, tripeptide transport system from *L. lactis* has recently been cloned through complementation of a proline auxotrophic *E. coli* mutant, negative for the di-, tripeptide transport system. The *dtpT* gene showed an ORF coding for a protein of 463 amino acids. The inferred protein, DptT, is very hydrophobic and showed 12 possible membrane-spanning α-helices. To further characterize the di-, tripeptide transport system, a deletion mutant is currently being made (W. N. Konings, personal communication).

In 1987, the cloning of a chromosomal DNA fragment from *L. lactis* in a proteinase negative strain, allowed this strain to grow in autoclaved but not in pasteurised milk (143). This observation suggested that the fragment coded for a peptide transport system. The cloned DNA fragment specified, at least, four proteins, three of which were essential to sustain peptide utilization (137). Recently, the cloned fragment has been completely sequenced. Five ORF's, associated in an operon-like structure *(oppFDCBA)*, have been found (S. Tynkkynen, personal communication). The deduced proteins of these ORF's showed homology with the oligopeptide transport proteins of *Salmonella typhimurium* and *B.*

subtilis. This system transports, among other peptides, tetra-, penta- and hexaalanine, but cannot transport oligopeptides containing proline or glutamate residues (W. N. Konings, personal communication). The uptake of these oligopeptides is directly driven by ATP.

Concluding remarks

We can conclude that we have a reasonably good understanding of the cell envelope-attached caseinolytic proteinase of lactococci, and preliminary notions of the organization of this proteinase in other microorganisms. There is a considereable amount of knowledge on the peptidase enzyme system in lactococci; to date, a set of at least 11 to 12 different types of aminopeptidases and endopeptidases are known, which acting in concert can potentially hydrolyse most of the oligopeptides generated from casein by the proteinase. The principal insights of the amino acid and peptide uptake systems have also been elucidated.

There are still some gaps which hamper the integration of all the currently available data into a plausible working model of the proteolytic system operating in cells growing in milk-based media. Most of the questions we have to answer have been elegantly related by Pritchard and Coolbear in a recent review (106). These are especially relevant: (i) how can the long peptides, that result from casein hydrolysis by the proteinases, be converted into short peptides that can be incorporated by the cells? It hast to be taken into account that all described peptidases but one probably have an intracellular location. (ii) How many peptidases are essential and how many dispensable? (iii) Which one of the peptidases supply di- and tripeptides to the essential di- and tripeptide transport system? (iv) Are there still some more peptidases not characterized or membrane carriers, facilitating the active transport of peptides greater than 6-7 amino acid residues, not yet detected? So far, the DtpT and OppFDCBA peptide transporters, together with the proteinase (PrtP), are the only components of the proteolytic system of lactococci that are essential for casein utilization and growth in milk.

Continued biochemical and genetic studies will complement each other in order to have a comprehensive picture of the proteolytic system by which lactic acid bacteria can grow in milk and participate in the flavouring and ripening of dairy products.

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Levaduras autóctonas aisladas en vinos de Tenerife y su influencia en las concentraciones de acetato de etilo y alcoholes superiores analizados por cromatografía de gases

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Summary

A taxonomic study of yeasts present on Tenerife wines, (Tacoronte-Acentejo Specific Denomination) has been carried out.

Nine species of the genera: *Saccharomyces, Torulaspora, Brettanomyces, Kluyveromyces, Debaryomyces, Saccharomycodes, Hansenula, Pichia* and *Candida* have been isolated.

Parallely we analysed volatile compounds of the wines such as ethyl acetate, methanol, isobutanol and amylic alcohols by gas chromatography.

Appreciable quantities of ethyl acetate were detected due to the low fermentative power of species such as *Candida glabrata* and *Debaryomyces hansenii*. The greatest concentration of amylic alcohols were found in wines containing yeast with high alcohol producing power like *Saccharomyces cerevisiae*.

Key words: Wine, yeasts, volatile compounds.

Resumen

Se ha realizado un estudio taxonómico de levaduras autóctonas en los vinos de Tenerife, concretamente de la Denominación Específica Tacoronte-Acentejo en la vendimia de 1989.

El número total de especies aisladas fue de 9, perteneciendo a los géneros: *Saccharomyces, Torulaspora, Brettanomyces, Kluyveromyces, Debaryomyces, Saccharomycodes, Hansenula, Pichia* y *Candida*.

Al mismo tiempo analizamos por cromatografía de gas ciertos compuestos volátiles presentes en el vino, como acetato de etilo, metanol, isobutanol y alcoholes amílicos.

Se detectaron apreciables cantidades de acetato de etilo debido a especies de bajo poder fermentativo como *Candida glabrata* y *Debaryomyces hansenii*. Por otro lado, las mayores concentraciones de alcoholes amílicos eran debidas a especies de elevado poder alcohológeno como *Saccharomyces cerevisiae*.

(*) A quien debe dirigirse la correspondencia.

Introducción

Resulta enormemente laborioso determinar las numerosas sustancias que contribuyen al aroma vínico, considerando que muchas de ellas se encuentran en trazas, y que su concentración no está directamente relacionada con su importancia en el aroma, sino con el umbral de apreciación sensorial de cada compuesto (13, 15).

Actualmente se conocen ciertos compuestos que por exceso o por defecto, pueden alterar el equilibrio organoléptico del vino tales como algunos ácidos orgánicos, aldehídos, ésteres y alcoholes superiores (5, 10, 12). En el presente trabajo se plantea la relación existente entre las concentraciones de ciertas sustancias volátiles como acetato de etilo, metanol, etanol, 1-propanol, isobutanol, alcoholes amílicos, y las levaduras autóctonas identificadas. En Tenerife existen aportaciones taxonómicas de interés en el campo de la flora blastomicética con importancia en Enología (Salvadores, M. P., 1991, Tesis doctoral. Universidad de La Laguna).

En este estudio, llevado a cabo durante la vendimia de 1989, se realizó el análisis taxonómico de las levaduras que intervienen de forma natural en la fermentación vínica de la zona muestreada, junto con el análisis cromatográfico de algunas sustancias volátiles de interés existentes en estos vinos.

El acetato de etilo es el éster principalmente implicado en el aroma vínico y contribuye al aroma afrutado (7), siendo en dosis elevadas perjudicial para el mismo (13, 14, 15), ya que puede desnaturalizarlo confiriéndole un olor picante desagradable.

Los alcoholes superiores han sido descritos como uno de los compuestos vínicos que tienen mayor influencia sobre los caracteres organolépticos del vino, esencialmente en el aroma (17).

Aunque se encuentran en todos los tipos de vino, sus concentraciones relativas dependen de la cepa que conduce la fermentación (16). Diversos autores señalan entre ellos a los alcoholes amílicos, el isobutanol y el 1-propanol (2, 11).

Su concentración en los vinos es considerada como un criterio de calidad, ya que se ha observado que los vinos mejor clasificados por la degustación corresponden a los de mayor contenido en alcoholes superiores (4).

Materiales y métodos

1) Recogida y manejo de las muestras

Se recogieron 21 muestras de 4 bodegas de la Denominación de Origen Tacoronte-Acentejo, en 2 de ellas (1 y 3) se tomaron muestras de 2 cubas diferentes (A y B).

Las bodegas 1, 2 y 3 están situadas en diferentes sectores de la localidad de El Sauzal, tomándose 4 muestras por bodega y cuba con niveles de sulfuroso entre 10 y 20 g/hl.

La bodega 4 está situada en la localidad de La Victoria, recogiéndose sólo una muestra que no fue sulfitada.

El grado alcohólico expresado en °Be de las muestras de vino correspondientes en cada una de las bodegas fue: 10,50 en la bodega 1A, 10,75 en la 1B, 11,60 en la 2, 13,70 en la 3A, 12,87 en la 3B y 12,10 en la 4.

Las muestras tomadas en las 4 bodegas son de vino tinto y corresponden a las variedades de uva siguientes: Listán negro 80% y Negra moll 20% en las bodegas 1 y 4, Tintilla en la 2, Negra moll en la 3B y Mezcla de Listán negro 60% y Listán blanco 40% en la 3A.

La recogida se realizó en envases estériles, y el transporte al laboratorio en recipientes refrigerados a una temperatura de –4 °C.

2) Técnicas de aislamiento e identificación de levaduras

En cuanto al aislamiento de levaduras, las muestras se diluyeron en función de la densidad celular en agua destilada estéril desde 10^{-1} a 10^{-6}. A partir de la dilución más conveniente en cada muestra, el aislamiento de levaduras se efectuó inoculando por extensión alícuotas de 0,1 ml sobre placas de Petri con medio Agar Y.P.D. Después de 3 a 7 días de incubación a 25 °C, la aparición de colonias de diferente morfología se hizo evidente, procediendo a su aislamiento en placas con el mismo medio ajustado a pH 5,7.

Los cultivos puros así obtenidos se conservaron por duplicado en viales que contenían 1 ml de glicerol al 15% manteniéndose a –20 °C.

La identificación de los cultivos aislados se llevó a cabo según los criterios de Barnett *y col.* (1) seleccionando las pruebas de identificación más discriminatorias para levaduras vínicas agrupándose en:

a) Pruebas morfológicas

Se hizo una siembra de los cultivos en medio Agar Y.P.D., y se observaron las características morfológicas en el microscopio de contraste de fases a los 3 ó 5 días de crecimiento a 25 ºC. Esto se completó con la observación de la morfología celular de las colonias, así como su textura y pigmentación.

b) Pruebas bioquímicas

Utilización anaerobia de compuestos carbonados (D-glucosa), asimilación de compuestos nitrogenados (Nitrato potásico), asimilación de compuestos carbonados (pentosas: L-arabinosa, D-arabinosa; hexosas: D-galactosa; disacáridos: lactosa, melibiosa; trisacáridos: rafinosa, melezitosa; polisacáridos: almidón; alcoholes: eritritol, D-manitol; ácidos orgánicos: succínico, cítrico).

Los diferentes medios de cultivo para la asimilación de los compuestos carbonados contenían 0,67% de Y.N.B., agar al 2,5% y la fuente de carbono correspondiente al 1%. Para la asimilación de compuestos nitrogenados, el medio contenía 1,17% de Y.C.B., agar al 2,5% y 1% de la fuente nitrogenada.

También se realizó el Test de la ureasa.

c) Pruebas fisiológicas

Resistencia a altas presiones osmóticas (Y.N.B.-glucosa al 50% y 60%), resistencia a la temperatura (Y.N.B.-glucosa incubado a 30° y 37°), resistencia a la cicloheximida (Sigma: 0,01%).

La lectura de los resultados se llevó a cabo después de tres y siete días, volviéndose a leer al cabo de 15 días de incubación a 25 °C, para considerar los cultivos con crecimiento lento o retardado, anotando la respuesta a cada prueba en función de la ausencia o presencia de crecimiento, comparando éste con el obtenido en placas de medio base Y.N.B. ó Y.C.B. según el caso, utilizadas como testigos.

3) Cromatografía de gases

Los componentes volátiles fueron determinados por Cromatografía en fase gaseosa, empleando una columna de 4 m de longitud por 1/8'' de diámetro interno, rellena de Carbowax 1500 al 15% sobre Chromosorb W de 80/100 mallas.

Las condiciones cromatográficas fueron: gas portador de nitrógeno 20 ml/min, temperatura del inyector y del detector 150 °C, temperatura del horno 80 °C. Detector: FID.

TABLA 1
NUMERO DE AISLAMIENTOS Y PORCENTAJES TOTALES DE LEVADURAS EN LAS DIFERENTES BODEGAS MUESTREADAS

Bodega	Especie	Nº Aislamientos	%
	Saccharomyces cerevisiae	247	72,60
	Toruslaspora delbrueckii	53	15,50
1A	*Brettanomyces lambicus*	25	7,40
	Candida glabrata	15	4,40
	Total	340	
	Saccharomyces cerevisiae	89	68,40
	Kluyveromyces marxianus	31	23,80
1B	*Hansenula anomala*	9	6,90
	Brettanomyces lambicus	1	0,76
	Total	130	
	Saccharomyces cerevisiae	310	83,70
	Kluyveromyces marxianus	23	6,30
	Pichia membranaefaciens	19	5,10
2	*Candida glabrata*	15	4,00
	Debaryomyces hansenii	3	0,80
	Total	370	
	Saccharomyces cerevisiae	183	70,40
	Toruslaspora delbrueckii	49	18,80
3A	*Hansenula anomala*	21	8,10
	Brettanomyces lambicus	7	2,70
	Total	260	
	Saccharomyces cerevisiae	179	85,20
	Pichia membranaefaciens	15	7,10
3B	*Saccharomycodes ludwigii*	13	6,10
	Candida glabrata	3	1,40
	Total	210	
	Saccharomyces cerevisiae	406	75,80
	Kluyveromyces marxianus	47	8,70
	Toruslaspora delbrueckii	27	5,00
4	*Hansenula anomala*	23	4,30
	Pichia membranaefaciens	19	3,50
	Saccharomycodes ludwigii	14	2,60
	Total	536	

El análisis de cada cromatograma se realizó en un tiempo aproximado de 35 minutos. Como patrón interno se utilizó el 4-metil-2-pentanol en solución de 5 g/l en etanol de 10°.

Resultados y discusión

De las muestras estudiadas en la vendimia de 1989 se obtuvieron los siguientes aislamientos: 340 en la bodega 1A, 130 en la 1B, 370 en la 2, 260 en la 3A, 210 en la 3B y 536 en la 4, que tras las

TABLA 2
VALORES MEDIOS DE COMPUESTOS VOLATILES (MG/L) DETECTADOS EN LAS MUESTRAS DE VINOS RECOGIDAS EN CADA BODEGA DURANTE LA VENDIMIA DE 1989 Y GRADO ALCOHOLICO EN (°BE) DE LAS MISMAS

Compuestos analizados	Bodegas						
	1A	1B	2	3A	3B	4	C*
Acetato de etilo	20,6	29,2	33,4	45,9	59,2	59,5	29,0
Metanol	31,7	36,3	42,4	40,0	49,6	55,8	40,0
1-Propanol	28,4	27,8	28,1	25,4	16,6	17,2	27,5
Isobutanol	75,9	76,6	72,4	80,6	86,0	60,0	77,5
Alcoholes amílicos	117,5	133,7	116,6	118,4	101,4	174,4	144,0
Grado alcohólico**	10,5	10,7	11,6	13,7	12,8	12,1	

*: Valores medios establecidos según bibliografía.
**: Valores obtenidos por el método oficial del M.A.P.A. (Ministerio de Agricultura, Pesca y Alimentación).

pruebas de identificación citadas en el apartado anterior pudieron agruparse en 9 especies diferentes (Tabla 1).

Saccharomyces cerevisiae, levadura fermentadora por excelencia fue la especie predominante en todas las muestras. Este hecho se produce en diversos estudios realizados en este mismo campo (5, 10, 18, 19).

Otras especies que se aislaron en considerables porcentajes aunque no en todas las bodegas, fueron *Kluyveromyces marxianus* y *Torulaspora delbrueckii*, ambas de poder fermentativo medio. Es de destacar, la presencia de un grupo de levaduras de marcado carácter oxidativo y poder fermentativo débil como son *Pichia membranaefaciens, Candida glabrata, Brettanomyces lambicus* y *Hansenula anomala* que fueron aisladas con frecuencias variables en algunas bodegas debido a las malas condiciones higiénico-sanitarias de éstas.

También notorio por sus implicaciones prácticas fue el aislamiento e identificación de *Saccharomycodes ludwigii* en dos bodegas (3B y 4). Esta especie apiculada de gran tamaño y cetogénica es considerada como perjudicial en la vinificación.

La Tabla 2 muestra las concentraciones medias obtenidas en mg/l por cromatografía en fase gaseosa de alcoholes superiores y acetato de etilo en las muestras de vino recogidas en cada bodega, frente a valores medios establecidos en la bibliografía (6, 15). Puede observarse que las concentraciones de acetato de etilo son las que presentan mayores fluctuaciones, siendo máximas en las muestras recogidas en las bodegas 3B y 4. Este hecho se correlaciona con la presencia de la especie apiculada fuertemente cetogénica *Saccharomycodes ludwigii* en los caldos de dichas bodegas (6,1% y 2,6% respectivamente, ver Tabla 1), y sugiere que esta especie podría ser la responsable de estos altos niveles.

En otras bodegas, los porcentajes relativamente altos de acetato de etilo (1B: 6,9%, 3A: 8,1%y 2: 5,1%) podrían atribuirse a la presencia de otras especies de metabolismo fundamentalmente oxidativo como *Pichia membranaefaciens* y *Hansenula anomala*, que aparecen en las mismas. En favor de esta hipótesis está el hecho de que ninguna de estas tres especies se aisló de la bodega 1A, cuyas muestras dieron el menor porcentaje en acetato de etilo.

Otras especies de bajo poder alcohológeno como *Candida glabrata* y *Debaryomyces hansenii*, las cuales forman apreciables cantidades de acetato de etilo podrían ser responsables en ciertos casos de la presencia de este compuesto. Así la primera se aisló de diferentes bodegas (1A: 4,3%, 2:4,0% y 3B: 1,2%) y la segunda se encontró en la bodega 2: 0,9% (ver Tabla 1).

La Tabla 2 muestra también las concentraciones de los alcoholes superiores. Los valores son, en general elevados, sobresaliendo de forma especial los relativos a alcoholes amílicos en las muestras de la bodega 4. En el caso del isobutanol las concentraciones medias fueron muy elevadas en las muestras de las bodegas 3B y 3A.

De los tres alcoholes, el 1-propanol fue el que dio concentraciones medias más bajas y uniformes en todas las bodegas. Los valores obtenidos por nosotros están de acuerdo con los reportados anteriormente por Soufleros *y col.* (20), quienes tras comparar la producción de estos alcoholes por diferentes especies de levaduras, concluyen que aquéllas de mayor poder alcohológeno, como *Saccharomyces cerevisiae*, producen las concentraciones más elevadas de los mismos.

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Species of the *Paramecium aurelia* complex in Spain

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Summary

The new habitats of *P. primaurelia*, *P. biaurelia*, and *P. triaurelia* of the *P. aurelia* complex were described in Spain. *P. pentaurelia* was also found, for the first time in that country.

Key words: The Paramecium aurelia species-complex, *occurrence in Spain.*

Resumen

En este trabajo se analiza la distribución de algunas especies del complejo de *Paramecium aurelia* en España. Se caracteriza *P. pentaurelia* como una nueva especie para la fauna de España. Se describen nuevas localizaciones de las siguientes especies *P. primaurelia*, *P. biaurelia* y *P. triaurelia*.

Introduction

Studies on the geographical distribution of the *Paramecium aurelia* species-complex carried out in Spain (14, 15, 17) revealed the presence there of 6 out of 8 species known in Europe (5, 17) and 15 species known world-wide (3, 23). Namely, *P. primaurelia*, *P. biaurelia*, *P. triaurelia*, *P. tetraurelia*, *P. sexaurelia*, and *P. novaurelia* were described in Spain. Of these, *P. primaurelia*, *P. biaurelia*, and *P. novaurelia* are common in Europe (5, 14, 20), while the occurrence of *P. tetraurelia* seems to be limited to certain environments (8, 12, 14, 17). In turn, the appearance of *P. triaurelia* is limited to certain regions (17). Two known European habitats of *P. sexaurelia* (15,16) have been described in Spain alone. Further sampling in Spain seemed worthwhile as habitants of the *P. aurelia* species-complex new for Europe might be found there, especially, as the Iberian Peninsula has geographical individuality (24).

The aim of the work was to study the occurrence of species of the *P. aurelia* complex in the samples collected in Cuenca. The town of Cuenca lies on the steep slopes of a hill, below which the River Júcar joints its tributary the River Huécar. The Júcar cuts a gorge in the lime-stone bedding. The town is surrounded by the Serranía de Cuenca from the west, and by Sierra de Palancares from the south-east. Cuenca province, part of the region of Castile, is very interesting from the geological (1), physiographical, and biological points of view.

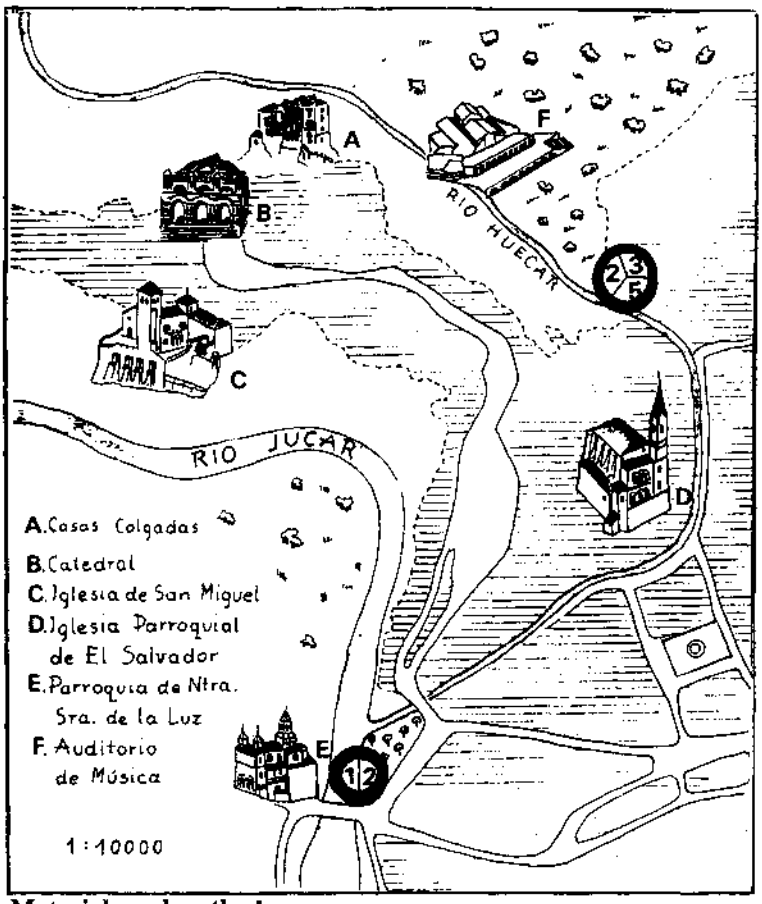

Fig. 1. Distribution of species of the *Paramecium aurelia* complex at the sampling points in Cuenca. Number 1 refers to *P. primaurelia*, number 2 to *P. biaurelia*, number 3 to *P. triaurelia*, and number 5 to *P. pentaurelia*.

Materials and methods

Samples of water were collected on 10th September, 1991 in Cuenca, from the River Júcar and its tributary, the Huécar. The sampling places (s.p.) are shown on the map (Fig. 1). Routine methods of collecting samples and establishing clones of species of the *P. aurelia* complex (6, 8, 17) were applied and their identification was carried out according to Sonneborn (19, 22).

The paramecia were cultivated on a lettuce medium inoculated with *Enterobacter aerogenes*.

Species of the *P. aurelia* complex were identified by mating the investigated clones with the mating types of standard strains of known species. The following were used: strain 90 of *P. primaurelia*; strain Rieff, Scotland of *P. biaurelia*; strain 324 of *P. triaurelia*; strain 87 of *P. pentaurelia*; strain 38 of *P. septaurelia*; and strain 138 of *P. octaurelia*, from the collection of Dr. J. R. Preer, Jr., Zoology Department, Indiana University, Bloomington, Indiana, U.S.A. The clones were determined as *P. primaurelia*, *P. biaurelia*, *P. triaurelia*, and *P. pentaurelia* on the basis of 95-100% conjugation between the complementary mating types of the examined clones with the appropriate standard ones. When determining the clones belonging to *P. triaurelia*, parallel cultures of the examined clone and the standard strains of *P. primaurelia*, *P. triaurelia*, *P. septaurelia*, and *P. octaurelia* were grown; also for determining *P. pentaurelia* the studied clone was cultivated parallely with *P. primaurelia*, *P. pentaurelia*, and *P. septaurelia*. The viability of the offspring of the hybrid exconjugant clones was also observed in the case of inter-strain crosses within *P. triaurelia* and *P. pentaurelia*.

TABLE I
CHARACTERISTICS OF THE STUDIED HABITATS IN CUENCA

Sampling point and kind of habitat	pH of water during sampling	Temperature (°C) of water during sampling	Ambient temperature (°C)	Number of designated clones of *Paramecium primaurelia*	Number of designated clones of *Paramecium biaurelia*	Number of designated clones of *Paramecium triaurelia*	Number of designated clones of *Paramecium pentaurelia*	Accompanying plankton organisms
Cuenca, River Huécar	7.2	20	30	—	12	1	1	*Dileptus anser, Paramecium bursaria, P. caudatum, Spirostomum ambiguum, Euplotes sp., Dendrocoelum lacteum, Rotatoria*
Cuenca, River Júcar	7.0	20	35	17	7	—	—	*Bodo sp., Paramecium caudatum, Euplotes sp., Dendrocoelum lacteum, Rotatoria, Cyclops s.p.*
Total number of designated clones				17	19	1	1	38

Results and discussion

As many as three species of the *P. aurelia* complex, i.e. *P. biaurelia*, *P. triaurelia*, and *P. pentaurelia*, were found at the sampling point (s.p.) in the River Huécar, and two species, *P. primaurelia* and *P. biaurelia*, at the s.p. in the River Júcar (Fig. 1). Characteristics of the habitats (s.p.) are given in the table (Table 1). The distribution of species of the *P. aurelia* complex at the studied sampling points is very interesting (Table 2). *P. aurelia* species were found in two out of ten samples (I-X) collected from the Huécar, 10 clones established from the first sample (I) were identified as *P. biaurelia*, while 4 clones established from the second (II) sample were determined as *P. biaurelia* (2 clones), *P. triaurelia* (1 clone), and *P. pentaurelia* (1 clone). This indicates the possible existence in the nearest proximity in nature of at least three species of the complex. According to Anderson (2) «..., protozoan competitive strategies are complex and vary with the demands of the environment, and in relation to their genetic potential to adapt to diverse biotic and abiotic variables of their habitat».

In turn, among ten samples collected from the River Júcar individuals belonging to he *P. aurelia* complex were found in seven, and the established clones were determined as *P. primaurelia* and *P. biaurelia*. The two species appeared in the same sample twice (samples I and IV).

Altogether, among 38 identified clones, 19 represented *P. biaurelia*, 17 *P. primaurelia*, 1 *P. triaurelia*, and also 1 *P. pentaurelia*. *P. primaurelia* and *P. biaurelia* are common in Europe (see Introduction) and they had also been found before in Spain (14, 17). *P. triaurelia* was described in Europe in Romania (13), Spain (14, 17), Russia (11), Poland (7), and Czecho-Slovakia (9, 18). The habitats in Poland and Czecho-Slovakia were limited to the Sudeten Mts only, and they might represent the northern limit of the species in Europe. *P. pentaurelia* was found before only in Hungary (10), Romania (13), and Russia (4). This is a new European habitat of that species, and the first record in Spain. The occu-

TABLE 2
DISTRIBUTION OF SPECIES OF THE *PARAMECIUM AURELIA* COMPLEX AT THE STUDIED SAMPLING POINTS IN CUENCA

Sampling point (s.p.) and kind of habitat	Number of		clones identified as			
	samples [+] in which *Pa* s.p. were found among 10 collected from one s.p	clones established from one sample	*Paramecium primaurelia*	*Paramecium biaurelia*	*Paramecium triaurelia*	*Paramecium pentaurelia*
Cuenca, River Huécar	I	10	—	10	—	—
	II	4	—	2	1	1
Cuenca, River Júcar	I	5	2	3	—	—
	II	1	—	1	—	—
	III	5	5	—	—	—
	IV	10	8	2	—	—
	V	1	1	—	—	—
	VI	1	—	1	—	—
	VII	1	1	—	—	—
Total	9	38	17	19	1	1

[+] designated by Roman numerals.

rrence of *P. triaurelia* and *P. pentaurelia* in Europe seems to be restricted to defined climatic zones, the existence of which was suggested by Sonneborn (21, 23).

The previous studies on the occurrence of some species of the *P. aurelia* complex in Spain (14, 16, 17) and the present results are summed up in Table 3. The most frequent species, as to the number of designated clones (c.), examined habitats (h.), and the ratio value (r.v.), was *P. primaurelia* (96 c., 11 h., r.v. 0.50). *P. biaurelia* was also characteristic for this country (91 c., 7 h., r.v. 0.32), though found

TABLE 3
FREQUENCY OF OCCURRENCE OF THE *PARAMECIUM AURELIA* SP. COMPLEX IN SPAIN

Name of region	Number of designated clones, examined habitats, and ratio value[+] of							Total number of designated clones, and studied habitats in the region
	Paramecium primaurelia	*Paramecium biaurelia*	*Paramecium triaurelia*	*Paramecium tetraurelia*	*Paramecium pentaurelia*	*Paramecium sexaurelia*	*Paramecium novaurelia*	
Andalusia	46 (5)[++] 0.55[+]	—	4 (1) 0.11[+]	11 (2) 0.22[+]	—	2 (2) 0.22[+]	—	63 (9)[+++]
Castile	50 (6) 0.46[+]	91 (7) 0.54[+]	5 (2) 0.15[+]	37 (2) 0.15[+]	1 (1) 0.77[+]	—	39 (4) 0.30[+]	223 (13)[+++]
Total	96(11)[++] 0.50[+]	91 (7) 0.32[+]	9 (3) 0.14[+]	48 (4) 0.18[+]	1 (1) 0.04[+]	2 (2) 0.09[+]	39 (4) 0.18[+]	286 (22)

[+] Ratio value = $\frac{\text{No of habitats of a defined species}}{\text{Total no of habitats of the area}}$

[++] In brackets number of studied habitats

[+++] Some species appeared together in one habitat

only in Castile. *P. tetraurelia* and *P. novaurelia* appeared in 4 habitats each, *P. triaurelia* in 3 habitats, *P. sexaurelia* in 2, and *P. pentaurelia* in 1.

It seems that further sampling, especially in Andalusia, is very promising.

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Riboflavin production by *Aspergillus terreus* from beet-molasses

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Summary

Aspergillus terreus was used for riboflavin (vitamin B_2) production in a medium containing beet molasses as the sole carbon source. Growth and the vitamin production of the fungus were markedly affected by the composition of the culture medium. A maximum riboflavin yield was achieved at the late exponential growth phase (16 day-old cultures) in the presence of (g/l): centrifuged beet-molasses, 90; L-asparagine, 1; $MgSO_4.7H_2O$, 0.5; K_2HPO_4/kH_2PO_4 (1:1), 5; and the medium initially adjusted to pH 8.

Key words: Aspergillus terreus, *Riboflavin.*

Resumen

Se ha utilizado *Aspergillus terreus* para la producción de riboflavina en un medio que contenía melazas de remolacha como única fuente de carbono. Tanto el crecimiento del hongo como la producción de la vitamina dependió de la composición del medio de cultivo. El rendimiento máximo de riboflavina se obtuvo al final de la fase exponencial (cultivo de 16 días) en presencia de (g/l) melazas de remolacha centrifugadas 90; L-asparagina 1; $MgSO_4.7H_2O$ 0,5; K_2HPO_4/KH_2PO_4 (1:1) 5; a un pH inicial de 8.

Introduction

Riboflavin can be synthesized by many microorganisms including bacteria, moulds, algae and protozoa. The two closely related ascomycetes *Eremothecium ashbyii* and *Ashbya gossypii* have been extensively studied (4, 13) However, few studies addressed the ability of aspergilli to produce riboflavin (8, 14, 17).

Aspergillus terreus has recently been reported to produce riboflavin from carbohydrates and hydrocarbons (15). The aim of the present work was to evaluate the appropriateness of beet molasses, a carbohydrate-rich by-product from the sugar industry, for the production of vitamin B_2 by *A. terreus*.

(*) Corresponding author

Materials and methods

Organism

Aspergillus terreus was isolated from El-Alameen crude oil sludge (11) and identified by the staff of the Commonwealth Mycological Institute at Kew, Surrey, England. The cultures were maintained on glucose-peptone slants with transfers at monthly intervals.

Molasses

Crude beet molasses (BM) was kindly supplied by the Delta Sugar Company, Egypt. Its chemical composition has been previously reported (3).

Culture conditions

The composition of the fermentation medium was as follws (g/l): BM, 30 (= 16.73 g total reducing sugars as glucose); peptone, 5; KH_2PO_4, 2.5; K_2HPO_4, 2.5; $MgSO_4.7H_2O$, 1.0, at pH 6. The BM used as the sole carbon source was prepared by diluting with water to a concentration of 3% sugar. The muddy precipitate was then removed by centrifugation at 4000 rpm for 20 min. The organism was allowed to grow in 100 ml aliquots of the medium dispensed in 250 ml Erlenmeyer flasks. The flasks were sterilized by autoclaving at 121 °C for 15 min., inoculated with 2 ml spore suspensión (3,6 x 10^5 spores/ml) from a 72 h old culture and incubated at 30 °C under stagnant conditions for 12 days unless otherwise stated.

Analysis

–Dry weight estimation

The mould mycelium was separated by centrifugation, washed with ethanol and distilled water and then dried at 90 °C until constant weight was achieved.

–Extraction and estimation of riboflavin

The cultures obtained at the end of the incubation period were heated in the dark in a water bath at 80 °C for 10 min in order to release the vitamin bound to the cells into the culture medium (9). The fungal mat was then removed by centrifugation. Decolorization was carried out by the addition of 0.1 g glucose and 2.0 ml acetic acid to 5 ml of the filtrate. A strong solution of $KMnO_4$ was added dropwise with continuous shaking until the colour of $KMnO_4$ persisted for at least 30 seconds. Excess permanganate was removed by the addition of few drops of 3% H_2O_2 solution (5).

Riboflavin identification was carried out by thin layer chromatography (TLC) using a solvent system of n-butanol: acetic acid: distilled water (4:1:5, v/v/v) (18). Quantitative estimation of the vitamin was determined spectrophotometrically at 450 nm (7).

Riboflavin productivity was calculated as:

$$\frac{\text{total riboflavin (mg/l)}}{\text{consumed sugar (mg/l)}} \times 100$$

TABLE 1
ASPERGILLUS TERREUS GROWTH, SUGAR UPTAKE AND RIBOFLAVIN PRODUCTION AS AFFECTED BY THE DIFFERENT TREATMENT OF BM SAMPLES.

Molasses treatment	Sugar uptake (%)	Dry weight (mg/100 ml)	Riboflavin content (mg/100 ml)	Productivity (%)
Crude (untreated)	58	520	4.20	0,4
Centrifuged (mud-free)	66	470	5.55	0.5
H_2SO_4 -treated	54	420	4.12	0.4
Decationized	45	370	3.50	0.3

Initial sugar content = 1765 mg/100 ml

–Sugar content

The original as well as the unassimilated sugars were determined in the culture filtrate as glucose (2).

Results and discussion

Utilization of treated BM

In order to minimize the deleterious effect of heavy metals and to remove the mud from the crude BM different treatments were carried out (3).

The data represented in Table 1 indicate that low riboflavin production was found with the decationized samples which were previously reported (3) to be completely free from Zn^{2+}, Cu^{2+}, phosphorus and Mn^{2+} and contain reduced concentrations of Na^+, Ca^{2+}, K^+, Mg^{2+} and Fe^{2+} supported the lowest growth yield and vitamin output. These results confirm those previously reported on the stimulatory effect of these elements on riboflavin production (6,16). On the other hand, the maximal riboflavin

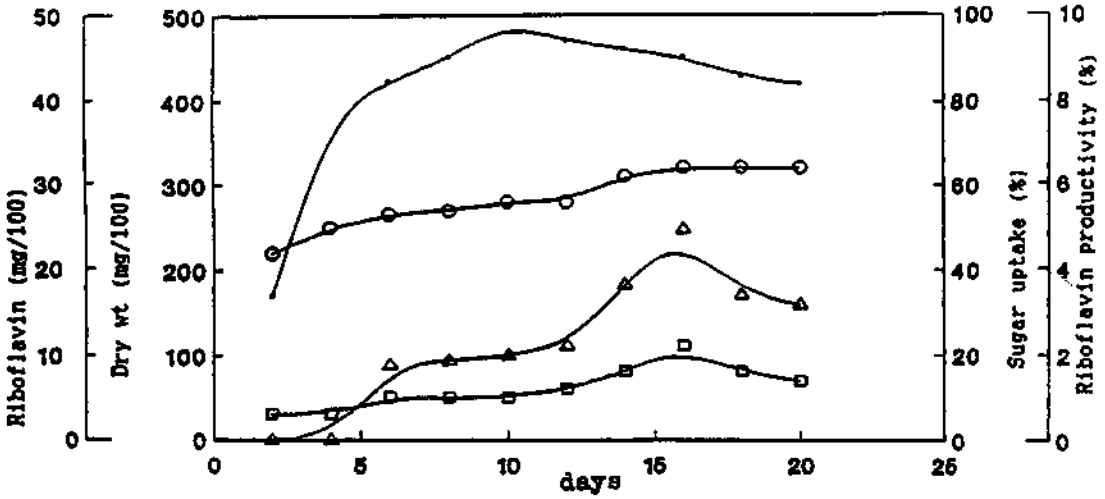

Fig. 1. *Aspergillus terreus* growth (•), sugar uptake (○), riboflavin production (△) and productivity (□) during 20 days of growth

TABLE 2
ASPERGILLUS TERREUS GROWTH, SUGAR UPTAKE AND RIBOFLAVIN PRODUCTION AS AFFECTED BY SOME SALTS IN THE FERMENTATION MEDIUM

Salt (g/l)	Sugar uptake (%)	Dry weight (mg/100 ml)	Riboflavin content (mg/100 ml)	Productivity (%)
$MgSO_4.7H_2O$:				
0.00	30	410	41.70	2.6
0.25	31	423	48.92	3.0
0.50	31	428	55.19	3.4
0.75	31	455	47,64	2.9
1.00	31	465	44.56	2.7
K_2HPO_4: KH_2PO_4 (1:1):				
0.00	24	285	39.79	3.1
1.00	29	370	50,51	3.3
3.00	30	398	52.53	3.3
5.00	31	429	55.21	3.4
7.00	36	438	53.59	2.8
9.00	38	446	48.45	2.4

output was recorded using centrifuged samples indicating that the iron content of BM was not inhibitory to the flavinogenesis enzymes of the fungus.

Time-course production

The changes in the growth and riboflavin yields of *A. terreus* during the different fermentation periods (2 to 20 days) were recorded. Fig. 1 shows that under static culture conditions a significant growth was observed after 4 days of incubation and reached its maximum value (490 mg/100 ml) at the end of the 10th day. On the other hand, riboflavin was only detected after 6 days and increased concurrently up to the 16th day (the end of the stationary growth phase) where it reached its highest level (about 12.4 mg/100 ml). These results coincide with those of other authors who have reported that organisms must first form enough biomass yield, after which flavinogenesis occurs (1, 10). In further experimentations, riboflavin was estimated in 16 days old cultures.

pH value

Different initial pHs (2 to 9) were tested. It is evident from Fig. 2 that adjustment of the basal medium to pH values below 3 seems to be inhibitory for growth and vitamin production. However, the fermentation parameters increased in a linear and regular fashion between pH 4 and 8. Thus, while maximal growth (445 mg/100 ml) was achieved at pH 6, the highest riboflavin output (18.07 mg/100 ml) was recorded at pH 8. Under all experimental conditions the final pH tended to remain alkaline. An initial pH of 7.5 has been reported to be optimal for vitamin production by *A. niger* (17). A similar situation has been observed with *Ashbya gossypii* (19). This author explained that during the growth phase sugar is converted to extracellular ethanol, acetone and acids such as pyruvate and acetate, thus lowe-

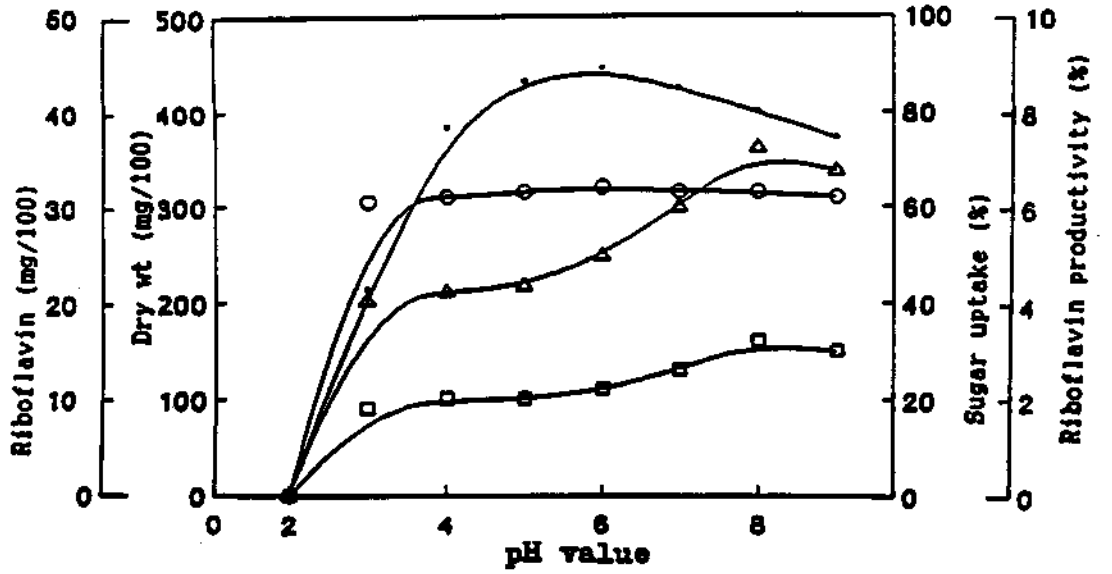

Fig. 2. *Aspergillus terreus* growth (•), sugar uptake (○), riboflavin production (△) and productivity (□) as affected by the initial pH value of the medium. Data at 16 days of growth.

ring pH close to neutrality. As growth slows down, accumulated intermediates are utilized and the pH rises. The rise in pH signals the start of the phase of riboflavin production.

Molasses level

Variation of the BM level, as the sole carbon source, between 1 and 10% (w/v) revealed that 9% (=50 g total reducing sugar as glucose/l) supported maximal riboflavin production by yeasts (7). A high glucose concentration (40 g/l) is optimal for vitamin productivity (16).

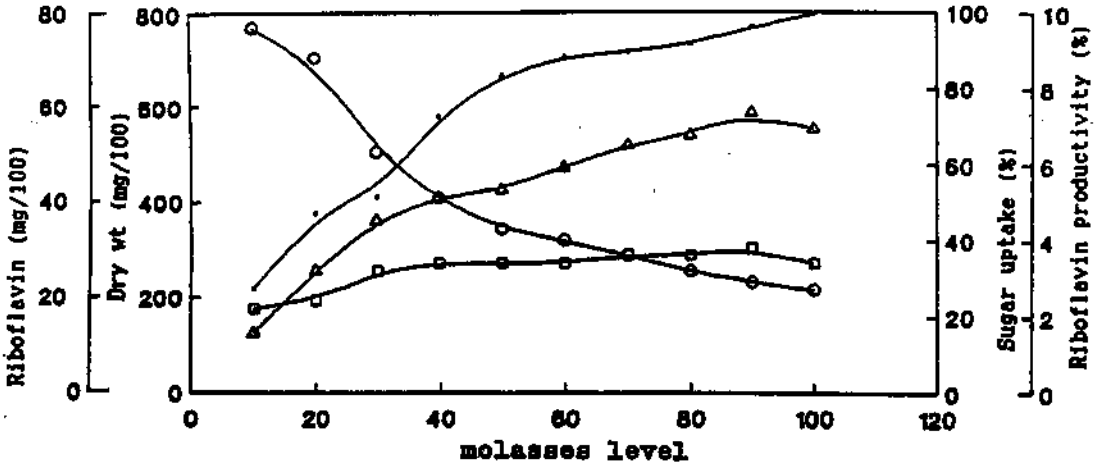

Fig. 3. *Aspergillus terreus* growth (•), sugar uptake (○), riboflavin production (△) and productivity (□) as affected by the molasses level. Data at 16 days of growth.

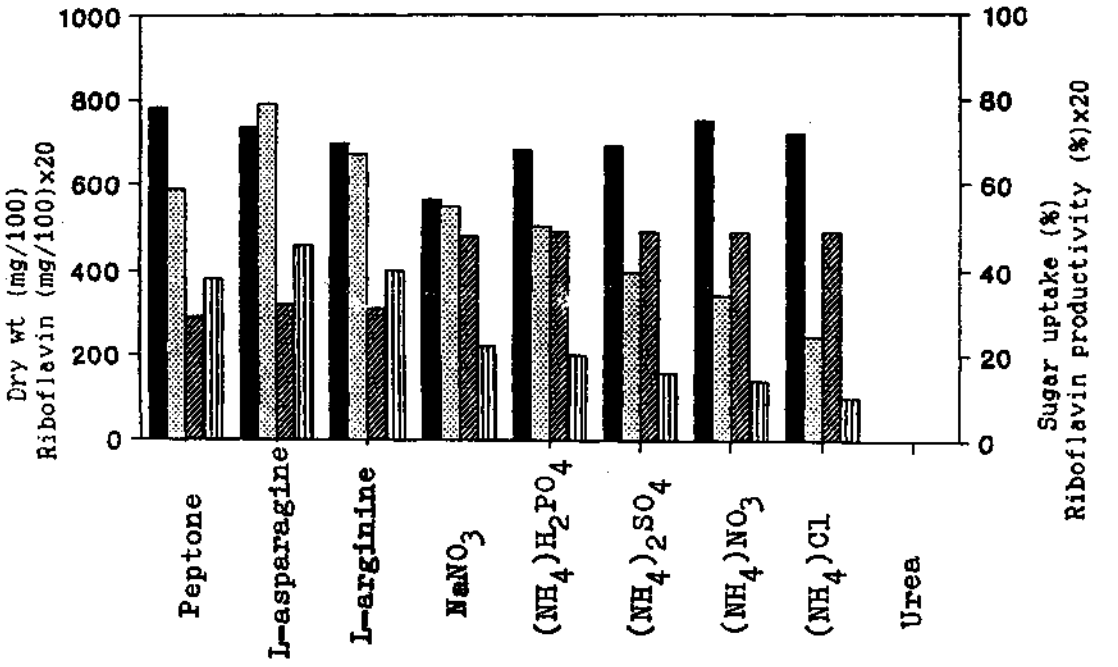

Fig. 4. *Aspergillus terreus* growth (■), sugar uptake (▩), riboflavin production (▨) and productivity (■) as influenced with the nitrogen source of fermentation medium.

Nitrogen requirements

Different nitrogen sources, at 127 mg/100 ml equivalent nitrogen were tested. The results (Fig. 4) indicate that nitrogen sources as peptone, asparagine and arginine were best assimilated and supported high growth rates as well as a strong riboflavin production. L-asparagine was the highest, yielding the maximal vitamin output (39.54 mg). The preference for asparagine and arginine by most riboflavin over-producing organisms has been previously reported (1).

Different levels of L-asparagine (0.0 - 6.8 g/l), as the best nitrogen source, were tested (Fig. 5) and the best level for maximum riboflavin output was found to be 1 g/l in the presence of 50 g glucose what gives a C/N ratio of about 110/1. A high C/N ratio and low nitrogen content has been already described for riboflavin production (1).

Effect of salts on riboflavin production

Good growth and riboflavin yields were achieved in the absence of $MgSO_4.7H_2O$ from the fermentation medium (Table 2). Maximum vitamin production was recorded in the presence of 0.5 g/l, indicating that the BM used had supplied adequate amounts of magnesium salts. In a similar way, a low concentration of this salt (0.2 g/l) has been employed in complex media for riboflavin formation by others (12).

The presence of a phosphate mixture in the fermentation medium led to marked increase in growth yield and riboflavin output (Table 2). Maximal riboflavin production was obtained on using a mixture of KH_2PO_4 and K_2HPO_4 (1:1) at a concentration of 5 g/l with an increase of about 39% as compared to the medium lacking phosphate.

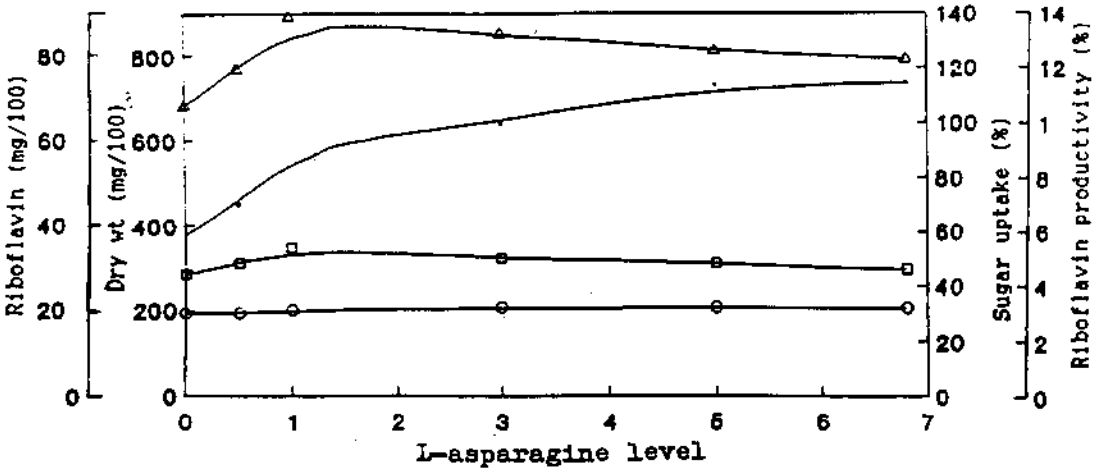

Fig. 5. *Aspergillus terreus* growth (•), sugar uptake (○), riboflavin production (△) and productivity (□) as affected by the L-asparagine level.

The requirement of phosphorus for vitamin formation depends on the organism. A phosphate deficiency stimulates riboflavin production by *Mycobacterium lacticolum*, but not in *Pichia guilliermondii* (16).

The work reported in this investigation shows that molasses is a satisfactory substrate for riboflavin production by *Aspergillus terreus*. The advantage of this fungus over the yeast is its independence of the iron content of the medium which suggests that it might have industrial applications.

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Protein-enrichment of wheat bran using *Aspergillus terreus*

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Summary

Wheat bran was fermented by *Aspergillus terreus* to increase the protein content for use as animal feed. Maximum protein content (55%) and conversion efficiency (59%) were achieved at the late growth phase (8 day-old cultures), when each flask containing 100 ml medium was inoculated with 4% (v/v) spore suspension (3.6×10^5 spore/ml) and shaked at 250 rpm. The best fermentation medium contained (g/l): wheat bran, 10; urea, 1.4; $MgSO_4.7H_2O$, 0.3; KH_2PO_4, 1.0; KC1, 0.1 and was adjusted to pH4.0. Under optimal growth condition, 4 fold increase in protein content was obtained compared to the protein content of the wheat bran.

Key words: Aspergillus terreus, *protein*.

Resumen

Se ha estudiado el enriquecimiento protéico de salvado de trigo destinado a pienso para animales mediante su fermentación con *Aspergillus terreus*. El máximo contenido en proteína (55%) y el mayor índice de conversión (59%) se logró en la última parte de la fase de crecimiento (cultivos de 8 días) cuando el medio se incubó con un 4% de una suspensión de esporas ($3,6 \times 10^5$ esp/ml) y se agitó a 250 rpm. El medio de fermentación más adecuado contenía (g/l) salvado 10, urea 1,4; $MgSO_4.7H_2O$ 0,3; KH_2PO_4 1,0; y KCl 0,1 siendo el PH de 4,0. Bajo condiciones óptimas de crecimiento, el contenido en proteína se multiplicó por un factor de 4 en solución con el del salvado sin fermentar.

Introduction

A great attention has been given to the use of cellulosic wastes for microbial protein production. Of the cellulolytic microorganisms, fungi have been the subject of the most intensive study because of their high cellulolytic activity, simple culturing and higher biomass production (5). A great attention has been recently given to *Aspergillus terreus* for the production of microbial protein, cellulases and xylanases (3,7,8,18).

Wheat bran is a lignocellulosic waste available in enormous amounts in cereal producing countries. In Egypt, it is used as a supplement for animal feeding. The objective of the present article is to increase the nutritional value of wheat bran to be a complete fodder by microbial fermentation with *Aspergillus terreus*. The study presents the effect of cultural factors such as pH, aeration, the size of ino-

TABLE 1
CHEMICAL COMPOSITION OF THE WHEAT BRAN USED IN THIS WORK

Constituents	Dry weight (%)	Elements	Dry weight (mg/g cell)
Moisture content	8.20	Potassium	11.74
Ash content	1.70	Calcium	16.63
Holocellulose	58.17	Sodium	1.14
Lignin	5.70	Magnesium	0.93
Pectin	1.68	Phosphorus	0.73
Crude protein	13.79	Iron	0.08
Total soluble sugars	1.38	Copper	0.04
Lipid	7.40	Manganese	0.03
Undetected	1.98	Cadmium	0.02
		Zinc	0.01
		Lead	0.003

culum and nutrients concentrations on the growth and protein production of *A. terreus* when grown on wheat bran as the sole carbon source.

Materials and methods

Microorganism

Aspergillus terreus used throughout the experiments was supplied by the Department of Botany and Microbiology, Alexandria University.

Wheat bran

Egyptian Wheat bran was dried, finely ground and passed through a 60 gauge mesh sieve to give an homogenous powder and was held in a desiccator containing $CaCl_2$.

The waste was analyzed according to the methods indicated in the references for ash (2), pectic substances (12), holocellulose (10), lignin (17) and lipids (11). Protein was determined using the micro-Kjeldahl method and is given as mg/mg dry wt. The elements were estimated using the Perkin. Elmer 2380 Atomic Absorption Spectrophotometer.

Culture conditions

Pure stock cultures of *A. terreus* were maintained on glucose/peptone agar slants containing in g/l glucose, 20.0; peptone, 5.0; $MgSO_4.7H_20$, 05.5; H_2KPO_4, 1.0 and agar, 15.0 with transfers at monthly intervals. Fermentation was carried out with the following medium (g/l): $NaNO_3$, 2.5; KC1, 0.5; $MgSO_4.7H_20$, 0.5 and KH_2PO_4, 1.0 (5). The wheat bran was incorporated as the sole cellulosic carbon source at a 1% concentration. The organism was allowed to grow in 250 ml Erlenmeyer flasks containing 100 ml medium. The flasks were sterilized by autoclaving at 121 °C for 15 min, inoculated with 2 ml spore suspension (3.6×10^5 spore/ml) of 72 h old cultures and incubated at 30 °C under shaken conditions (250 shakes/min, amplitude 7 cm) for 8 days, unless otherwise stated.

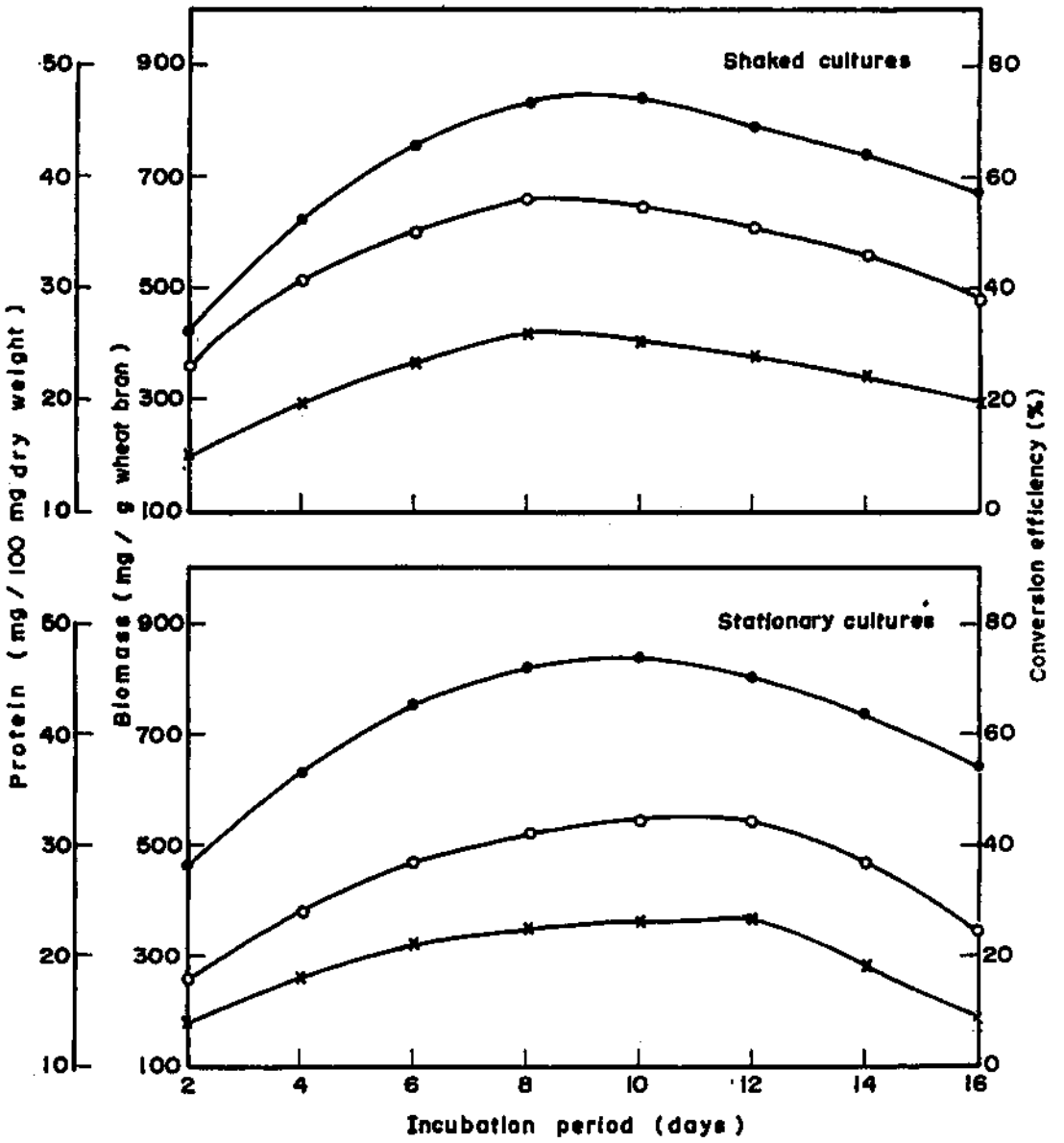

Fig. 1. Biomass (•), protein percentage of dry weight (o) and conversion efficiency (x) of *A. terreus* at different incubation periods under static and shaked conditions.

Analysis

The fermentation residue (residual wheat bran + fungal growth) separated by centrifugation at 1500 x g for 20 min, washed with distilled water and dried at 60 °C to constant weight is referred to as the biomass. The biomass was analysed for its content of crude protein by the microKjeldahl technique (total nitrogen x 6.25). Each treatment was carried out in triplicate and the results obtained throughout this work were the arithmetic mean. The conversion efficiency is estimated as:

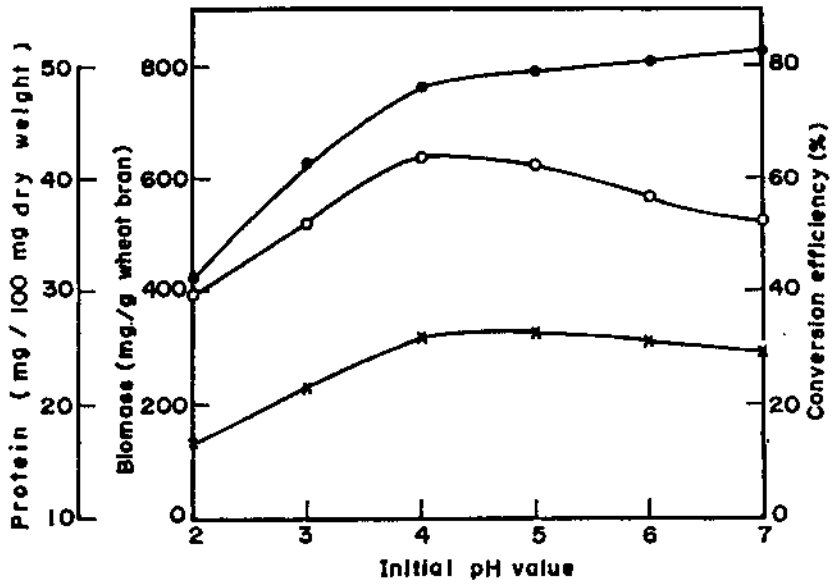

Fig. 2. Biomass (•), protein percentage of dry weight (o) and conversion efficiency (x) of *A. terreus* as influenced by the pH of the medium.

$$\text{Conversion efficiency} = \frac{\text{mg produced protein}}{\text{mg initial holocellulose}} \times 100$$

The optimal conditions from each experiment were used in the subsequent one.

Results and discussion

Analysis of wheat bran

The chemical composition and elements content of wheat bran are represented in Table 1.

Growth Phase Relations

The activities of *A. terreus* were estimated during the different phases of growth under static and shaked culture conditions. The data (Fig. 1) indicate that the crude protein content in shaken cultures reached its maximal value (38%) after 8 days of growth, whereas the crude protein content in stationary cultures reached its maximal value (32%) after 12 days of growth.

The shaken cultures supported higher conversion efficiency (32%) and protein fold increase (2.8) as compared to the stationary cultures. Therefore, in all futher experimentations, an incubation period of 8 days is employed for shaked cultures. *A. terreus* has been used by several workers for the production of protein from cellulosic substrates (3,4,15). In accordance with our results, Broderick and Rhodes (1) stated that the optimal incubation period for maximum protein recovery for *A. terreus* grown on wheat pollard and bran was 16 days under static conditions. Shaked cultures proved to be best for protein production and seven days was the optimal time (4).

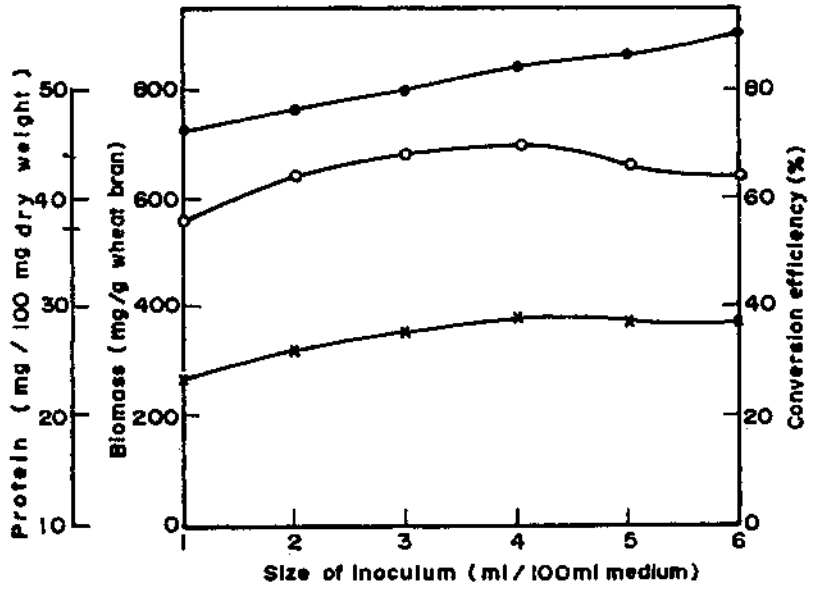

Fig. 3. Biomass (•), protein percentage of dry weight (o) and conversion efficiency (x) of *A. terreus* as influenced by the inoculum size.

pH relation

Aliquots of the basal medium were initially adjusted to pH values from 2 to 7. The analysis was carried after incubating the cultures at 30 °C for 8 days under shaked condition. The data in Figure 2 reveal that the highest crude protein content (42%) was obtained at pH 4.0 as compared to the protein content of 13.29% of the wheat bran, even though maximum biomass production (8-10 mg) was at pH 6.0. The optimum pH for culturing *A. terreus* on wheat bran for protein production was 4 as maximum conversion efficiency (32%) was achieved and three fold increase in protein content was reached. Similar observations have been reported for protein production by *A. terreus* from bagasse (4,17). Other investigators (14) reported an optimal pH range (5.0-5.5) for protein production depending on the fungal species and substrate used.

Size of inoculum

The data represented in Figure 3 show the effect of the size of inoculum on growth and protein production by the tested fungus. At 4% inoculum size, maximum protein yield (45%), conversión efficiency (38%) and three fold increase in crude protein were recorded. Inoculum size has been reported as an important factor for the fermentation of cellulosic substrates, microbial growth and protein production. Similarly, with 5% inoculum, highest protein content was achieved with *A. terreus* grown on alkali-treated bagasse (4) whereas, maximum protein yield and conversion efficiency were recorded with 8% inoculum of *Trichoderma reesei* grown on beet pulp (6).

Effect of wheat bran level

The effect of wheat bran level was tested by using a fixed volume of the nutrient medium (100 ml/flask) and increasing the wheat bran level.

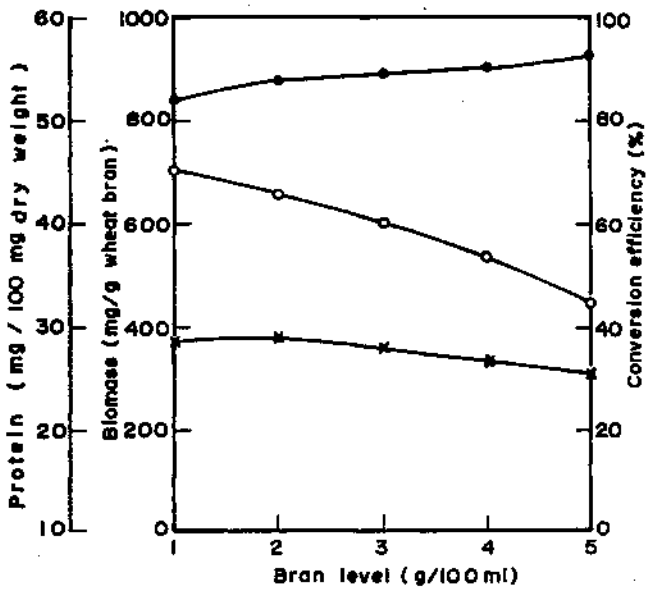

Fig. 4. Biomass (•), protein percentage of dry weight (o) and conversion efficiency (x) of *A. terreus* as affected by the bran level in the medium.

The data in Figure 4 indicate that lg wheat bran/100 ml provide the optimal C/N ratio for protein content (45%) and conversion efficiency (38%) accompanied with a 3.6 fold increase in protein compared to the protein in the wheat bran. The increase in bran level and C/N ratio was accompanied with low protein content. These results agree with those reported by Ghanem *et al.* (6). Kintsurashvili et al (9) reported a 5% substrate as optimal for protein production.

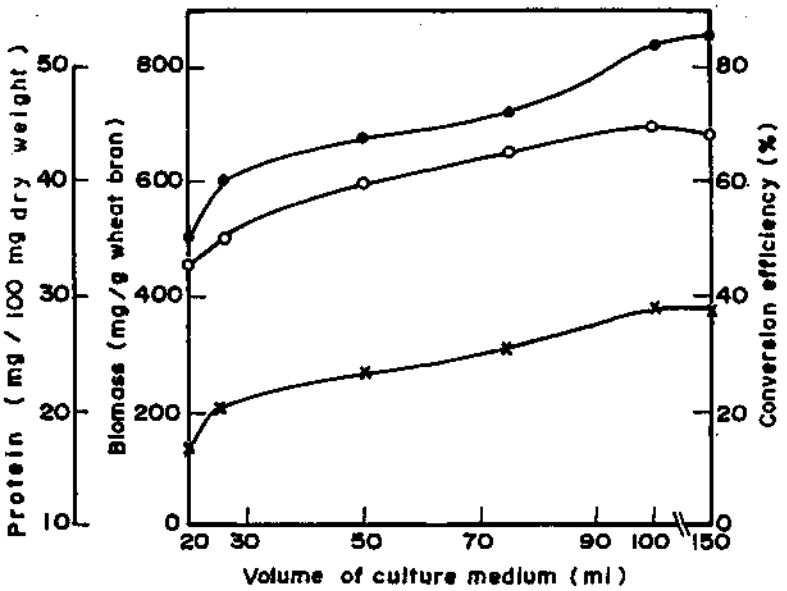

Fig. 5. Biomass (•), protein percentage of dry weight (o) and conversion efficiency (x) of *A. terreus* as affected by the volume of culture medium in flask.

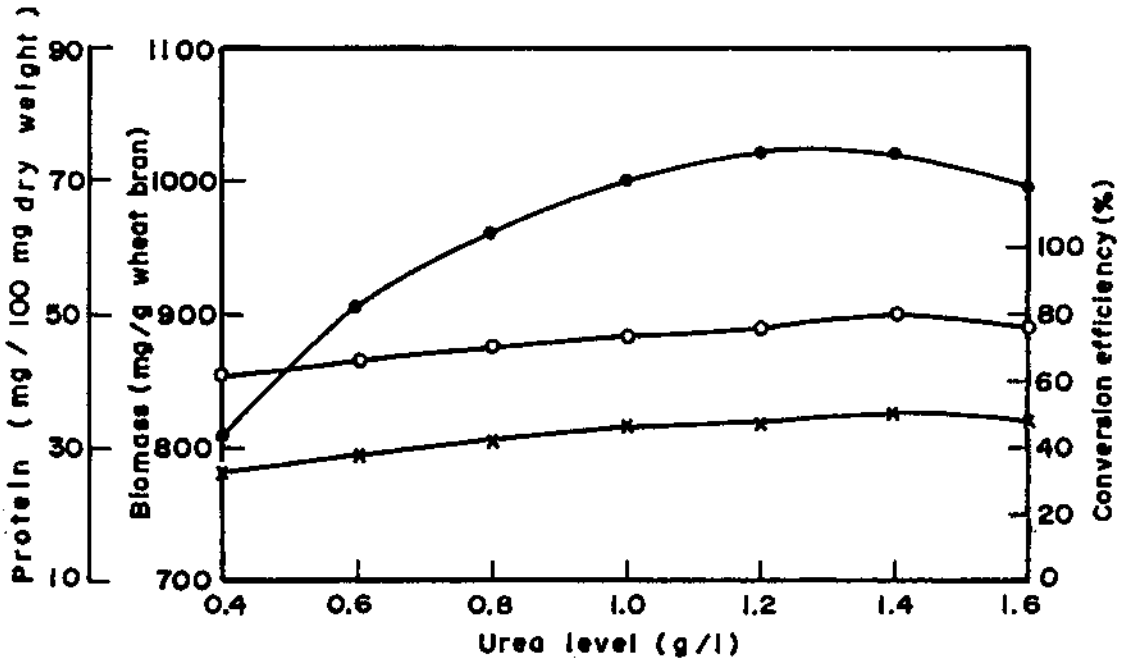

Fig. 6. Biomass (•), protein percentage of dry weight (o) and conversion efficiency (x) of *A. terreus* as influenced by the nitrogen level in the medium.

Effect of volume of culture medium

The effect of aeration was tested by using a constant wheat bran level (1g/100 ml) and increasing volumes of medium.

The data presented in Figure 5 show that as the volume of the broth was increased in the flask, there was considerable increase in crude protein content. However, the highest protein content and conversion efficiency were obtained in cultures grown with 100 ml/250 ml Erlenmeyer flasks (2/5 v/v). Similar results were reported for *A. terreus* grown on alkali-treated bagasse (4). The results also revealed that increasing aeration (decreasing medium volume/flask) is not in agreement with fungal growth and assimilation of wheat bran to proteins.

Nitrogen source and concentration

On an equivalent nitrogen basis, the sodium nitrate in the basal medium was replaced by NH_4NO_3, $NH_4H_2PO_4$, $(NH_4)_2SO_4$, NH_4Cl and urea, one at a time Urea was the best nitrogen source yielding the highest protein content (47%) and conversion efficiency (44%) with a 3.4 fold increase in protein compared to the protein present in wheat bran (13.79%). Urea was previously reported as the optimal N source for protein production by *A. terreus* (9,14). NO_3, NH_4 and peptone were also used (9).

The optimal level of urea supporting highest protein content and conversion efficiency is 1.4 g/L (Fig. 6). Similarly 350 mg N was optimum for *A. terreus* as diammonium hydrogen phosphate (14).

Higher concentration (> 1.4 g/L) has an inhibitory effect on growth and protein content. This might be due to changes in osmotic pressure leading to higher solute molecular concentration outside the fungal cells (6,14). The toxic effect of higher concentrations of urea is probably due to the accumulation of NH_4^+ ions released by the breakdown of urea by the fungal urease.

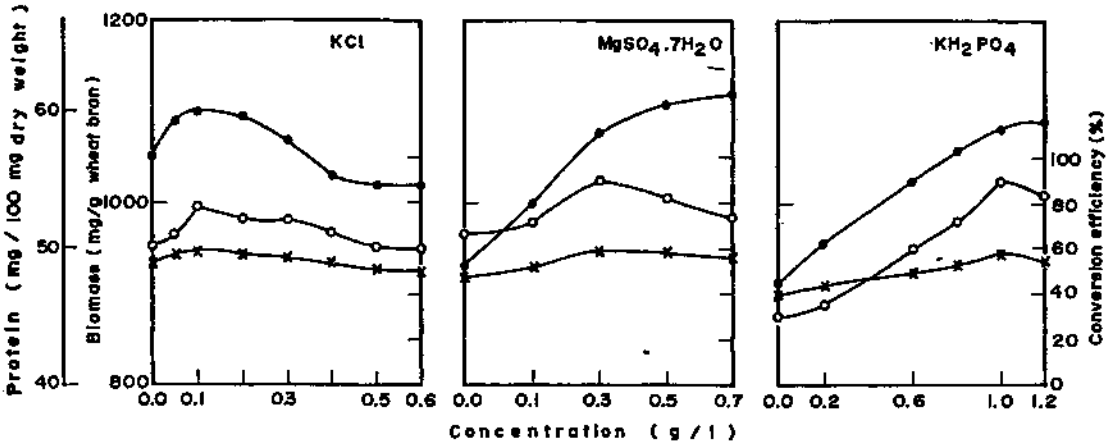

Fig. 7. Biommas (•), protein percentage of dry weight (o) and conversion efficiency (x) of *A. terreus* as influenced by KCl, $MgSO_4.7H_2O$ and KH_2PO_4 concentrations in the medium.

The previous results reveal that by using wheat bran (1%) as substrate, and allowing *A. terreus* to grow under optimum cultural conditions in a medium composed of (g/L): urea, 14; KCl, 0.1; $MgSO_4.7H_2O$, 0.3 and KH_2PO_4, 1.0 and pH initially adjusted to 4, a maximum biomass crude protein content of 55% could be obtained with 4 fold increase in protein compared to that present in wheat bran.

The protein content obtained under our experimental condition is higher than those reported with *A. terreus* grown with other substrate where only 13-15.6% was obtained with solid waste from tea manufacter (9), 23% from sugar cane trash blended (7), 12.2% from corn cobs (14) and 29.8% from alkali-treated bagasse (5). This indicates the possible fermentation of wheat bran using *A. terreus* to increase its protein content to be used as animal feed.

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Lipase production by *Aspergillus niger* under various growth conditions using solid state fermentation

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Summary

Ricinus seed litters were chosen as a cheap carbon source for lipase production by *A. niger* under solid state fermentation (SSF). Maximum lipase production was achieved upon using an enriched (potassium citrate and casein) waste at pH 7.8 and 30°C for 8 days incubation. Nitrogen sources as NH_4Cl, NH_4NO_3, $(NH_4)_2SO_4$, urea and amino acids repressed the lipolytic activity. The chloride salts of Ba^{2+}, Co^{2+}, Cu^{2+}, Fe^{3+}, Hg^{2+}, K^+, Mg^{2+}, Mn^{2+}, Na^+ and Sn^{2+} inhibited, while Zn^{+2} did not affect lipase production. Compounds containing hydrolizable ester group, such as Tween(s), were found to inhibit lipase activity. When the effect of different additives such as EDTA, gum acacia, span(s), mineral and vitamins, were studied, it was found that they all exhibit decreased lipase production by the tested fungus.

Key words: Aspergillus niger, *lipase*.

Resumen

Para la producción de lipasa por *A. niger* se eligieron desechos de semillas de ricino como fuente de carbono barata. La producción máxima de la enzima se logró a pH 7,8 y temperatura de 30°C durante 8 días de vinculación en un sustrato de desechos enriquecidos con caseína y citrato potásico, siendo la relación de desechos/tampón de 3:2 (p/v). Diversos compuestos nitrogenados (NH_4Cl, NH_4NO_3, $(NH_4)_2SO_4$, urea y aminoácidos inhibieron la actividad lipolítica. Asimismo, diversos cloruros (Ba^{2+}, Co^{2+}, Cu^{2+}, Fe^{3+}, Hg^{2+}, K^+, Mg^{2+}, Mn^{2+}, Na^+ y Sn^{2+}) inhibieron la producción de lipasa pero el $ZnCl_2$ no la afectó. Los compuestos con grupo éster hidrolizable, Tweens, inhibieron también la actividad lipásica. Cuando se estudió el efecto de diferentes aditivos (CDTA, goma de acacia, span, minerales y vitaminas) se observó que todos ellos disminuyeron la producción de lipasa.

Introduction

Carbohydrates are generally used as substrates in microbial fermentation for lipase production. However, the utilization of wastes as substrates for lipase production has required an intensive search

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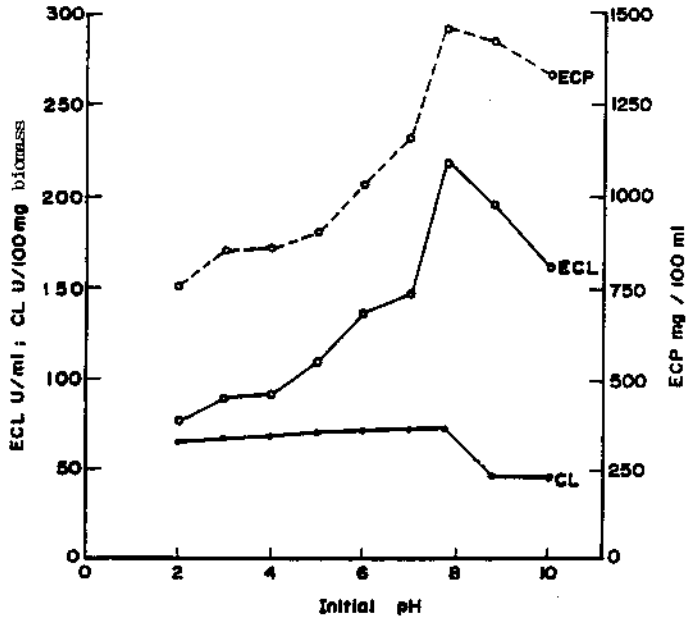

Fig. 1. Production of extracellular lipase (ECL), cell bound lipase (CL) given by 100 mg biomass and extracellular protein (ECP) by *Aspergillus niger* grown for 8 days on ricinus seed litters basal medium adjusted to different initial pH values.

for proper microbial strains capable of supplying large amounts of lipase from oil rich seed litters. The relationship of certain fungi to sun-flower seed and other oil rich seeds and to free fatty acids have been studied by several authors (18).

Lipases are produced throughout the world by submerged fermentation (SF). The presence of the product in low concentration and the consequent handling, reduction and disposal of large volumes of water during down-stream processing in SF are known as cost intensive, highly problematic and poorly understood unit operations (5). Solid state fermentation (SSF) is very effective as the yield of the product is many times higher as compared to that in SF (1) and it also offers many other advantages (10). Our objective was to elaborate the best conditions for production of extracellular lipase (ECL) and cell bound lipase (CL) by *A. niger* V. Tieghem NO: 339340 using a cheap and useless carbon source (rich oil seed litters) under SSF.

Materials and methods

Organism maintenance

The fungus used through out the present work was isolated from ricinus seeds and was identified as *Aspergillus niger* V. Tieghem NO: 339304 by C.A.B. International Mycological Inst. (Surrey, UK). The fungus was maintaned with periodic transfer on glucose peptone agar slants. *Inoculum preparation:* The 5 day old cultures were transferred aseptically into 10 ml sterile distilled water and the spore suspensions were homogenized by shaking with glass beads. The spore suspension was used as the standard inocula at a level equivalent to 1 x 10^7 spores/ml.

TABLE 1
LIPASE ACTIVITIES AND PROTEIN CONTENT AS AFFECTED BY DIFFERENT CARBON SOURCES UNDER SSF USING RICINUS SEED LITTERS.

Carbon sources		Lipase activities				
		Extracellular (ECL) U/ml	Production (%)	Cell bound (CL) U/100 mg biomass	Production (%)	Extracellular protein (ECP) mg/100 ml
Control		356.78		144.73		2027.25
Fructose	1%	344.42	94.2	114.59	79.2	2981.25
Galactose	1%	397.82	108.8	109.83	75.4	3543.75
Glucose	1%	291.02	79.6	170.57	117.9	2543.75
Mannitol	1%	371.12	101.5	115.71	79.9	3331.25
Mannose	1%	371.12	101.5	111.79	77.2	3081.25
Ribose	1%	408.50	111.7	92.26	63.7	3568.75
Xylose	1%	413.84	113.1	65.11	44.9	3581.25
Lactose	1%	392.48	107.3	115.92	80.0	3393.75
Maltose	1%	339.08	92.7	152.44	105.3	2937.50
Sucrose	1%	371.12	101.5	114.19	78.9	3256.25
Raffinose	1%	416.50	113.9	129.92	89.8	3587.50
Starch	1%	389.81	106.6	107.82	74.5	3381.25
Potassium citrate	0.1%	555.85	152.0	152.84	105.6	3700.00
Sodium acetate	0.1%	301.69	82.5	114.35	79.0	2581.25
Sodium malate	0.1%	381.79	104.4	39.32	27.2	3300.00
Sodium succinate	0.1%	349.76	95.6	44.28	30.6	2995.00
α-keto glutaric acid	0.1%	403.16	110.2	29.33	20.3	3475.00
F ratio significance		1.05		8.95*		13.84*
LSD		N.S.		57.58		0.76

Average of three trials * Significant ($P < 0.01$)

Waste material

Ricinus seed litters were obtained from Ricinus and Linum Oils Company (Alexandria, Egypt) after oil extraction. The oil rich seed litters were dried at 40°C in an oven, ground in a willey Mill and the material that passed through 60 mesh sieve collected. The elemental content of ricinus seed litters had been reported (13).

Cell growth and Culture Condition

Enzyme production was carried out by solid state fermentation as reported by Olama and EL-Sabaeny (15). This growth medium had the following components in 250 ml flask: Ricinus seed litters, 15 g; casein, 2 g; potassium citrate, 0.15 g and 10 ml of 0.1 M sodium phosphate buffer (pH 7.8). The flasks were autoclaved for 15 min at 121°C and inoculated with $1x10^7$ spores/ml. The culture flasks were incubated at 30°C for 8 days without shaking. The extracellular lipase (ECL) was extracted at the end of the fermentation period, by the addition of 90 ml of 0.01 M sodium phosphate buffer (pH 7.0)

TABLE 2
LIPASE ACTIVITIES AND PROTEIN CONTENT AS AFFECTED BY DIFFERENT NITROGEN SOURCES UNDER SSF USING RICINUS SEED LITTERS.

Nitrogen sources	Lipase activities				Extracellular protein (mg/100 ml)
	Extracellular (U/ml)	Production (%)	Cell bound (U/100 mg biomass)	Production (%)	
Control	620.80		165.19		3925.00
Ammonium Cl (50 mg N/100 ml) medium	345.85	55.7	60.10	36.0	3537.50
Urea (")	315.78	50.9	62.21	37.0	2262.50
Yeast extract (2%, w/v)	626.54	100.9	184.03	111.4	4825.00
Casein (4%, w/v)	741.10	119.4	192.27	116.4	4400.00
Corn steep liquor (8%, w/v)	350.41	56.4	65.83	39.9	2362.50
F ratio significance	39.63**		2.96**		74.87**
LSD	0.07		35.02		0.42

Average of three trial
* Significance ($P < 0.05$)
** Significance ($P < 0.01$)

to the medium followed by centrifugation at 8.000 rpm for 20 min at 4°C. The supernatant represents the extra-cellular lipase (ECL).

Estimation of lipolytic activity

Lipase activity was measured by a titration method (21). One unit of lipase was defined as the amount of enzyme which liberates 1 μ mole of free fatty acids per min. under test conditions.

Protein content

Protein was estimated by the microKjeldahl technique (17).

Chemicals and Reagents

Vitamin mixture I contained P-amino beinzoic acid, 13.7 mg; Ca-Panthothenate, 47.6 mg; nicotinic acid, 10 mg; pyridoxine HCl, 20.6 mg; riboflavin, 37.6 mg; thamine, 33.7 mg; biotin, 1.0 mg; folic acid, 5.0 mg and distilled water to make 100 ml. Vitamin mixture II contained 0.1 mg of each: Ascorbic acid, biotin, folic acid, riboflavin, and thiamin-HCl, dissolved in 1 ml of 0.1 M phosphate buffer (pH 7.8) and diluted to 100 ml.

Results and discussion

The chemical analysis of ricinus seed litters indicate that, they contain 14.65% lipid; 40.50% holocellulose; 20.31% protein; 10.81% lignin, 4.38% ash; 0.48% sugars, and 0.21% pectin.

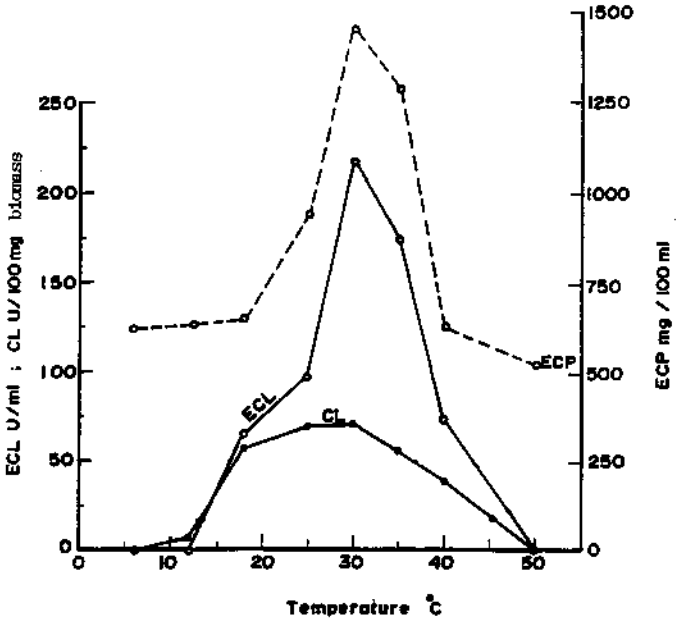

Fig. 2. Production of extracellular lipase (ECL), cell bound lipase (CL) given by 100 mg biomass and extracellular protein (ECP) by *Aspergillus niger* incubated at different temperatures for 8 days.

Data shown in (Fig. 1) revealed that optimal lipase synthesis (ECL) as well as protein production (ECP) were obtained at initial pH 7.8. A noticeable decrease in both lipase (ECL & CL) and protein production were observed at pH 8.8. Higher optima pH were recorded with *Penicillium camemberti* and *Gleosporium olivarium* (6). On the contrary maximum amount of lipase was produced by *Aspergillus wentii* at pH 6.0 (5).

Maximal activities (ECL, 219.10 U/ml; CL, 72.60 U/100 mg biomass; ECP, 1440.00 mg/100 ml) were achieved by incubating the culture at 30°C (Fig. 2). Lower or higher temperatures led to lower productions. These results are in agreement with those obtained with *Penicillium roqueforti* but differs from those of *Mucor mucedo* (20).

The maximum values were reached after 8 days of incubation (Fig. 3). These activities appeared to be related not only with the cell activity but also with the number of cells (23). There was a measurable decrease upon the extension of the fermentation period to 15th day, the loss of enzyme activities may be due to poisoning, denaturating and/or degradation of lipase as a result of interactions with other components or metabolites in the medium. Upon increasing the waste: buffer ratio from 1:1 to 3:2 (60%, w/v), the *A. niger* V. Tieghem ECL values increased under SSF using ricinus seed litters as a waste material, the increase is not significant beyond this ratio (Fig. 4). This ratio 3:2 (%, w/v) is higher as compared to 1:0.6 - 1:1.07 usually employed in SSF involving other metabolites (9).

By addition (0.2 g/30 g waste) of each glucose, fructose or maltose to the medium, the ECL and CL synthesis, correlated with ECP production were slightly repressed (Table 1). This in accordance with the existence of catabolic repression of lipase (3). Other carbohydrates like galactose, mannitol, mannose, ribose, xylose, lactose, sucrose, raffinose and starch increase slightly lipase production. These findings are in accordance with the data obtained with *Penicillium roqueforti* (8). Lipase synthesis by *A. niger* V. Tieghem showed maximum values (52.0% production for ECL and 5.6% for CL) with

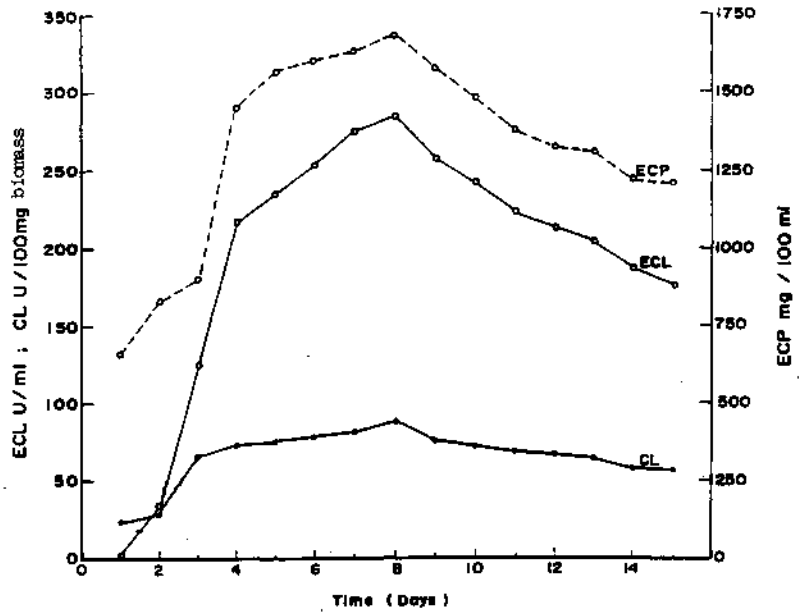

Fig. 3. Production of extracellular lipase (ECL), cell bound lipase (CL) given by 100 mg biomass and extracellular protein (ECP) by *Aspergillus niger* at different time of incubation on ricinus seed litters of the basal medium.

potassium citrate which agrees with the results reported for lipase synthesis by *Syncephalastrum racemosum* which also stimulated by calcium, potassium or sodium citrate (3).

Equivalent amounts of different nitrogen sources were used (Table 2), each in the basal medium to provide 0.5 mg N/ml buffer and the pH was adjusted to 7.8. It was found that NH_4Cl, NH_4NO_3, $(NH_4)_2SO_4$ and urea repressed the lipolytic productivity. The latter two N-sources did not stimulate lipase production by *P. citrinum* (22). None of the tested amino acids was able to enhance lipase secretion, all were repressive to different extents. With the exception of glutamic acid and methionine, those amino acids that supplied N but not C for growth were also found to repress lipase secretion by *Pseudomonas fluorescens* (7). It is suggested that the accumulation of metabolic intermediates may cause repression of lipase production. Among the tested natural nitrogen sources casein (4 g/30 g waste) offered the best fermentation yields with increases of production of 119.4% in ECL and 116.4% in CL% respect of the control. Corn steep liquor at 8% (w/v) was the poorest nitrogen source. Other nitrogen sources tested (peptone, yeast extract, gelatin, oat, ground pea nut, wheat bran and soy bean) were not effective in increasing the production. Peptone has been reported to enhance lipase secretion is *Aspergillus wentii* (2). Corn steep liquor at 8% (v/v) exhibited maximum growth and lipase production of *Syncephalastrum racemosum* while ground nut protein was the poorest nitrogen source (3).

The effect of cations Ba^{2+}, Ca^{2+}, Co^{2+}, Cu, Fe^{3+}, Hg^{2+}, K^+, Mg^{2+}, Mn^{2+}, Na^+, Sn^{2+} and Zn^{2+}) added to the basal medium as chloride salts at 1 mg/ml concentration on lipase production was tested. Only Zinc chloride has no effect on both ECL and CL lipase, whereas the other tested chloride salts significantly inhibited lipase activity. These results differ from those obtained with *Syncephalastrum racemosum* in which the salts of Na^+ and Ca^{2+} stimulated lipase productivity whereas salts of Cu^{2+}, Fe^{3+}, Li^+ and Zn^{2+} were lipase inhibitors (4).

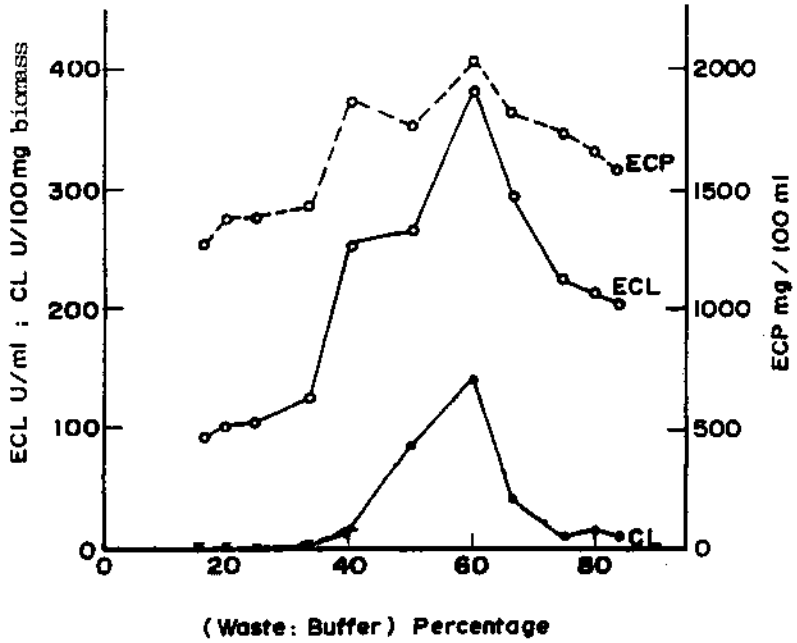

Fig. 4. Production of extracellular lipase (ECL), cell bound lipase (CL) given by 100 mg biomass and extracellular protein (ECP) by *Aspergillus niger* as influenced with ricinus seed litters to phosphate buffer ratio (%, w/v).

Other additives at 0.1% concentration were tested on lipase activities and protein production, EDTA (0.5%), gum acacia (0.7%), span(s), tween(s), minerals and vitamin mixtures I and II at 0.1% concentration were found to decrease lipase production in *Aspergillus niger*. Mates and Sudakevitz (11), suggested that compounds containing a hydrolyzable ester group such as Tweens, inhibit lipase activity of microorganisms which agrees with our results. Contradictory evidence has also been presented by Tabak and Shchelokova (24) who reported that tweens are able to increase the lipase activity of *Oospora lactis*. Nahas (1988) proved that Tweens significantly enhanced lipase production in *Rhizopus oligosporus*. Other authors have reported that tweens are able to induce lipase production (16). The gum acacia at 0.7% concentration reduced lipase synthesis in a 43% (ECL) and 14% (CL) respectively. This is in accordance with the report of Nahas (14) indicating that oils supported good growth and enzyme production when emulsified with gum acacia while gum acacia on its own did not support growth.

The variation in the extracellular lipase (ECL), cell bound lipase (CL) and total extracellular protein (ECP) was significant as evaluated by F-test. The probability that these variations were due to random sampling was lower either at 0.01 or 0.05. LSD test was then applied to evaluate the differences between the members of pairs of production.

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Solid State Fermentation	: SSF
Submerged Fermentation	: SF
Extracellular lipase	: ECL
Cell bound lipase	: CL
Extracellular protein	: ECP
Biomass	: BM

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α-amylase production in lactose medium by *Bacillus circulans* ACB

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Summary

α-amylase production by *Bacillus circulans* ACB was studied in various cultural conditions. During nutrient optimisation, it was found that 2% lactose can be utilized by the strain as source of carbon providing better growth and enzyme yields than starch. Ammonium sulfate of the basal medium (10) can be replaced by ammonium nitrate for better growth and α-amylase activity. The strain demonstrated significant enhancement in α-amylase production when grown at pH 6.6.

Key words: α-amylase, Bacillus circulans, *lactose.*

Resumen

La producción de α-amilasa por *Bacillus circulans* ACB ha sido estudiada en varias condiciones de cultivo. Durante la búsqueda de los nutrientes óptimos, se ha encontrado que lactosa al 2% es una fuente de carbono que produce mejor crecimiento y poder enzimático que el almidón. El sulfato amónico del medio base (10) puede ser reemplazado por nitrato amónico para un mayor crecimiento y actividad de la α-amilasa. La cepa mostró la máxima producción de α-amilasa a pH 6,6.

Introduction

Amylolytic enzymes find wide application in various starch processing industries. α-amylase is an endoenzyme, which attacks the starch molecule randomly at the α-1,4 glycosidic linkages, producing dextrin from amylopectin, and maltose, maltooligosaccharides or glucose from amylose. Amylases are widely used in the drink, food, textile, biogas and pharmaceutical industries.

Enzymatic starch hydrolysis has clear advantages over acid hydrolysis, as it does not need high temperature, pressure nor very low pH. Many amylase producing microorganisms have been tested for industrial starch hydrolysis. Among them, those from the genus *Bacillus* are of immense importance for its thermostability.

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TABLE 1
SUITABILITY OF DIFFERENT TYPES OF MEDIUM FOR GROWTH AND α-AMYLASE PRODUCTION BY *B. CIRCULANS* ACB

Medium	Growth (OD 650)	Amylase activity Unit/ml
Robinson	0.103	3.5
Makula and Finnerty	0.115	3.7
Tokoro	0.100	3.3
Takasaki	0.087	2.3
Sata and Maruyama	0.105	3.6
Groom and White	0.108	3.5

Many fungi including *Rhizopus* (24), *Aspergillus* (3, 29), *Penicillium* and bacteria including *Bacillus* (11, 25), *Clostridium* (23) are known to produce amylases extracellularly. Most of previous work on *Bacillus* amylases had been done on *B. subtilis* (12, 14, 16, 27), *B. amyloliquefaciens* (8, 19), *B. licheniformis* (7, 11, 31). Comparatively few studies have been done on *B. circulans*. This communication tries to improve cultural conditions for α-amylase production by *Bacillus circulans* strain ACB.

Materials and methods

Microorganism used

The microorganism used in this study is a soil isolate, identified as *Bacillus circulans* ACB (4). Stock cultures were maintained in agar slants at 4°C.

Culture conditions

The selected strain was tested in six different media (8, 10, 17, 21, 25, 26). For further use, the medium of Makula and Finnerty (10) was chosen. It contained (g/l), $(NH_4)_2SO_4$ - 2.0, KH_2PO_4 - 4.0, Na_2HPO_4 - 4.0, $MgSO_4 : 7\ H_2O$ - 0.2, $CaCl_2$ - 0.001, $FeSO_4 : 7\ H_2O$ - 0.004, Glucose - 10.0, pH 7.0. To study the effect of pH Sodium-phosphate buffer (20 mM) was used to regulate the pH of the medium between 6.0 and 8.0. All six media were supplemented with 0.1% soluble starch to stimulate the production of α-amylase.

TABLE 2
SUPPLEMENTATION OF THE SELECTED MEDIUM FOR GROWTH AND α-AMYLASE PRODUCTION BY *B. CIRCULANS* ACB.

Medium supplemented	Growth (OD 650)	Amylase activity Unit/ml
Basal medium + 0.1% yeast extract	0.120	3.9
Basal medium + 0.1% casamino acid	0.130	4.6
Basal medium + 0.1% yeast extract + 0.05% casamino acid	0.122	4.0
Basal medium + 0.05% yeast extract + 0.1% casamino acid	0.126	4.4
Control (Basal medium only)	0.116	3.7

TABLE 3
SUITABILITY OF CARBON SOURCE FOR GROWTH AND α-AMYLASE PRODUCTION BY *B. CIRCULANS* ACB

Carbon source (1% W/V)	Growth (OD 650)	Amylase activity Unit/ml
Glucose	0.130	4.6
Ribose	0.135	5.0
Fructose	0.107	3.6
Galactose	0.124	4.2
Arabinose	0.102	3.3
Xylose	0.158	5.9
Mannitol	0.140	5.1
Maltose	0.104	3.3
Sucrose	0.128	4.5
Lactose	0.235	7.9
Mesoinositol	0.100	3.2
Starch	0.190	6.8
Control	0.005	0.0

In the supplementation experiment, the stock solution of casamino acids and yeast extract were separately sterilized by bacterial filter and aseptically added to the medium as required.

The carbon source solutions were also sterilized separately by filtration. pH of the medium was regulated using sodium-phosphate buffer as mentioned before. The cultures were incubated for 48 hours at 30 °C in a rotary shaker (180 rpm). Growth of the organism was determined at 650 nm in Systronics (India) colorimeter.

Assay methods

α-amylase activity was determined by cell-free culture fluids obtained by centrifugation of the culture medium (at 4500 x *g* for 15 minutes) by using the modified method of Bernfeld (8). Absorbance was recorded at 650 nm. The samples reducing groups concentration was calculated from a maltose standard curve.

TABLE 4
SUITABILITY OF NITROGEN SOURCE FOR GROWTH AND α-AMYLASE PRODUCTION BY *B. CIRCULANS* ACB

Nitrogen source (1% W/V)	Growth (OD 650)	Amylase activity Unit/ml
$(NH_4)_2SO_4$	0.240	8.4
NH_4Cl	0.236	8.2
NH_2-CO-NH_2	0.237	8.2
KNO_3	0.235	7.9
NH_4NO_3	0.262	9.3
$NaNO_3$	0.230	7.6
Control	0.005	0.0

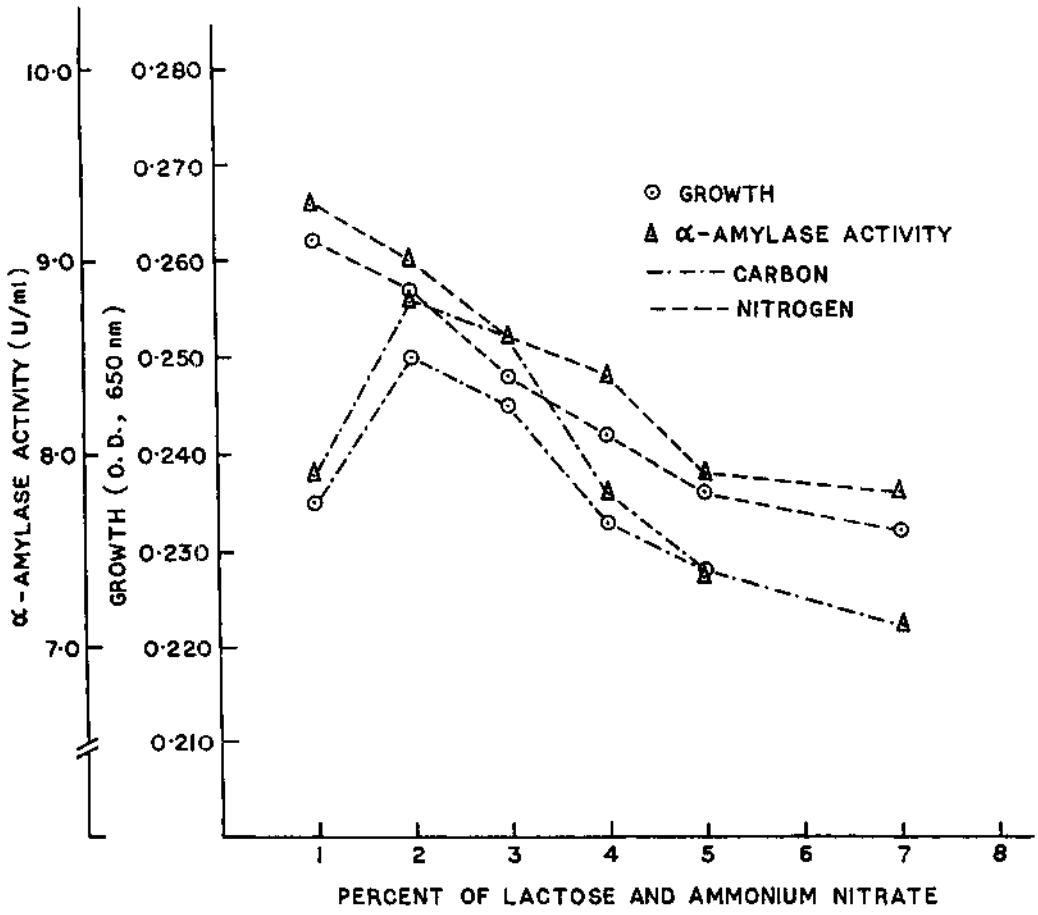

Fig 1. Effect of different concentration of Lactose and Ammonium nitrate on growth and α-amylase activity.

α-amylase activity in the culture broth was expressed as unit/ml. One unit (U) of α-amylase activity is defined as the amount of enzyme releasing 1 mg of reducing sugar expressed as maltose equivalent during a three minute reaction under standard conditions (8).

Results and discussion

Bacillus circulans ACB was grown in the six different media for growth and α-amylase production, it was found (Table 1) that the medium of Makula and Finnerty (10) was the most suitable for the *B. circulans* ACB strain. The remaining media except that of Takasaki showed slightly lower activity.

Saha *et al* (20) and Shen *et al* (23) have shown that supplementation (0.5% maltose and 0.5% soluble starch respectively along with their regular growth medium) could help for enhanced production of β-amylases. To determine whether growth factor supplementation could affect the α-amylase yields, the selected basal medium was enriched with yeast extract and casamino acids in varying concentrations and combinations. The experimental results in Table 2, showed casamino acids were more effec-

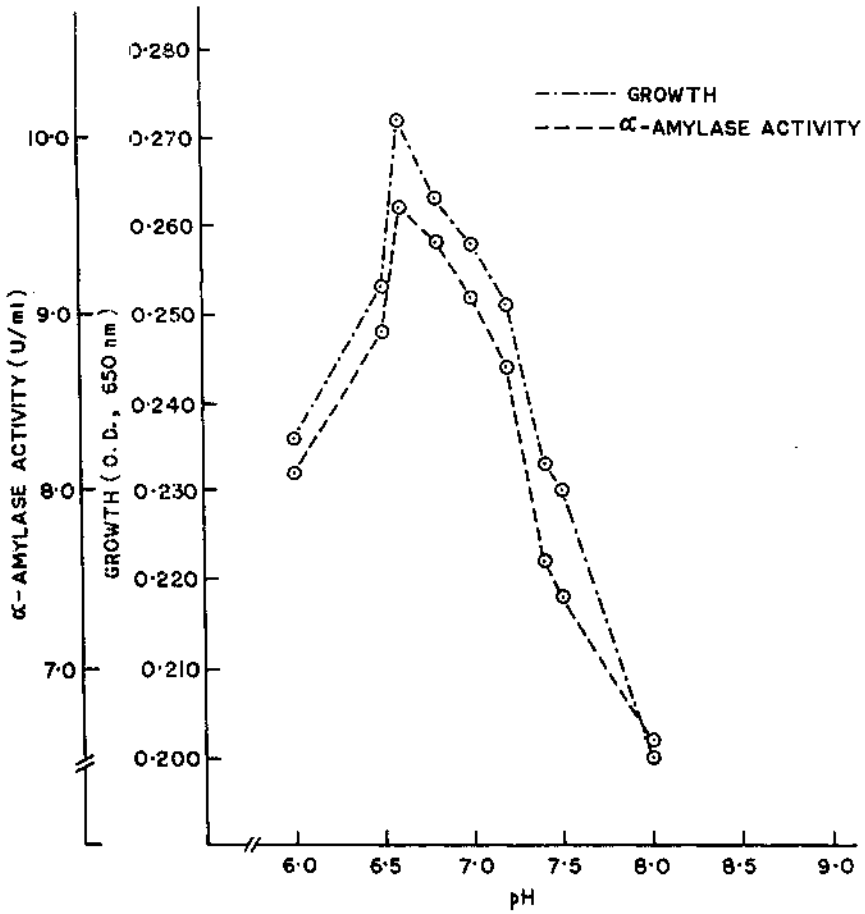

Fig 2. Effect of pH on growth and α-amylase activity.

tive for both growth and amylase production than yeast extract. 0.1% casamino acids be able to provide 24% increase in the extracellular α-amylase activity.

Eleven carbon sources were then tested to find out the most suitable one as shown in Table 3, all the sugars used were able to support growth and amylase production, lactose giving the best results especially when used at a concentration of 2% (Fig. 1). Starch also behaved as a good inducer. These results indicate that production of amylase by the strain ACB is constitutive and not catabolically-repressible. In most of the published studies on carbon sources in this context, starch was found to be the optimal carbon source (8, 9, 18, 27). Maximum yield by utilizing maltose was observed in *Bacillus subtilis* (30), *B. circulans* F-2 (22) and glucose was observed in *B. cereus* (13).To check whether the alternative nitrogen source does have any relevant effect, several kinds of inorganic nitrogen compounds (six) were tested (Table 4), substituting the original nitrogen source of the basal medium and using 2% lactose as carbon source. All nitrogen compounds were added in such an amount to match the nitrogen content of the ammonium sulfate initially present in the basal medium. Of the several nitrogen sources tested, ammonium nitrate was the only one giving better result, near about 11%, than the original nitrogen source. Optimal ammonium nitrate concentration was found to be 1% (nitrogen content equivalent, Fig. 1). Some authors

have reported, for in some strains of *Bacillus (B. subtilis, B. amyloliquefaciens, Bacillus* sp. H-167) peptone as the best nitrogen source for amylase production (2, 9, 16). Other strains, in the presence of different carbon sources do prefer ammonium sulfate as a nitrogen source (1, 15, 19, 21).

To find out the optimum pH for α-amylase production by the strain ACB, it was grown at different pH range of 6.0 to 8.0. The results (Fig. 2) showed that at pH 6.6, the strain produced the maximum α-amylase activity. α-amylase activity decreased when increasing de pH. It has been reported that bacterial α-amylases are most active in the pH range 5.0-8.0 (6, 28, 31) *B. circulans* ACB can withstand a wide range of pH, i.e. from 6.0 - 8.0, but maximum activity was registered when grown at pH 6.6.

It is worth mentioning that the strain can withstand a wide range of changed physicochemical conditions. It is further to note that the production is growth dependent. As the lactose emerged for the best source of carbon, it may be recommended that the whey or whey permeate can provide good growth hence production.

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MICROBIOLOGIA SEM 9 (1993), 149-152 **NOTA**

Propiedades tóxicas de los *Escherichia coli* enteroinvasivos

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Summary

Up to now enteroinvasive *Escherichia coli* (EIEC) has not been extensively studied for toxin production. We have investigated 15 EIEC strains belonging to ten representative serotypes (O28ac:H-, 029:H-, O112ac:H-, O124:H-, O136:H-, O143:H-, O144:H-, O152:H-, O164:H- and O167:H-) for their ability to produce enterotoxins (LT and STa), Shiga-like toxins (SLT-I and SLT-II), cytotoxic necrotizing factors (CNF1 and CNF2) and other toxic products with necrotic or lethal activity. None of these strains were toxigenic.

Key words: E. coli, *enteroinvasive, toxins, virulence.*

Resumen

La producción de toxinas en los *Escherichia coli* enteroinvasivos (ECEI) ha sido poco investigada. En este estudio hemos ensayado 15 cepas de ECEI pertenecientes a diez serotipos representativos (O28ac:H-, O29:H-, O112ac:H-, O124:H-, O136:H-, O143:H-, O144:H-, O152:H-, O164:H- y O167:H-) para la producción de enterotoxinas (LT y STa), citotoxinas relacionadas con la toxina Shiga (SLT-I y SLT-II), factores necrosantes citotóxicos (CNF1 y CNF2) y otros productos tóxicos con actividad necrótica o letal. Ninguna de las cepas ensayadas resultó ser toxigénica.

Algunas cepas de *Escherichia coli*, que pertenecen a un reducido número de serogrupos (O28ac, O29, O112ac, O124, O136, O143, O144, O152, O164, O167, O171 y O173), son capaces de causar una disentería clínicamente semejante a la provocada por *Shigella;* caracterizada por dolor abdominal, fiebre y frecuentes deposiciones sanguinolentas o mucoides. A estos *E. coli* se les denomina enteroinvasivos ya que, tras establecer contacto con el epitelio del intestino grueso, invaden los enterocitos ocasionando una infección ulcerativa localizada (2,5). Los *E. coli* enteroinvasivos (ECEI) pueden ser confundidos con *Shigella spp.* debido a que normalmente no son móviles (H-), son lisina descarboxilasa negativos, algunos tampoco fermentan la lactosa y además suelen poseer antígenos O que están relacionados inmunológicamente con los de *Shigella* (8, 14, 15). Sansonetti *et al.* (13) y Hale *et al.* (10) demostraron que la capacidad invasora de *E. coli* y de *Shigella spp.* depende de plásmidos no conjugativos, de 120 a 140 Md, que codifican para una serie de proteínas de la membrana externa que interac-

(*) A quien debe dirigirse la correspondencia.

cionan con los receptores de las células intestinales y facilitan la entrada del microorganismo en los enterocitos. La producción de toxina Shiga es un determinante de la virulencia, fundamentalmente en *Shigella dysenteriae* Tipo 1. Aunque algunas cepas de *E. coli* producen toxinas relacionadas funcional e inmunológicamente con la toxina Shiga, las cuáles se denominan *Shiga-like toxins* (SLT-I y SLT-II) o verotoxinas (VT1 y VT2) (11), los estudios realizados por Cleary y Murray (6) y Fasano *et al.* (9) indican que los ECEI no sintetizan estas citotoxinas. Recientemente, nosotros hemos demostrado que aislados clínicos de *E. coli* de origen humano y animal pueden producir dos nuevas citotoxinas con actividad necrosante a las que hemos denominado CNF1 y CNF2 (7). Como la producción de toxinas ha sido muy poco estudiada en los ECEI, hemos decidido investigar la síntesis de CNF1 y CNF2 y otros productos tóxicos en 15 cepas enteroinvasivas pertenecientes a diez serotipos representativos.

Hemos ensayado 15 ECEI de referencia que nos fueron amablemente cedidos por L. R. Trabulsi (Escola Paulista de Medicina, Sao Paulo) y por F. Ørskov e I. Ørskov (Statens-Seruminstitut, Copenague). Como cepas control se emplearon cuatro *E. coli* toxigénicos que producían LT, STa, SLT-I, SLT-II, CNF1 y/ó CNF2. Todas las cepas se conservaron a temperatura ambiente en agar nutritivo con un 0,75% (p/v) de agar. Para la producción de toxinas las cepas se sembraron en 5 ml de caldo triptona-soja (pH 7,5) (Oxoid) contenidos en matraces erlenmeyer de 50 ml y se incubaron durante 20 h a 37 °C con una agitación mecánica de 200 rpm. Los extractos sónicos, los fluidos extracelulares y los extractos con mitomicina C se obtuvieron como describimos previamente (3, 4). Estos tres tipos de preparaciones fueron inoculadas en monocapas de células Vero y HeLa para la detección de la enterotoxina termolábil (LT), *Shiga-like toxins* (SLT-I y SLT-II) y factores necrosantes citotóxicos (CNF1 y CNF2) (4). En el momento del ensayo se renovó el medio de cultivo (0.5 ml por pocillo) y se añadieron 50 µl de extracto sónico, fluido extracelular o extracto con mitomicina C. Tras 24 y 48 h de incubación (37 °C/5% CO_2) se realizó la lectura en fresco con un microscopio de contraste de fases (4). Para la actividad necrótica y la modificación de la permeabilidad vascular, se inyectaron intradérmicamente en la piel de cuatro conejos albinos de Nueva Zelanda 0,1 ml de extracto sónico. Tras 24 h de incubación dos de los consejos se sacrificaron y se evaluó la existencia de necrosis. A los otros dos conejos se les administró intravenosamente colorante azul de Evans al 2% (p/v) en solución salina (ClNa 0,85%) hasta alcanzar una concentración de 2 mg de colorante por Kg de peso. Se dejó difundir el colorante durante 30 min y seguidamente se sacrificaron los animales y se midieron las zonas de acumulación del colorante (4). Los extractos sónicos también se administraron intraperitonealmente a lotes de cinco ratones adultos (25-30 g) de la raza BALB/c, a razón de 0,5 ml por ratón; y durante un período de una semana se contabilizó el número de muertes (3). La detección de la enterotoxina termoestable STa se llevó a cabo administrando oralmente 0,1 de fluido extracelular a ratones lactantes menores de tres días de edad (1). Por último, la producción de α-hemolisina se realizó en agar sangre base con un 5% (v/v) de eritrocitos de carnero lavados dos veces. Todos estos ensayos se han descrito con detalle en publicaciones previas (1, 3, 4, 7).

Como puede verse en la Tabla 1, ninguno de los 15 ECEI examinados en este estudio sintetizó las enterotoxinas LT y STa, las *Shiga-like toxins* SLT-I y SLT-II, los factores necrosantes citotóxicos CNF1 y CNF2, ni otros productos con actividad necrótica o letal. Al igual que nosotros, Cleary y Murray (6) tras evaluar 30 ECEI no detectaron la producción de citotoxinas. Sin embargo, Fasano *et al.* (9) encontraron que los 35 ECEI ensayados por ellos producían bajos niveles de verotoxinas (citotoxinas que destruyen las células Vero) genética e inmunológicamente diferentes a las SLT-I y SLT-II producidas por los *E. coli* enterohemorrágicos. Además, encontraron que los fluidos extracelulares y los extractos sónicos de sus cepas enteroinvasivas poseían una moderada actividad enterotóxica en asas ligadas de intestino de conejo. Después de fraccionar el material en una columna de cromatografía líquida de alta presión, observaron que la máxima actividad enterotóxica quedaba retenida en la fracción de 68.000 d a 80.000 d, mientras que las verotoxinas citotóxicas eran detectadas en la fracción menor de

TABLA 1.
PRODUCCION DE TOXINAS EN CEPAS DE *E. COLI* ENTEROINVASIVAS

Cepa	Serotipo	Hly[a]	Ensayo en piel de conejo[b]	Ratones inyectados intraperitonealmente[c]	Ratones lactantes[d]	Células Vero	Células HeLa
E. coli enteroinvasivos							
22-84-HM	028ac:H-	—	0/4	2/5	0,073	—	—
127-82 FAV	029:H-	—	0/4	0/5	0,072	—	—
GUANABARA	0112ac:H-	—	0/4	1/5	0,068	—	—
280-83 FAV	0124:H-	—	0/4	1/5	0,074	—	—
1111-55	0136:H-	—	0/4	0/5	0,071	—	—
WMP-83 HSP	0136:H-	—	0/4	2/5	0,060	—	—
4608-58	0143:H-	—	0/4	0/5	0,063	—	—
122-83 LIAC	0143:H-	—	0/4	0/5	0,057	—	—
1624-56	0144:H-	—	0/4	0/5	0,075	—	—
88-82 FAV	0144:H-	—	0/4	0/5	0,073	—	—
1184-68	0152:H-	—	0/4	0/5	0,069	—	—
288-83 HV	0152:H-	—	0/4	2/5	0,065	—	—
DRL 145/46	0164:H-	—	0/4	0/5	0,061	—	—
SAIGON	0164:H-	—	0/4	0/5	0,070	—	—
226-83 FAV	0167:H-	—	0/4	0/5	0,079	—	—
Cepas control de referencia							
m452-C1	063:H-	—	4/4 (MP)	0/5	0,135 STa	LT	—
H19	026:H11	—	0/4	5/5	0,065	SLT-I	SLT-I
933	0157:H7	—	0/4	5/5	0,060	SLT-I y II	SLT-I y II
MR249	06:K-	+	4/4 (NE)	5/5	0,071	CNF1	CNF1
B26a	0123:H16	—	4/4 (NE)	5/5	0,068	CNF2	CNF2

[a] Hly = α-hemolisina.

[b] Número de conejos positivos / Número de conejos inyectados intradérmicamente. MP = modificación de la permeabilidad y NE = necrosis.

[c] Número de ratones muertos / Número de ratones inyectados intraperitonealmente. Se considera que existe actividad letal cuando mueren al menos tres de los cinco ratones inyectados.

[d] Los coeficientes de enterotoxigenicidad superiores a 0,100 son indicativos de la producción de la enterotoxina STa.

30.000 d. Tal vez si nosotros hubiésemos concentrado nuestras preparaciones habríamos podido detectar las citotoxinas y enterotoxinas encontradas por Fasano *et al.* (9). No obstante, cabe preguntarse cuál es el significado real de una producción de toxinas a tan bajo nivel como la detectada por estos investigadores. Nuestra experiencia nos indica que si concentramos las preparaciones, al final tanto las procedentes de cepas patógenas como las de cepas no patógenas terminan poseyendo actividad toxigénica.

Los ECEI son raramente aislados en nuestro país de casos esporádicos de diarrea (12). No obstante, han provocado dos brotes importantes de disentería en Cataluña en la década de los ochenta (Marne, C. *et al.* 1988. III Congr. SEIMC p. 225).

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