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NITRATE LEVELS AND STAGES OF GROWTH IN HYPERNODULATING MUTANTS OF *LUPINUS ALBUS*. I. N_2 FIXATION POTENTIAL

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ABSTRACT

This work aimed to evaluate physiological parameters, nodulation response and N_2 fixation rate in mutants of *Lupinus albus* in comparison with the standard Multolupa cultivar. Two nitrate levels (0 and 5mM) and two evaluation periods (7 and 10 weeks) were used. Significant differences were observed among genotypes, in relation to fresh nodule weight, nitrate levels and growth stages. The overall average for nitrate level differed between them where 5mM severely inhibited the number of nodules, reaching a 49.5% reduction in relation to treatment without nitrate. There were no behaviour differences among genotypes, nor among evaluation periods. Although the level of nitrate did not influence the production of shoot dry matter in relation to the average among levels applied, the L-135 genotype, being an inefficient mutant, reached very low values. There were no significant differences in electron allocation coefficient (EAC) among nitrate levels, nor among genotypes studied. However, the evaluation periods revealed differences, where the EAC for the seventh week had a higher value than that for the tenth week, when a 5mM aplication was evaluated. The N_2 fixation rate (N_2 FIX) showed the existence of the nitrate interference in fixation, given that the application of 5mM severely reduced. However, there were no differences among the genotypes and it was noted that the fixation rate was much higher in those that received nitrate. The L-88 and L-62 genotypes were the ones that have shown best adaptability in this experiment, thus being able to be recommended for new studies with higher nitrate levels and different evaluation periods. The nitrate (5mM) interferes in the nitrogen fixation rate, given that all the genotypes were affected by the level applied.

Key words: Mutants, electron allocation coefficient, nitrogen fixation rate, *Bradyrhizobium* sp (*Lupinus*).

INTRODUCTION

Multiple effects of combined N on the legume nodulation function are conveniently divided into three classes: (a) The detrimental effect on the infection of legume roots by *Rhizobium*; (b) The negative effect of N on nitrogenase activity; c) The influence on nodule weight per plant (14). However, application of combined N to legumes may be required for maximization of yields and, therefore,

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economically justified. The mechanisms ruling the adverse effects on nodulation must be understood and controlled in such away that a manner of correcting the problem may be devised. Several forms of environmental stress and the supply of mineral N reduce nodulation and nitrogenase activity without altering the total N concentration in the plant tissue.

The inhibitory effects of nitrate could be accounted for by alterations in the partitioning of reducing sugars in soybean (16), alleviation of nitrate inhibition on nitrogenase activity by sucrose has been for intact pea plant. The reduction in carbohydrate transport to nodules could simply be a result of the loss of nitrogenase activity, i.e. the decline in photosynthates might lag behind the loss of activity in nitrate treated plants. According to Streeter (14), carbohydrate composition was similar in both high nitrate and zero nitrate treatments, suggesting that nitrogenase inhibition is linked to the availability of carbohydrates. Otherwise, it is well known that nitrate limits the infection process, the development of nodules, the subsequent expression of nitrogenase activity in bacteroids as well as hastening the breakdown of nodule tissue (6). The NO_3^- inhibited nodulation in nts (nitrate tolerant symbiosis) mutants. The difference is probably due to the different nitrate levels used, cultivation conditions, NO_3^- addition methods and growth period (4). The same authors showed that all mutants were sensitive to nodulation and N_2 fixation repression at high NO_3^- levels and suggest that the NO_3^- tolerant phenotype is the result of a failure in the self-regulation mechanism and not the mechanism responding to the NO_3^- in itself.

The influence of nitrate remains unclear, especially in the initial stages of nodulation, where there is no evidence as to whether the effects are due to the external presence of nitrate or to the events that occur following uptake and/or metabolism. Cho and Harper (3) provided evidence that the site of N-application primarily controls nodulation inhibition, probably through a decrease in the internal levels of root isoflavonoids. Also, the authors provided clear evidence that nitrate has an adverse effect on roots isoflavonoid compounds. A possible direct effect of nitrate on nodulation has been indicated by split-root studies with soybean, and by excised root experiments with common bean. In both kinds of research, Raggio *et al.* (12) demonstrated a positive nitrate and carbohydrates interaction on rhizobial root nodule formation. In another work, the incorporation of ammonium nitrate near the nodule zone (crown region) was more inhibitory to nodulation than was its placement below this zone (7).

This report compares the selected physiological parameters, nodulation response and N_2-fixation rate of the parent wild type *Lupinus albus* cv. Multolupa to two hypernodulating mutants, L-280 and L-88, one nitrate resistent mutant L-62, and one inefficient mutant L-135. Comparisons among cv. Multolupa and mutants at two growth stages are also reported.

MATERIALS AND METHODS

The following *Lupinus albus* plant materials were used (Table 1), cultivar Multolupa (Wild-type, nod^+ fix^+) and mutants selected from H_2 families derived from Multolupa seed treated with ethyl methanesulfonate (0.04M), designed L-280, L-88, and treated with sodium azide (0.002M), designed L-62 and L-135. According to previous work done by C.I.D.A., Centro de Investigación y Desarrollo Agrario, Sevilla – Spain, the main characteristics of

Table 1. Characteristics of the *Lupinus albus* cv. Multolupa mutants.

Species	Selection year	Product utilized for mutagenesis process	Genotypes	Characteristics
Lupinus albus	1988	[1]EMS	280	High nodulation and Nif^+
Lupinus albus	1988	EMS	88	High nodulation and Nif^+
Lupinus albus	1989	[2]NNA_3	62	Resistant to NO_3^-
Lupinus albus	1989	NNA_3	135	Inefficient (f)
Lupinus albus cv. Multolupa	-	-	-	Control

[1]EMS - Ethyl methane sulfonate
[2]NNA_3 - Sodium azide

the cv. Multolupa mutants used in the study were: L-280 nod^+ fix^+; L-88 nod^+ fix^+; L-62 nod^+, resistant to NO_3^- and inefficient L-135 (nod^- fix^-).

Seeds of *Lupinus albus* cultivar Multolupa and selected mutants were scarified and surface sterilized by treating with ethanol (70%) for 20 min followed by 2 washes in sterile distilled water, before being planted in 2.5l Leonard jars using perlite as growth medium. After uniform germination of seedlings, three plants per jar remained for the experimental period. Innoculation was carried out twice, at planting and one week later, by placing 5 ml of a suspension containing 10^7-10^8 cells per ml of a strain mixture of *Bradyrhizobium* sp (*Lupinus*), namely L-750 and L-18C_2, with constitutive nitrate reductase, which belong to the C.I.D.A. collection.

The experiment was carried out during Autumn 1992, in a greenhouse at a temperature of 25°C during the day and 15°C at night. A N-free solution (4) was periodically supplied to the jars, and the nitrate level was built up by adding the appropriate volume of 5mM KNO_3. Additions of nutrient solution and KNO_3 were necessary for maintenance of the rooting medium as well as the combined N at the desired level.

The experiment was conducted according to a completely randomized block design, with four replications. Treatments were formed by a factorial arrangement with five genotypes, two growth stages (7 and 10 weeks) and two nitrate levels (0 and 5mM). F-tests for significance (0.05 level) were based upon ANOVA; where F-tests were significant, the LSD values were calculated to compare treatment means.

The plants were harvested at two growth stages, 7 and 10 weeks after emergence. At each harvest, the apparent nitrogenase activity (ANA) and total nitrogenase activity (TNA) were determined by measurements of H_2 evolution in air and ArO_2, respectively, of the whole root system incubated for 30 min., using a FI detector and a Poropak R column in a Shimadzu GC model RIA, as described in Chamber-Perez and Iruthaythas (1988). The N_2 fixation rate (NFR) was calculated = TNA-ANA/3 and also the electron allocation coefficient of nitrogenase (EAC)=(1-TNA/ANA) according to Hunt *et al.* (1987). Shoots and roots were dried (70°C for 72 hours) and weighed, and their total nitrogen measured with a Technicon 300B analyzer.

RESULTS AND DISCUSSION

In the fresh nodule weight (FNW) data shown in Table 2, significant differences among genotypes, nitrate levels and growth stages can be observed. The average nitrate level (5mM NO_3^-) significantly reduced the weight of the nodules, resulting in a value of 1.02g, compared to 1.74g in absence of nitrate. Comparing the behaviour of the genotypes at the 0mM level, L-88 was the one which produced the greatest nodule weight, similar to L-62, indicating that the inherent characteristics of these genotypes influenced the results, given that L-88 is a hypernodulating mutant and that L-62 is nitrate resistant. In spite of the lower values of FNW at 5mM in all genotypes, L-62 and L-88 showed the weights of 1.46 and 1.65g, respectively. Another factor that deserves attention is that the innefficient L-135

Table 2 -Effect of nitrate level and growth stage on number of nodules (NN) and fresh nodule weight (FNW) in five genotypes of *Lupinus albus*

		Control		L-62		L-135		L-88		L-280		Mean		LSD[1]	
		NN	FNW	NN	FNW	NN	FNW	NN	FNW	NN	FNW	NN	FNW	NN	FNW
N-level	0mM	116.8aA	1.5cdA	101.9aA	2.2abA	170.9aA	1.0dA	134.2aA	2.5aA	160.1aA	1.6bcA	135.6A	1.7A	10.6	0.7
	5mM	56.6aA	0.5bB	43.6aA	1.5aB	95.4aA	0.7bA	66.4aA	1.6aB	80.0aA	0.8bB	67.2B	1.0B		
	LSD[2]	3.4	0.2												
Growth stage	7 weeks	79.8bA	0.8cA	69.9bA	1.7aA	206.2aA	0.6cA	95.6abA	1.6abB	117.8abA	1.0bcA	109.4A	1.2B		
	10 weeks	88.3aA	1.2cA	69.6aA	1.9abA	71.9aB	1.0cA	99.1aA	2.5aA	115.5aA	1.4bcA	88.0A	1.6A		
	LSD[3]	3.4	0.2												
	Overall Mean	84.0a	1.0b	69.7a	1.8a	130.5a	0.8b	97.3a	2.1a	116.6a	1.2b				
	LSD[4]	7.5	0.5												

[1] LSD (0.05), between N levels and growth Stages, averaged across *Lupinus* genotypes
[2] LSD (0.05), between two N levels, within a *Lupinus* genotype.
[3] LSD (0.05), between two Growth Stages within a *Lupinus* genotype
[4] LSD (0.05), between *Lupinus* genotypes, averaged across N levels and Growth Stages

mutant did not differ in relation to the levels applied. These results are similar to those obtained by Carrol *et al.* (1), in which some Bragg soy mutants nodulated in the presence of a continuous supply of 5.5 mM NO_3^- in sand culture. These mutants, however, are intermediate or extremely supernodulating and have an altered self-regulating response. Similar behaviour was observed by Jacobsen and Feenstra (9), who studied a new mutant of *Pisum sativum* var Rondo with efficient nodulation in the presence of nitrate and noted that the use of 15mM KNO_3 strongly inhibited the cv. Rondo nodulation. These authors observed the behaviour of the mutant in relation to the cv. Rondo, and noted that without nitrate the mutant presented better nodulation. However, the concentration of nitrate was three times higher than that used in the present study. They also observed that the "high nodulation" mutant characteristic is monogenic and recessive. Nodulation behaviour in the nitrate medium differed from the *nod1 nod2* lineage, which showed great inhibition by 15mM nitrate, similar to the standard strain from which our mutant was derived. The mutant, however, was highly resistant to nitrate. The mutant gene was designated *nod3*. The analysis of growth stages showed that 10 weeks produced the highest values of FNW, perhaps due to the culture cycle, being this period the best time for the evaluation of this parameter. In 10 weeks, the L-88 genotype was the one which presented the highest value, which was different of the value at 7 weeks. The other genotypes showed no differences. On comparing the genotypes at the 7 week period separately, the nitrate resistant L-62 showed the highest value, not differing from L-88. However, at the tenth week, these genotypes showed higher values than the others, and L-88 presented a higher FNW than L-62.

Table 2 also shows that the nitrate average level differed among genotypes and 5mM strongly inhibited the number of nodules (NN), reaching a reduction of 49.5% in relation to treatment without nitrate addition. This Table also shows that there were no differences, in the behaviour of genotypes, and in the periods evaluated.

Table 3 indicate that the nitrate level did not influence the production of shoot dry matter (SDM). However, a difference among the genotypes was observed: at 0mM, the L-88 and L-62 genotypes presented SDM of 6.70 and 6.40g, respectively. Being an inefficient mutant, the L-135 genotype reached extremely low values of SDM. At 5mM level, genotypes showed similar behaviour, demonstrating that even in the absence of significant differences, the application of 5mM in some way affects the production of dry matter in *Lupinus*. These data disagree with Lee *et al* (10), who, in studying regulation in the nodules development in supernodulating mutants and soy standard, characterized the supernodulating mutants by lower root and shoot dry matter and high nodulation. In the nitrate tolerant mutants that showed lower root and shoot growth, with high number of nodules and dry nodule weight, they suggested that nitrate tolerant mutants are supernodulating due to the absence of a

Table 3 - Effect of nitrate level and growth stage on shoot dry weight (SDW) and root dry weight (RDM) in five genotypes of *Lupinus albus*, a control genotype (Multolupa); two hypernodulating (L-88; L-280); one nitrate resistant (L-62) and one inefficient genotype (L-135).

		Control		L-62		L-135		L-88		L-280		Mean		LSD[1]	
		SDW	RDW	SDW	RDW	SDW	RDW	SDW	RDW	SDW	RDW	SDW	RDW	SDW	RDW
N-level	0mM	3.8 bA	0.8bcA	6.4 aA	0.9abA	1.6 cA	0.4cA	6.7 aA	1.2 aA	3.9 bA	0.8abcA	4.5 A	0.8 A	1.8	0.4
	5mM	2.6 bA	0.6 cA	5.8 aA	1.1abA	2.2 bA	0.5 cA	6.6 aA	1.5 aA	3.0 bA	0.8bcA	4.0 A	0.9 A		
	LSD[2]	0.6	0.1												
Growth stage	7 weeks	2.2 bB	0.6 ca	5.6 aA	1.3abA	1.4 bA	0.6 cA	5.7 aB	1.5 aA	3.2 bA	0.9 bcA	3.6 B	1.0 A	1.8	0.4
	10 weeks	4.1 bA	0.7 bA	6.6 aA	0.8bB	2.5 bA	0.4 bA	7.6 aA	1.3 aA	3.8 bA	0.8 bA	4.9 A	0.8 A		
	LSD[3]	0.6	0.1												
	Overall Mean	3.2 bc	0.7 cd	6.1 a	1.0 b	1.9 c	0.5d	6.7 a	1.4 a	3.5 b	0.8 bc				
	LSD[4]	1.3	0.3												

[1] LSD (0.05), between N levels and growth Stages, averaged across *Lupinus* genotypes
[2] LSD (0.05), between two N levels, within a *Lupinus* genotype.
[3] LSD (0.05), between two Growth Stages within a *Lupinus* genotype
[4] LSD (0.05), between *Lupinus* genotypes, averaged across N levels and Growth Stages

nodulation inhibitor instead of the presence of a nodule development activator. However, this inhibitor has not been clearly identified. For SDM, the evaluation of growth stages revealed differences, where in the second period, the value of dry matter in grams was greater than when evaluated in the first period. Among the genotypes, L-88 showed an overall average of 6.66g and L-62 6.09g, clearly showing that these genotypes were those that adapted best to the treatments used in the experiment. In Table 3 with regard to root dry matter (RDM), behaviour was very similar in relation to the level of nitrate applied, although among genotypes at 0mM, L-62, L-88 and L-280 did not differ among themselves, the hypernodulating genotypes having obtained the highest values of RDM. At the level of 5mM, L-88 also behaved equally towards the non-application of nitrate, followed by L-62. The analysis of growth stages showed higher SDM values in the first evaluation period than in the second. This would suggest that the reduction in SDM had been due to the limitation that the roots underwent as a consequence of the size (only 2.5l) of the vessel in which the plants were cultivated.

Table 4 indicates that the electron allocation coefficient (EAC) was a parameter that showed no significant difference among nitrate levels, nor among the genotypes studied. In compensation, the evaluation periods revealed differences, where the EAC in the seventh week showed a higher value than in the tenth week, when it was evaluated on application of 5mM. These results can be considered when Serrano and Chamber (13) report that the lack of homology in the DNA sequence *Hup* in *Bradyrhizobium* sp (*Lupinus*) can reflect different catalytic or physical properties among the enzymes. Some of these may represent a different period of *Hup* activity over the N_2 fixation cycle and persistence under stress conditions, such as the presence of a high nitrate level in the medium. According to Evans *et al.* (5), the average relative efficiency (RE) in the fixation of nitrogen (percentage of electron flow) through the nitrogenase which is allocated by N_2 to the 22 strains tested in symbiosis with *L. augustifolius* was 0.53, which is similar to the average value reported for other symbiotic pairs. With regard to the inability of some *Hup+ in Lupinus* rhizobium to induce hydrogenase activity in symbiosis with *Lupinus*, Murillo *et al.* (1989) interpreted this as an effect of the host observed in the phenotypical expression of hydrogenase. According to these authors, the interpretations were based on the idea that genes other than the hydrogenase structural genes are involved in the availability of H_2.

Table 4 also presents the N_2 fixation rate (N_2 FIX), which shows the existence of the interference of nitrate in fixation, since the application of 5mM greatly reduced the rate, there being no differences among the genotypes. It was also noted that the fixation rate in the genotypes was higher in those that received no nitrate. Specific nitrogenase activity,

Table 4 - Effect of nitrate level and growth stage on electron allocation coefficient (EAC) and nitrogen fixation rate (N_2FX) in five genotypes of *Lupinus albus*, a control genotype (Multolupa); two hypernodulating (L-88; L-280); one nitrate resistant (L-62) and one inefficient genotype (L-135).

		Control		L-62		L-135		L-88		L-280		Mean		LSD[1]	
		EAC	N_2FX	EAC	N_2FX	EAC	N_2FX	EAC	N_2FX	EAC	N_2FX	EAC	N_2FX	EAC	N_2FX
N-level	0mM	0.9 aA	1.0 aA	0.7 aB	0.9 aA	0.9 aA	0.8 aA	0.8 aAB	1.2 aA	0.8 aAB	1.2 aA	0.8 AB	1.0 A	0.2	1.0
	5mM	0.8 aAB	0.4 bA	0.7 aB	0.4 bA	0.9 aA	0.3 bA	0.8aAB	0.6 bcA	0.8 aAB	0.3 cA	0.8 AB	0.4 A		
	LSD[2]	0.1	0.3												
Growth stage	7 weeks	0.9 aA	0.8 aA	0.8 aA	0.7 abA	0.9 aA	0.7 aA	0.9 aA	0.8 bA	0.8 aA	0.9abA	0.8 A	0.8 A	0.2	1.0
	10 weeks	0.8 aA	0.7 aA	0.7 aA	0.6 bA	0.8 aA	0.5 abA	0.8 aA	1.0 abA	0.8 aA	0.6 bcA	0.8 A	0.7 A		
	LSD[3]	0.1	0.3												
	Overall Mean	0.8 a	0.7 a	0.7 a	0.7 ab	0.9 a	0.6 ab	0.8 a	0.9 ab	0.8 a	0.8 b				
	LSD[4]	0.1	0.7												

[1] LSD (0.05), between N levels and growth Stages, averaged across *Lupinus* genotypes
[2] LSD (0.05), between two N levels, within a *Lupinus* genotype.
[3] LSD (0.05), between two Growth Stages within a *Lupinus* genotype
[4] LSD (0.05), between *Lupinus* genotypes, averaged across N levels and Growth Stages

in nodules of supernodulating plants, is reduced and there is little information available, that is an increase in nodulation results in an increase in fixation (4). According to Jacobsen and Feenstra (9), the reduction of acetylene per plant in a medium without nitrate was greater in the mutant than in the cv. Rondo, this also occurring with the genotypes used in the present work. According to Jacobsen and Feenstra (9), the effect of nitrate on the reduction of acetylene was related to competition for energy and/or carbohydrates. However, studies have shown that the balance between nitrogen fixation and the reduction of nitrate in the mutant is different from the standard strain. Neither did the evaluation period interfere in the N_2 fixation rate, possibly owing to the periods in which they were analysed not having been the best for the genotypes studied.

From these results we can observe that L-88 and L-62 genotypes were those that adapted best in this experiment, thus being recomendable for new studies with higher nitrate levels and different evaluation periods. Generally speaking it has become clear that the nitrate (5mM) interferes in the N_2 fixation rate, given that all the genotypes were affected by the level applied.

RESUMO

Influência dos diferentes níveis de nitrato e estágio de crescimento em mutantes hipernodulantes de *Lupinus albus* I. potencial de fixação de N_2

Este trabalho teve como objetivo avaliar parâmetros fisiológicos, resposta da nodulação e a taxa de fixação de N_2 em mutantes de *Lupinus albus* comparando com a cultivar padrão Multolupa. Foram utilizados dois níveis de nitrato (0 e 5mM) e dois diferentes períodos de avaliação (7 e 10 semanas). No peso fresco dos nódulos, diferenças significativas entre genótipos em relação aos níveis de nitrato e estágios de crescimento foram observadas. Nível de nitrato de 5mM inibiu fortemente a produção de nódulos, chegando a ter uma redução de 49,5% em relação ao tratamento onde não se adicionou o nitrato. Em relação aos genótipos, os desempenhos não diferiram entre si, o mesmo acontecendo com os períodos avaliados. O nível de nitrato não influenciou na produção de matéria seca da parte aérea em relação à média entre os níveis aplicados. Entretanto, entre os genótipos existiu diferença, onde 0mM, os genótipos L-88 e L-62 apresentaram 6,7 e 6,4g, respectivamente. O genótipo L-135, por ser um mutante ineficiente alcançou valores extremamente baixos de matéria seca da parte aérea. Para o coeficiente de alocação de elétrons (EAC), não houve diferença significativa entre os níveis de nitrato, nem entre os genótipos estudados. Em compensação os períodos avaliados tiveram diferenças: na sétima semana a EAC apresentou valor superior à EAC na décima semana, quando foi avaliada na aplicação de 5mM. A taxa de fixação de N_2 (N_2 FIX) mostrou que existe interferência do nitrato na fixação, uma vez que, a aplicação de 5mM reduziu muito a fixação, apesar de que entre os genótipos não houve diferença entre si. Com estes resultados observamos que os genótipos L-88 e L-62 foram os que melhor se adaptaram podendo desta forma ser recomendados para novos estudos com maiores níveis de nitrato aplicados e diferentes períodos de avaliação. De uma forma geral ficou nítido que o nitrato (5mM) interfere na taxa de fixação de N_2, uma vez que, todos os genótipos foram afetados pelo nível aplicado.

Palavras-chave: Mutantes, coeficiente de alocação de elétrons, taxa de fixação de N_2, *Bradyrhizobium* sp (*Lupinus*).

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NITRATE LEVELS AND STAGES OF GROWTH IN HYPERNODULATING MUTANTS OF *LUPINUS ALBUS*. II. ENZYMATIC ACTIVITY AND TRANSPORT OF N IN THE XYLEM SAP

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ABSTRACT

The enzymatic study and transport of N in the xylem sap was carried out with a view to observing the influence of different nitrate levels and growth stages of the plant in chemically treated mutants of *Lupinus albus*. Several stresses induce a reduction in plant growth, resulting in the accumulation of free amino acids, amides or ureides, not only in the shoot, but also in the roots and nodules. Although enzyme activity is decisive in avoiding products that inhibit nitrogenase by ammonium, little is known about the mechanism by which the xylem carries these products. However, this process may be the key to the function of avoiding the accumulation of amino acids in the cells of infected nodules. The behaviour of the enzymes nitrate reductase (NR), phosphoenolpyruvate carboxylase (PEPC), glutamine synthetase (GS) and nitrogen compounds derived from fixation, such as N-α-amino, N-ureides and N-amide in mutant genotypes were observed. The NR enzyme activity was highly influenced by the application of nitrate showing much higher values than those in the non-application of nitrate, independently of genotype, being that the NR, the best evaluation period was in the tenth week. The L-62 genotype characterized with nitrate- resistance, clearly showed that the enzyme PEPC is inhibited by presence of nitrate. The L-135 genotype (nod$^-$ fix$^-$) showed GS activity extremely low, thus demonstrating that GS is an enzyme highly correlated with fixation. With regard to the best growth stage for GS, *Lupinus albus* should be evaluated in the seventh week.

Key words: nitrate reductase, phosphoenolpyruvate carboxylase, glutamine synthetase, N-α-amino, N-ureide, N-amide, *Bradyrhizobium* (*Lupinus*), nodule cytosol.

INTRODUCTION

Stresses inducing reduction in plant growth result in the accumulation of free amino acids, amides or shoot, root and nodule ureides which may be responsible for the regulation of nodulation and nitrogenase activity through a system of regeneration (19). The same author reports that the ammonium produced by nitrogenase in symbiosis with legumes is exchanged inside the cytosol of the host cell, where

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it is incorporated as amino acids and amides. It is for this reason that GS/GOGAT enzyme activity is decisive in avoiding inhibitive products from nitrogenase by ammonium. Although no information is available as to the mechanism by which the xilema carries amides or ureides, this process may be the key to the function of avoiding the accumulation of amino acids in cells of infected nodules.

On studying the reduction of nitrate in *Rhizobium sp.*, Serrano and Chamber (20) observed that this includes disassimilatory and defective processes, besides the assimilatory reduction. Alcantar-Gonzales *et al.* (2) reported an increase in the reduction of nitrate in stirps (NR^+) nitrate reductase and that this generally occurs with a decrease in acetylene reduction activity. In bacteroids of some *B. japonicum* which has a high level of constitutive NR, they showed no reduction of nitrate in nodules owing to this anion not having access to the bacteroid zone (12). Silsbury *et al.* (22) showed that the nitrate reductase and the fixation of N_2 work in a complementary way by supplying reductive sources of nitrogen in the plants, consequently exhibiting a regulatory system involving a level of soluble N in the plant. Temporary treatments with high nitrate inhibit the acetylene reduction activity without any relation to nitrate reductase activity in bacteroids of *B. japonicum*.

There is a report in which a partially nitrate reductase deficient mutant of *Pisum sativum* (L.) was less susceptible to the influence of nitrate application on symbiotic N_2-fixation than the wild type (11). In contrast, Ryan *et al.* (17) reported that a nitrate reductase mutant did not show improved nodulation compared to the wild type, so these results support the suggestion that the metabolism of nitrate was involved. The results of Burity *et al.* (7), suggest that *Lupinus mutabilis* mutants have a greater capability of assimilating symbiotically fixed N with greates available carbohydrates supply, and the partial tolerance to nitrate demonstrated by some mutants are apparently associated with the hypernodulated phenotype. Gibson and Harper (13) showed another type of pea mutant, whose nodulation demonstrated a greater tolerance to nitrate, althought it had normal nitrate reductase. These observations indicate that the adverse effect of nitrate on nodulation and N_2-fixation can be overcome by other mechanisms, such as limited carbon supply to the nodule or cultivars with altered nitrate metabolism associated with hypernodulated characteristics.

Concerning PEP carboxilase, Vance and Heichel (28) propose that the reductive fermentative path in the cytosol of nodules involves its synthesis and that this enzyme is also inhibited by the nitrate. Streeter (25) noted the possibility that nitrate reductase activity may increase GS, and it is already known that glutamine absorbed by bacteroids controls nitrogenase activity. According to Milic *et al* (16), studying the symbiosis of soybean with *Bradyrhizobium japonicum*, the activity of glutamine synthetase (GS) enzyme in the plants is correlated with nitrogen fixation in relation to the different varieties studied.

With respect to ureides, according to Atkins *et al.* (3), they are formed by the oxidation of purine xantina and hipoxantina bases which return with derivatives for a new synthesis of purine nucleotids. The application of alopurinol (AP) which has a similar structure to hipoxantina, in nodulating roots result in rapid inhibition of the activity of xantina hidrogenase (XDH) in the nodules. When the isolated bacteroids of inhibited nodules show nitrogenase activity rates with small differences in relation to the control, the direct application of AP (or xantina) on the isolated bacteroids had no effect (4). These data indicate that the effect of the AP on the nitrogenase was indirect, and a consequence of the interference of certain processes essential for the functioning of the nitrogenase, more localized on the outside of the bacteroid. In this same work it was observed that the production of H_2 was inhibited after 1 or 2 hours, whilst the accumulation of purines and inhibition of the synthesis of ureides in the nodules was detected after 1 hour. There are several paths by which the synthesis of ureides and nitrogenase can interact. The primary paths are intermediate (purines and ureides) which serve to regulate or aid the respiratory reactions that support the nitrogenase, and the second possibility is that respiratory substrates used by the bacteroids depend on ureide synthesis for their formation. From the disappearance of the relation between the abundance of ureides and nitrogen fixation which corresponds to the beginning of the formation of grains and the remobilization of these compounds at maturity in early genotypes, according to Aveline *et al.* (1), it was suggested that the interference of ureide synthesis derived from other products during senescence or even the release of these composites from any source group can quite clearly explain the lack of correlation with later genotypes. Furthermore, research into the

measurement concerning the origin of ureides in different growth stages should be carried out.

The aim of the present work was to observe enzymatic activity and the transport of N in the xylem sap in the growth of *Lupinus albus* cv. Multolupa (standard), of two hypernodulating mutants and (L-280 and L-88), one resistant to nitrate (L-62) and one inefficient mutant (L-135) inoculated with *Bradyrhizobium* sp *(Lupinus)*.

MATERIALS AND METHODS

The *Lupinus albus* plant material was used, and according to previous work done by C.I.D.A., Centro de Investigación y Desarrollo Agrario, Sevilla - Spain the main characteristics of the cv. Multolupa mutants that was used in the study were: L-280 nod^+ fix^+; L-88 nod^+ fix^+; L-62 nod^- with resistent to NO_3^- and L-135 inefficient (nod^- fix^-). The planting methodology, procedure and statistical guidelines are cited in Burity *et al.* (part I).

The number and fresh weight of nodules were determined and 1.5g of the nodules from each treatment were homogenized under N_2 stream at 4°C in a phosphate buffer (24). The homogenate solution was passed though a cheese-cloth layer and the suspension was collected in tubes which were then centrifuged at 200g for 20 min. The supernatants obtained were centrifuged at 8.000g for 20 min. at 4°C to separate bacteroids from cytosol. Soluble protein was measured according to Goa (14), and samples of cytosol suspension were analyzed for nodule glutamine synthetase (GS) (EC 6.3.1.2) and phosphoenolpyruvate carboxilase (PEPC) (EC 4.1.1.31) activities. Nodule GS activity was determined using the ADT- transferase reaction that measures the formation of γ-glutamylhidroxamate (21), while nodule PEPC activity was determined according to the method described by Briand *et al.* (6). The nitrate reductase (NR) activity in the cytosol nodule was assayed spectrophotometrically (540 nm) according to Sanchez and Heldt (18).

The determination of nitrogen compounds in the sap derived from fixation were: N-ureides - alantoic acid and alantoin estimated by glyoxylate hydrolysis (29); N-α-amino using the modified method described by Matheson *et al* (15), and the reagent hydridantine was prepared in accordance with Connel *et al.* (10) and N-amide measured through the glutamine (26), in which the amide was estimated after hydrolysis.

RESULTS AND DISCUSSION

On analysing Table 1, significant differences can be observed, in both the nitrate levels applied and the evaluation periods, the highest rates of activity being in accordance with the mean among genotypes of nitrate reductase (NR) at the level of 5mM of 9.04 μmoles of $NO_3.h^{-1}.\ mg.^{-1}$protein., that is almost double that of treatment not applied with nitrate. Despite there being no differences among genotypes, the L-135 obtained the activity of 11.17, the highest in relation to the other genotypes.

The phosphoenolpyruvate carboxilase (PEPC) (Table 2), did not show significant differences, neither for 0 to 5mM nitrate concentrations nor for 7

Table 1 - Effect of nitrate levels and growth stage on nitrate reductase activity (NR) (μmoles $NO_3.h^{-1}.\ mg^{-1}$.protein) in five genotypes of *Lupinus albus*, a control genotype (**Multolupa**), two hypernodulating (**L-88; L-280**); one nitrate resistant (**L-62**) and one inefficient genotype (**L-135**).

		NR (μmoles $NO_3.h^{-1}.\ mg^{-1}$.protein)						
		Control	L-62	L-135	L-88	L-280	Mean	LSD[1]
N	0mM	5.6aA	6.8aA	5.1aB	4.9aA	5.4aA	5.5B	
level	5mM	9.7aA	7.7aA	11.2aA	8.7aA	7.9aA	9.0A	6.1
	LSD[2]	1.9						
Growth	7 weeks	5.0aB	4.2aB	5.8aB	5.7aA	6.7aA	5.5B	
Stage	10 weeks	10.3aA	10.3aA	10.4aA	7.9aA	6.5aA	9.1A	6.1
	LSD[3]	1.9						
	Overall Mean	7.6a	7.2a	8.1a	6.8a	6.6a		
	LSD[4]	4.3						

[1] - LSD (0.05), between N levels and Growth Stages, averaged across *Lupinus* genotypes.
[2] - LSD (0.05), between two N levels, within a *Lupinus* genotype.
[3] - LSD (0.05), between two Growth Stages within a *Lupinus* genotype.
[4] - LSD (0.05), between *Lupinus* genotypes, averaged across N levels and growth stages.

Table 2 - Effect of nitrate levels and growth stage on phosphoenolpyruvate carboxylase (PEPC) (μmoles de PEPC.h^{-1}.mg^{-1}. protein) and glutamine synthetase (GS) (μmoles GH. h^{-1}.mg^{-1}. protein) activities in five genotypes of *Lupinus albus*, a control genotype (**Multolupa**), two hypernodulating (**L-88; L-280**); one nitrate resistant (**L-62**) and one inefficient genotype (**L-135**).

		Control		L-62		L-135		L-88		L-280		Mean		LSD[1]	
		PEPC	GS	PEPC	GS	PEPC	GS	PEPC	GS	PEPC	GS	PEPC	GS	PEPC	GS
N	0mM	25.1aA	2.3aA	25.5aA	5.5aA	18.8aA	3.7aA	22.3aA	5.6aA	14.4aA	2.8aA	21.2A	4.0A		
														25.6	3.7
level	5Mm	41.4aA	4.5aA	15.6bA	4.8aA	20.5abA	0.3bB	29.1abA	2.6abB	19.0abA	2.0abA	25.1A	2.8A		
	LSD[2]	8.1	1.17												
Growth	7 weeks	45.7aA	3.9bcA	12.8bA	8.8aA	16.6bA	3.2cA	22.7abA	7.2abA	19.8bA	3.9bcA	23.5A	5.4A		
											0.9aB			25.6	3.7
Stage	10 weeks	20.8aB	2.9aA	28.3aA	1.6aB	22.6aA	0.9aA	28.7aA	1.1aB	13.6aA		22.8A	1.5B		
	LSD[3]	8.1	1.2												
	Overall Mean			33.3a	3.4ab	20.5a	5.16a	19.6a	2.0b	25.7a	4.1ab	16.7a	2.4b		
	LSD[4]	18.1	2.6												

[1] - LSD (0.05), between N levels and Growth Stages, averaged across *Lupinus* genotypes.
[2] - LSD (0.05), between two N levels, within a *Lupinus* genotype.
[3] - LSD (0.05), between two Growth Stages within a *Lupinus* genotype.
[4] - LSD (0.05), between *Lupinus* genotypes, averaged across N levels and growth stages.

and 10 weeks. With regard to differences among genotypes at the 5mM level, we note that the control treatment obtained the greatest activity of the PEPC enzyme, of 41.40 μmoles of PEPC. h^{-1}. mg^{-1} protein, whilst the L-62, a nitrate-resistant genotype, showed the lowest value (15.55 μmoles). When nitrate was not applied, the activity of PEPC did not differ among genotypes. The behaviour of the L-62, which without receiving nitrate showed the highest activity of PEPC, corroborates results obtained by Vance and Heichel (28) and also resemble those of Vance and Stade (27), who observed that the nitrate inhibits the PEPC and reduces the formation of proteins (23).

Table 2 shows data for the activity of glutamine synthetase (GS) and shows that there were no significant differences with respect to the mean among the nitrate levels, although within the genotypes at the 5mM level, the genotypes differed in relation to the activity of GS, where the control (Multolupa), together with the L-62 showed values of 4.49 and 4.79 μmoles GS. h^{-1}.mg^{-1}.protein. The L-135 genotype, with inefficient characteristics, obtained the extremely low value of 0.34. For the growth stages, the difference among means in relation to the first period of 7 weeks and in relation to the second of 10 weeks was 265%, suggesting 7 weeks as the ideal period for the determination of GS in this culture. Among the genotypes, the L-62 was the one which obtained the highest value, not differing from the L-88, a hypernodulator, for the first period. Streeter (25) considers the possibility of the NR increasing the GS, however, in the present work, this did not occur, given that the genotypes showing the highest values of GS in the evaluated period of 7 weeks, were not outstanding in relation to the concentration of NR, nor did they differ among themselves. Another example of differential behaviour was observed in relation to the determination of NR in these genotypes, where the best period was 10 weeks, that is, later on.

The parameters N-α-amino, N-ureides and N-amide (Table 3) were studied over only one cycle, the first two having been evaluated in the seventh week, the last in the tenth week. For the mean values of N-α-amino, there were no differences among the levels of nitrate applied, this behaviour being repeated for the other nitrogen compounds from the sap derived from fixation. Only in the case of N-ureide levels did the L-135 genotype obtain a high value when nitrate was not applied compared to the value reached at the 5mM level. This behaviour is logical since the L-135, being inefficient, shows that the nitrate clearly interferes in fixation because, despite its inefficiency, the concentration of N-ureides was 84% higher when nitrate was not applied. As there are innumerable paths by which the synthesis of ureides and nitrogenase can interact, according to the reports of Atkins *et al.* (5), this behaviour may be a form of interaction in relation to the intermediate paths which aid the respiratory reactions that support nitrogenase or the respiratory substrates that the bacteroids use to synthesise ureides. Or, as Aveline *et al.* (1) observed on studying the best method for evaluating N-ureides in soybean, that there is a need to investigate different growth stages of the plant in order to quantify the ureides

Table 3: Effect of nitrate levels and growth stage on the concentrations, N-α-amino and ureide-N at first cylcle and amide-N at second cycle in five genotypes of *Lupinus albus*, a control genotype (**Multolupa**), two hypernodulating (**L-88; L-280**); one nitrate resistant (**L-62**) and one inefficient genotype (**L-135**).

NO_3^- Levels	Genotypes					
	Control	L62	L135	L88	L280	**Mean$_N$**
			N-α-amino (μmoles N/100μl of exuded sap) (7 weeks)			
0	348.6aA	275.1aA	672.8aA	472.6aA	804.6aA	**514.7A**
5	639.0aA	112.2aA	195.5bA	433.2aA	502.8aA	**376.5A**
Mean$_G$	**493.8ab**	**193.7b**	**434.2ab**	**452.9ab**	**653.7a**	
			N-urcide (μmoles N/100μl of exuded sap) (7 weeks)			
0	255.7aA	140.7aA	304.9aA	160.3aA	209.7aA	**214.3A**
5	169.6aA	174.4aA	165.0aB	172.9aA	186.4aA	**173.6A**
Mean$_G$	**212.6a**	**157.5a**	**235.0a**	**166.6a**	**198.0a**	
			N-amide (μmoles N/100μl of exuded sap) (10 weeks)			
0	20.7ab	13.2b	26.5a	17.1b	21.2ab	**19.7A**
5	18.5a	18.0a	24.9a	21.7a	25.4a	**21.7A**
Mean$_G$	**19.6ab**	**15.6b**	**25.7a**	**19.4ab**	**23.3a**	

Mean$_G$ = Means between genotypes; Mean$_N$ = Means between nitrate levels
N-α-amino - LSD (%)$_{nitrate}$ = 180.3767; LSD (%)$_{genotypes}$=408.1235
Ureide-N - LSD (%)$_{nitrate}$=55.7239; LSD (%)$_{genotypes}$=126.0819.
Amide-N - LSD (%)$_{nitrate}$=2.9020; LSD (%)$_{genotypes}$=6.4891

derived from fixation.

For the N-amide data, the tenth week evaluation showed significant differences among the averages of the genotypes where the L-135 obtained the highest value of 25.73 μmoles/100μl of sap extracted. In contrast, N-ureides show similar behaviour to those obtained with this genotype, not differing statistically, however, from the L-280 genotype, whilst the L-62 showed the lowest value.

From these results it can be concluded that the nitrate reductase enzyme was highly influenced by the application of nitrate, showing much higher values in relation to the non-application of nitrate, irrespective of genotype, and that for NR, the best evaluation period was in the tenth week. Concerning PEPC, the nitrate-resistant L-62 genotype clearly demonstrated that this enzyme is inhibited by the presence of nitrate. With respect to GS, the L-135 genotype (nod$^-$ fix$^-$) showed an extremely low value, thus demonstrating that the GS is an enzyme highly correlated with fixation. In relation to the best growth stage for the GS, *Lupinus albus* should be evaluated in the seventh week. For the nitrogen compounds derived from fixation, we suggest that a deeper study concerning the best evaluation period would be of great importance, given that each genotype demonstrates differential behaviour for the synthesis of these compounds.

RESUMO

Diferentes níveis de nitrato e estágio de crescimento em mutantes hipernodulantes de *Lupinus albus* II. Atividade enzimática e transporte de N na seiva do xilema

O estudo enzimático e o transporte de N na seiva do xilema foi realizado visando observar a influência de diferentes níveis de nitrato e estágios de crescimento da planta em mutantes tratadas quimicamente. Vários estresses induzem a redução no crescimento da planta da qual resulta na acumulação de aminoácidos livres, amidas ou ureídos, tanto na parte aérea como nas raízes e nódulos. A atividade de enzimas é decisiva em evitar produtos inibidores da nitrogenase pelo amônio, porém poucas são as informações deste mecanismo com o qual o xilema carrega estes produtos, em compensação, este processo, pode ser a chave da função em evitar acumulação de aminoácidos nas células dos nódulos infectados. O comportamento das enzimas nitrato redutase (NR), fosfoenolpiruvato carboxilase (PEPC), glutamina sintetase (GS) e dos compostos nitrogenados derivados da fixação como: N-α-amino, N-ureídos e N-amida em genótipos mutados foi observado. A atividade da enzima nitrato

redutase foi altamente inflenciada pela aplicação de nitrato apresentando valores bastante superiores em relação a não aplicação do nitrato, independentemente dos genótipos, sendo que o melhor período de avaliação foi na décima semana. O genótipo L-62, com caracteríticas de resistência ao nitrato, mostrou de forma bem evidenciada que a enzima PEPC é inibida pela presença de nitrato. O genótipo L-135 (nod$^-$ fix$^-$) apresentou atividade da GS extremamente baixo, mostrando desta forma que a GS é uma enzima altamente correlacionada com a fixação. Em relação ao melhor estágio de crescimento para a GS, o *Lupinus albus*, deve ser avaliado na sétima semana.

Palavras-chave: nitrato redutase, fosfoenolpiruvato carboxilase, glutamina sintetase, N-α-amino, N-ureidos, N-amida, *Bradyrhizobium* (*Lupinus*), citosol nodular.

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TOXIC METABOLITES FROM CULTURE FILTRATE OF *FUSARIUM OXYSPORUM* AND ITS EFFECTS ON CUCUMBER CELLS AND PLANTLETS

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SHORT COMMUNICATION

ABSTRACT

Resistance of cucumber plantlets to culture filtrate of *Fusarium oxysporum* is correlated with resistance of single cells from callus. Single cells and plantlets of two cultivars of cucumber were incubated with culture filtrates. Rapid cell death occurred, as assessed by the stain fluorescein diacetate. More cell death ocurred in the cells of the cultivar *Aodai* than in to cells of the cultivar *Caipira,* which presented high level of resistance. Maximum toxic activity of culture filtrates was attained after 21-25 days of growth of the fungus.

Key words: *Fusarium oxysporum*, single cells, cucumber, toxic metabolites

Fusarium oxysporum causes wilt and is a major pathogen of cucumber in greenhouse condition in São Paulo State, Brazil. In young seedlings the cotyledons lose their green color, droop, and wither. In old plants, leaves wilt during the day for several successive days, and then wilt permanently. There is no resistance in commercial cultivars under those conditions and little is known of the inheritance of resistance. The use of resistant cultivars is the most efficient way to control the fungus.

Although effective, the currently used root inoculation and soil infestation with the pathogen for screening procedures are laborious, time consuming and some times permit many scapes.

Correlation of resistance to a parasite and resistance to its toxins is a necessary prerequisite for such use of phytotoxins. (1, 3, 5, 6, 7) Phytotoxins are useful tools for selection techniques in callus cultures and seedlings.

In this paper, the killing of seedlings and single cells of cucumber has been used to detect phytotoxic activity of *F. oxysporum in* two cultivars, *Aodai* and *Caipira.* The cultivar *Caipira* has been considered to be more resistant than *Aodai* by farmers.

Culture of the fungus (isolated from infected cucumber plants) was maintained on PDA slopes (potato-dextrose-agar) at 28°C and stored at 8°C. The fungus was grown on a defined liquid medium, Czapek-Dox. The medium was adjusted to pH 5.5. For bulk production of culture filtrate, 200 ml of medium in an 1 L erlenmeyer flask was inoculated with three mycelial plugs taken from the ege of 7-day old cultures grown on PDA. The flasks were incubated on a rotary shaker (150 rpm at 28°C). At

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harvesting the mycelium was collected and filtered through whatman 3 mm filter paper. The broth cultures were then filter sterilized by passing them, under vaccum, through millipore filter (pore diameter of 0.2 μm).

Callus was initiated from leaves of two cultivars of cucumber (*Aodai* and *Caipira*) on Murashige and Skoog (MS) medium supplemented with 11.40 mg/ml NAA and 20 g/L sucrose. Cell suspensions were initiated from the rapidly growing callus cultures of both cultivars on MS supplemented with 20 g/L sucrose and three different combinations of hormones. Medium 1 with 11.40 mg/L NAA, Medium 2 with 5 μM 2.4-D and 5 μM BAP and Medium 3 with 5 μM NAA and 5 μM BAP.

Toxic metabolites from *Fusarium oxysporum* were produced and interacted *in vitro* with two cultivars of cucumber: *Aodai* and *Caipira*.

A time course experiment for the accumulation of toxic metabolites was performed. Culture filtrates of different ages were assayed for toxic activity with three week old plantlets of the two cultivars.

Seven day old cell suspensions of cucumber were interacted with 21 day old toxic metabolites from *Fusarium oxysporum*. Fusaric acid (5-Butylpicolinic acid) (Sigma) was included in the experiments as control. Cell viability was estimated by epifluorescence microscopy, using fluorescein diacetate staining.

Cucumber seeds of both cultivars were sterilized and planted in an equal mixture of autoclaved soil and vermiculite. Pots of 800 ml, containing the mixture were maintained in greenhouse for three weeks.

Healthy, vigorous seedlings were aseptically removed from the soil. The soil adering to the roots was removed by washing with tap water and root system was immersed in different concentrations of toxic metabolites or in distilled water.

Disease incidence was evaluated in terms of severity of wilt as compared with control plants.

Maximum toxic activity of culture filtrate of *F. oxysporum* was attained after 14-25 days of growth of the fungus (Table 1). Therefore, 25 days old culture was used in all subsequent experiments. In this trial, it was observed that the cultivar *Caipira* presented a good level of resistance to *F. oxysporum* when compared to cultivar *Aodai*. Percent of wilt increased with age of *F. oxysporum* culture filtrates.

The severity of wilt in *Aodai* and *Caipira* plantlets was shown to be concentration dependent (Table 2). The autoclaved culture filtrate produced the same level of disease symptoms as the non-autoclaved culture filtrate; indicating the presence of heat resistant toxic metabolites.

Table 1. Capacity of *Fusarium oxysporum* culture filtrates to cause wilt in cucumber plantlets.

Age of culture (days)	Cultivars wilt	
	Caipira	***Aodai***
25	0.7	2.8
21	0.5	2.3
14	0.3	2.0
8	0.3	1.4
PD (control)	0.0	0.0
PD and FA	1.1	1.6
W and FA	0.3	0.6

PD = Potato Dextrose Media W = water FA = Fusaric acid

[1]Wilt scored on a 0-4 scale; 0 indicating no wilt and 4 indicating severe wilt. Symptom score is a mean of 17 plants.

Table 2. Relationship of different concentrations of toxic metabolites from *Fusarium oxysporum* to cause wilt in *Aodai* and *Caipira* cultivars of cucumber.

	Symptoms	
Concentration %	***Aodai***	***Caipira***
100	3.42	0.85
75	3.00	0.71
50	2.71	0.14
25	2.28	0.14
10	2.71	0.10
50 autoclaved	2.85	0.14
100 autoclaved	3.00	1.00

Symptoms score is expressed as a mean of 17 plants.

Growth of cucumber cells was good on medium 3 and produced cells of a single nature in comparison to media 1 and 2. Hence, cucumber cell suspensions were maintained on medium 3. Seven days old cell suspensions were interacted with 21 days old toxic metabolites from *F. oxysporum*. More cell death occurred in the cells of the cultivar *Aodai* in comparison to cells of the tolerant cultivar *Caipira* (Table 3). This cultivar could be included in breeding programs to introduce resistance in commercial cultivars.

Table 3. Interaction of 21 days old culture filtrates of *Fusarium oxysporum* with 7 days old cell suspension of the cucumber cultivars, *Aodai* and *Caipira*.

	% Cell Death	
	Aodai	***Caipira***
21 days culture filtrate	23.6	8.4
Czapek Dox (control)	2.5	3.0

Note: 3 repetitions with 10 readings in each repetition.

The advantages of using cells and protoplasts over whole plants for assaying toxic have been discussed in detail elsewhere (2, 4, 9). An effective screening procedure has to be amenable for testing a large number of plants, and it should be simple, relatively rapid, and significantly differential. All assays evaluated satisfied these criteria and could be used in breeding programs. The action of the culture filtrate on cell suspensions of cucumber, closely reflect the action of the filtrate in plantlets suggesting a role fungal extracellular toxic compounds in the disease.

RESUMO

Metabólitos tóxicos de filtrado de cultura de *Fusarium oxysporum* e seus efeitos em células e plântulas de pepino

Plântulas de pepino e células isoladas obtidas de calos, das cultivares *Aodai* e *Caipira* foram incubadas com filtrado de cultura de *Fusarium oxysporum*, em condições assépticas. As reações de murchamento das plântulas frente à ação do filtrado evidenciaram que as cultivares *Aodai* e *Caipira* se comportaram como suscetível e resistente, respectivamente. Após avaliação da reação de células isoladas, sob microscópio acoplado com epifluorescência, utilizando-se de acetato de fluoresceína, discriminou-se a porcentagem de células mortas. A cultivar *Aodai* se comportou como extremamente suscetível e a *Caipira* como resistente.

Estes resultados sugerem que compostos tóxicos extracelulares produzidos pelo patógeno servem para utilização em "screening" de cultivares, como também para seleção de células sobreviventes quando submetidas ao filtrado tóxico do fungo com vistas à obtenção de regenerantes resistentes.

Palavras-chave: *Fusarium oxysporum*, células isoladas, pepino, metabólitos tóxicos.

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PROTEASE PRODUCTION DURING GROWTH AND AUTOLYSIS OF SUBMERGED *METARHIZIUM ANISOPLIAE* CULTURES

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ABSTRACT

The growth and autolysis of two strains of the entomopathogenic deuteromycete fungus *Metarhizium anisopliae* var. *anisopliae* were evaluated in medium containing casein or glucose as carbon source. Parameters such as economic coefficient and degree of autolysis were determined for each strain. Protease production was determined throughout the growth and autolysis phases of the cultures on medium under conditions of protease induction (in the presence of casein as sole source of carbon and nitrogen). The fungus was shown to utilize casein as a carbon/energy source in a more efficient manner than glucose. The autolysis shown by the strains was intense under both types of growth conditions, reaching up to 62.7% of the dry mass produced and started soon after the depletion of the exogenous carbon source. The relationship between the proteolytic activities of the two strains evaluated varied significantly (a maximum of 19.78 on the 5th day and a minimum of 2.03 on the 16th day of growth) during the various growth and autolysis phases, clearly showing that the difference between the growth curves and the difference in the kinetics of enzyme production may decisively affect the process of strain selection for protease production.

Key words: *Metarhizium anisopliae*, protease production, fungal growth, fungal autolysis, entomopathogenic fungus

INTRODUCTION

Fungi have been extensively used as producers of different substances of economic interest such as enzymes, antibiotics, vitamins, amino acids and steroids. In many cases, the metabolites produced in culture play an important role in the physiological processes of the microorganism in the environment. This is the case for the hydrolytic enzymes and the toxines produced by the entomopathogenic deuteromycete fungus *M. anisopliae* which, in addition to being of economic interest, are important during the process of host penetration and death. However, the evaluation of the production of these substances and the use of these parameters in programs of selection and genetic breeding of different strains depend on the understanding of the basic aspects related to their development in culture.

Several studies have associated protease production (more specifically chymoelastases acting on insect cuticles or proteases with a toxic action) with the virulence of *M. anisopliae* (4, 6, 7, 8, 23).

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The existence of wide genetic variability in the production of these enzymes has also been observed (2, 13, 19). For practical reasons, most of the studies on enzyme production are limited to a single evaluation performed after a given period of growth. On this basis, we may assume that factors such as the differences between the growth curves of the isolates or the difference in the kinetics of enzyme production by the strains may hamper comparison and selection for the trait of interest.

The objective of the present study was to determine the kinetics of protease production by *M. anisopliae* strains during culture growth and autolysis.

MATERIALS AND METHODS

Origin and maintenance of the strains

Strains CLII and 22 of *M. anisopliae* var. *anisopliae* were obtained from the germplasm bank of the laboratory of entomopathogenic fungi of the State University of Campinas - UNICAMP. Strain CLII was isolated in the state of Alagoas, Brazil, from the sugar cane leaf spittlebug *Mahanarva posticata* (Homoptera: Cercopidae) and strain 22 was isolated in the state of Espírito Santo, Brazil, from pasture spittlebug *Deois flavopicta* (Homoptera: Cercopidae). To obtain spores, the strains were grown on complete solid medium (CSM) (14) at 28°C for 12 days.

Growth conditions

Fifty ml Erlenmeyer flasks containing 30 ml culture medium were inoculated with 3 ml of a suspension containing 3 x 10^7 conidia ml^{-1} in an aqueous solution containing 0.85% NaCl (w/v) and 0.002% Tween-20 (v/v). The flasks were then incubated at 28°C with shaking at 150 rpm for variable periods of time (1 to 16 days). After each incubation period, the content of each flask was filtered through Inlab type 10 paper, previously washed and tared, in order to retain the mycelial mass produced. The collected filtrate was divided into 1 ml aliquots and stored at -70°C. The presence of possible contaminants was monitored throughout the experiment by microscopic observation of the filtrate and by plating aliquots of the medium onto dishes containing CSM. Controls containing culture medium only were used to determine the changes in medium volume over the 16 days of incubation. All experiments were carried out in triplicate.

The two carbon sources (glucose and casein, Hammarsten, vitamin free) were added individually to basic salt medium (14) at a concentration of 0.5% (w/v). When casein was used, $NaNO_3$ was eliminated. The media were sterilized by autoclaving at 121°C for 20 min.

Determination of proteolytic activity against casein

Caseinolytic activity was determined as shown by Söderhäl and Unestam (21), with the following modifications: test tubes containing 1 ml of the filtrate were incubated in a water bath at 30°C. After 5 min, 1 ml of a 2% (w/v) vitamin-free casein (Hammarsten) solution in 0.05 M Tris-HCl buffer, pH 8.0, of the same temperature was then added to each tube. The mixture was incubated at 30°C for 15 minutes without shaking. The reaction was stopped by the addition of 5 ml of a 10% (w/v) trichloroacetic acid and samples were allowed to stand at 25°C for 1 hour. After centrifugation at 3,000 *g* (r_{av} = 7.0 cm) for 15 min, the supernatant was filtered through Inlab type 10 paper and its absorbance was determined at 280 nm. Controls in which trichloroacetic acid was added to the filtrate before adding the substrate were prepared in parallel for all treatments. A mixture containing 1 ml buffer was used as blank and treated in the same manner. Enzymatic activity was calculated as the variation in supernatant absorbance during the period of incubation x 10.

Determination of proteolytic activity against elastin

The elastolytic activity of the filtrate was determined by the method used by St. Leger *et al.* (22), with the following modifications. A reaction mixture containing 4 ml of an elastin Congo red (Sigma) suspension, 1.5 mg ml^{-1} in 0.05 M Tris-HCl buffer, pH 8.0, and 1 ml of the filtrate was prepared. The mixture was shaken vigorously for 10 sec, incubated at 30°C for 30 min without shaking, and then centrifuged at 5,000 *g* (r_{av} = 7.0 cm) for 10 min. The supernatant was filtered through Inlab type 10 paper and absorbance was determined at 450 nm. Controls in which the substrate was excluded from the reaction mixture were carried out in parallel for all treatments. Buffer was used as the blank. Enzymatic activity was determined as the variation in supernatant absorbance during the incubation period x 100.

Determination of residual glucose

Residual glucose present in the medium throughout growth was determined by the method of Nelson (12).

Determination of dry mass and autolysis

The mycelium separated by filtration was dried at 70°C for 72 h. Autolysis was determined by the methods of Reyes and Lahoz (16). The degree of autolysis was defined as percent loss of mycelial dry mass from the day of maximum growth, taken as the initial point, to the day when the sample was evaluated.

Efficiency of growth

The efficiency of growth was estimated by the economic coefficient (EC) according to the following formula:

EC = Mycelial dry weigh (mg) / Quantity of carbon source consumed (mg)

RESULTS AND DISCUSSION

Growth and autolysis

Table 1 shows the production of biomass by strains 22 and CLII growing on medium containing glucose as the only carbon source. Maximum biomass production ocurred on the 5th day of incubation for both strains. The maximum biomass production of strain CLII (71.5 mg/flask) was lower, corresponding to 81.7% of the maximum production reached by strain 22 (87.5 mg/flask). Exogenous glucose depletion ocurred at about the 5th day of growth and on the 7th day both strains presented autolysis process. The beginning of autolysis after depletion of the exogenous carbon source has been observed to a greater or lesser degree in various species of filamentous fungi (1, 9, 10, 11, 17, 18). After 16 days of growth (11 days of autolysis), strain 22 presented 52.4% autolysis and strain CLII presented 61.4% autolysis. These rates of autolysis are observed only when degradation of cytoplasm and cell walls occurs (16). Table 2 shows the efficiency of the utilization of the substrate from the calculation of the economic coefficient. It can be seen that the economic coefficient varied considerably as a function of the time of culture development. The highest economic coefficient was observed on the 5th day for strain 22 when the highest biomass production and total utilization of exogenous substrate occurred. For strain CLII, the highest economic coefficient was observerved on the 3rd day, when the biomass production was 86.0% and the utilization of exogenous substrate was 70.0% of the total.

Table 1. Variations in pH, glucose availability and degree of autolysis during incubation of strains 22 and CLII in medium containing 0.5% (w/v) glucose as the only carbon source.

Time (days)	Dry mass (mg)	Degree of autolysis (%)[a]	Glucose in the medium (mg/ml)	pH of the medium
		Strain 22		
0	-	-	5.19 ± 0.00	6.50 ± 0.00
1	20.5 ± 5.1	-	3.66 ± 0.19	6.63 ± 0.02
2	31.5 ± 6.1	-	3.08 ± 0.15	6.73 ± 0.02
3	45.3 ± 7.4	-	1.72 ± 0.23	6.86 ± 0.07
5	87.5 ± 8.7	-	0.04 ± 0.00	7.82 ± 0.06
7	82.6 ± 4.6	5.6	0.03 ± 0.00	7.94 ± 0.04
9	63.9 ± 0.5	27.0	0.03 ± 0.00	8.14 ± 0.04
13	51.8 ± 4.1	40.8	0.03 ± 0.00	8.27 ± 0.02
16	41.6 ± 2.5	52.4	0.02 ± 0.00	8.40 ± 0.05
		Strain CLII		
0	-	-	5.19 ± 0.00	6.50 ± 0.00
1	18.6 ± 3.1	-	3.77 ± 0.19	6.64 ± 0.01
2	36.3 ± 2.0	-	2.75 ± 0.12	6.73 ± 0.02
3	61.5 ± 12.0	-	1.57 ± 0.56	7.82 ± 0.06
5	71.5 ± 8.5	-	0.03 ± 0.00	8.02 ± 0.01
7	66.2 ± 11.8	7.4	0.02 ± 0.00	8.22 ± 0.04
9	37.2 ± 1.0	48.0	0.02 ± 0.00	8.33 ± 0.12
13	26.7 ± 0.6	62.7	0.02 ± 0.00	8.63 ± 0.07
16	27.6 ± 1.7	61.4	0.02 ± 0.00	8.74 ± 0.04

Each result corresponds to the mean of three replicates.

[a] Autolysis was calculated with respect to the highest production of dry mass.

According to Reyes and Lahoz (16), during growth there is a balance between the synthesis and lysis of the polymers that form the walls. The lack of nutrients may cause the cessation of synthesis, provoking a shift of this balance toward lysis of hyphal walls, with the consequent beginning of autolysis. Although glucose, when present in the medium, represses the synthesis of hydrolytic enzymes, it has been shown that, after the beginning of autolysis, the production of hydrolytic enzymes is induced by the presence of soluble oligomers and monomers present in the medium. These substances are derived from the degradation of the cell wall and are found at low concentrations in the culture fluid due to the turnover of substances that occurs during autolysis (15).

Table 2. Glucose utilization by strains 22 and CLII in submerged cultures.

Incubation time (days)	Strain 22			Strain CLII		
	Glucose utilized (mg)	Mycelium formed (mg)	Economic coefficient	Glucose utilized (mg)	Mycelium formed (mg)	Economic coefficient
1	50.5 ± 6.4	20.5 ± 5.1	40.2 ± 6.2	46.8 ± 6.4	18.6 ± 3.1	40.0 ± 2.0
2	69.6 ± 5.0	31.5 ± 6.1	45.0 ± 5.5	80.6 ± 4.0	36.3 ± 2.0	45.5 ± 4.5
3	114.6 ± 7.7	45.3 ± 7.4	39.5 ± 6.3	119.6 ± 18.5	61.5 ± 12.0	51.0 ± 4.9
5	170.0 ± 0.1	87.5 ± 8.7	51.5 ± 5.1	170.5 ± 0.0	71.5 ± 8.5	41.9 ± 5.0
7	170.2 ± 0.0	82.6 ± 4.6	48.5 ± 2.7	170.5 ± 0.1	66.2 ± 11.8	38.8 ± 6.9
9	170.3 ± 0.1	63.9 ± 0.5	37.5 ± 0.3	170.6 ± 0.0	37.2 ± 1.0	21.8 ± 0.6
13	170.5 ± 0.0	51.8 ± 4.1	30.4 ± 2.4	170.6 ± 0.1	26.7 ± 0.6	15.6 ± 0.4
16	170.5 ± 0.1	41.6 ± 2.5	24.4 ± 1.5	170.8 ± 0.1	27.6 ± 1.7	16.1 ± 1.0

Each result represents the mean of three replicates.

Table 3. Variations in pH and in the proteolytic activity of the filtrate and degree of autolysis during incubation of strains 22 and CLII in medium containing 0.5% (w/v) casein as the only carbon and nitrogen source.

Incubation time (days)	Dry mass (mg)	Extent of autolysis (%)[a]	Proteolytic activity vs. casein[b]	Proteolytic activity vs. elastin[c]	pH of the medium
			Strain 22		
1	27.2 ± 0.5	-	0.14 ± 0.08	0.20 ± 0.16	6.45 ± 0.00
3	72.6 ± 0.3	-	0.35 ± 0.15	0.40 ± 0.14	-
5	103.4 ± 0.6	-	0.51 ± 0.13	0.34 ± 0.22	7.78 ± 0.08
7	102.9 ± 7.2	0.4	1.33 ± 0.17	1.03 ± 0.14	8.41 ± 0.03
9	90.5 ± 3.2	12.5	1.50 ± 0.07	1.40 ± 0.18	8.43 ± 0.06
13	70.3 ± 4.4	32.0	1.17 ± 0.14	1.14 ± 0.14	8.53 ± 0.02
16	58.4 ± 1.5	43.5	1.15 ± 0.18	1.22 ± 0.43	8.57 ± 0.09
			Strain CLII		
1	32.0 ± 1.8	-	0.05 ± 0.03	0.19 ± 0.04	6.46 ± 0.02
3	78.9 ± 4.2	-	0.38 ± 0.24	0.22 ± 0.17	-
5	91.7 ± 4.2	-	10.05 ± 0.22	3.93 ± 0.12	8.14 ±0.10
7	58.7 ± 1.5	36.0	7.21 ± 0.92	3.25 ± 0.39	8.32 ± 0.12
9	45.2 ± 4.6	50.7	5.48 ± 0.84	2.79 ± 0.19	8.59 ± 0.05
13	43.7 ± 1.6	52.3	3.22 ± 0.14	2.12 ± 0.25	8.77 ± 0.05
16	39.0 ± 1.7	57.5	2.33 ± 0.48	1.84 ± 0.13	8.67 ± 0.06

Each result represents the mean of three replicates.

[a] Autolysis was calculated with respect to the highest dry mass production.
[b] Activity determined by the variation in absorbance at 280 nm during the period of incubation (5 min.).
[c] Activity determined by the variation in absorbance at 450 nm during the period of incubation (30 min.).

Table 3 shows the production of biomass by strains 22 and CLII growing on medium containing casein as the only source of carbon and nitrogen. Again, the maximum production of strain 22 (103.4 mg/flask) was higher than the production of strain CLII (91.7 mg/flask). Both presented a production peak on the 5th day of growth. The biomass production data presented in Fig. 1 show that casein is a carbon source that can be used more efficiently by *M. anisopliae* than glucose, favoring a more rapid growth and a greater biomass production. Similar results have been reported for other species of filamentous fungi (5). Using respirometric experiments, we observed that casein and hydrolyzed casein accelerate germination, reduce the duration of the lag phase and increase the growth rate compared to glucose (umpublished data).

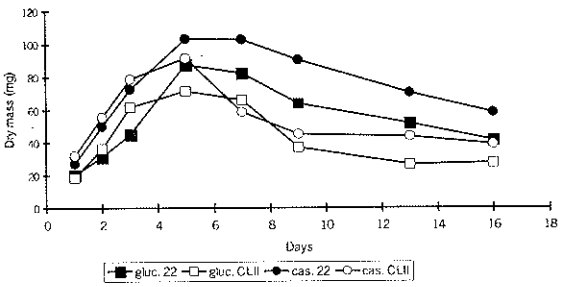

Figure 1. Growth curves for strains 22 and CLII in media containing 0.5% glucose or casein (w/v). Each point represents the mean of three replicates. The standard errors of the means were 3.1 for strain 22 in glicose, 3.9 for strain CLII in glicose, 2.0 for strain 22 in casein and 1.8 for strain CLII in casein.

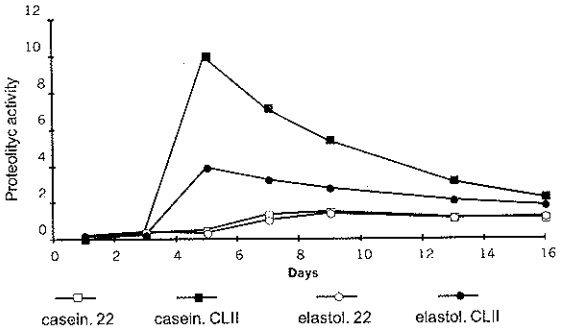

Figure 2. Caseinolytic and elastolytic activities present in the culture filtrates of strains 22 and CLII grown on medium containing 0.5 % casein (w/v) as the single carbon source.Caseinolytic activity was calculated as the variation in absorbance at 280 nm x 10 during the incubation period (15 min). Elastolytic activity was calculated as the variation in absorbance at 450 nm x 100 during the incubation period (30 min). Each point represents the mean of three replicates.

Tables 1 and 3 show that in the culture media used there was a progressive increase in pH both during the growth phase and the autolysis phase of the two strains. Santamaria and Reyes (20), in a study of protease production during autolysis in different species of filamentous fungi, observed that autolysis occurred at pH values between 6.5 and 8. According to these investigators, the fact that autolysis occurs in these fungi at slightly alkaline pH may be related to the production of neutral and alkaline proteases by fungi from the major taxonomic groups. The main protease produced by *M. anisopliae*, Pr1, has a pH optimum of about 8 (22). The progressive increase in medium pH during autolysis of *M. anisopliae* cultures was previously observed by Campbell *et al.* (3).

Protease production

The caseinolytic activity of the *M. anisopliae* culture filtrates is mainly due to the presence of two proteases, Pr1 and Pr2 (22), both capable of hydrolyzing casein. The elastolytic activity is due to the action of Pr1 which, in addition to having caseinolytic and elastolytic activity, has a strong activity against insect cuticles. Pr1 is being indicated as one of the factors responsible for the virulence of the fungus (4, 23).

In a previous study we determined the proteolytic activity of 16 *M. anisopliae* strains after a fixed time of growth (2). The results permitted us to identify two contrasting strains: strain CLII, with high proteolytic activity, and strain 22, with low proteolytic activity. However, we could not determine whether this difference persisted throughout growth and autolysis or whether there was variation in the kinetics of enzyme production between strains. This information was obtained only by monitoring protease production throughout the steps of strain growth and autolysis. Table 3 and Fig. 2 show the variations in the proteolytic activities present in the culture filtrates of the two strains grown on medium containing casein as the only carbon and nitrogen source. Up to the 2nd day the strains presented similar proteolytic activities. Starting at that time, the proteolytic activities of strain CLII were rapidly increased, reaching a maximum at about 5 days, when the highest production of biomass was also observed. A progressive decrease in proteolytic activity occurred thereafter. Protease production by strain 22 increased in a discrete manner, reaching a peak at about 9 days, already during the autolysis phase, and decreasing slowly until the end of the experiment. Studies on other species of fungi have shown that in most cases proteolytic activity increases with the beginning of autolysis; however, it is not uncommon for the maximum proteolytic activity to coincide with the growth phase (5, 20). The highest caseinolytic activity achieved by strain CLII was on average 6.7 times higher than that achieved by strain 22. The highest elastolytic activity observed in strain CLII was on average 2.8 times higher than that observed in strain 22. Fig. 2 shows that the ratios of the proteolytic activities of the two strains varied considerably along the various physiological stages of the cultures. The ratio of the caseinolytic activities of strains CLII and 22 on the 5th day was 19.78 (maximum), and was decreased to 2.03 (minimum) on the 16th day. Although strain 22 never presented a higher proteolytic activity than strain CLII, the variation in the ratio of their activities along

development clearly shows that the difference in the growth curves and in the kinetics of enzyme production may impair the evaluation of the trait if proteolytic activity is determined only once during culture development.

ACKNOWLEDGMENTS

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RESUMO

Produção de protease durante o crescimento e análise de culturas submersas de *Metarhizium anisopliae*

O crescimento e a autólise de duas linhagens do deuteromiceto entomopatogênico *Metarhizium anisopliae* var. *anisopliae* foram avaliados em meio contendo caseína ou glicose como fonte de carbono. Foram determinados parâmetros como o coeficiente econômico e o grau de autólise apresentado pelas linhagens. A produção de protease foi determinada durante todas as fases do crescimento e da autólise das culturas, em meio indutor da produção de proteases (meio contendo caseína como única fonte de carbono e de nitrogênio). Pôde-se verificar que o fungo foi capaz de utilizar a caseína como fonte de carbono/energia de maneira mais eficiente do que a glicose. A autólise apresentada pelas linhagens foi intensa em ambas as condições de crescimento, alcançando até 62,7% da massa seca produzida, e se iniciou logo após o esgotamento da fonte exógena de carbono. A relação entre as atividades proteolíticas apresentadas pelas duas linhagens avaliadas variou de maneira significativa (máxima de 19,78 no 5° dia e mínima de 1,55 no 16° dia de incubação) durante as diversas fases do crescimento e da autólise, deixando claro que as diferenças verificadas entre as curvas de crescimento e entre as cinéticas da produção das enzimas podem influenciar decisivamente no processo de seleção de linhagens para a produção de proteases.

Palavras-chave: *Metarhizium anisopliae*, produção de protease, crescimento de fungo, autólise de fungo, fungo entomopatogênico.

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PURIFICATION AND CHARACTERIZATION OF A LOW MOLECULAR WEIGHT XYLANASE FROM SOLID-STATE CULTURES OF *ASPERGILLUS FUMIGATUS* FRESENIUS

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ABSTRACT

A xylan-degrading enzyme (xylanase II) was purified to apparent homogeneity from solid-state cultures of *Aspergillus fumigatus* Fresenius. The molecular weight of xylanase II was found to be 19 and 8.5 kDa, as estimated by SDS-PAGE and gel filtration on FPLC, respectively. The purified enzyme was most active at 55°C and pH 5.5. It was specific to xylan. The apparent K_m and V_{max} values on soluble and insoluble xylans from oat spelt and birchwood showed that xylanase II was most active on soluble birchwood xylan. Studies on hydrolysis products of various xylans and xylooligomers by xylanase II on HPLC showed that the enzyme released a range of products from xylobiose to xylohexaose, with a small amount of xylose from xylooligomers, and presented transferase activity.

Key words: xylan, xylanase, *Aspergillus fumigatus*

INTRODUCTION

Xylan is one of the major constituents of lignocellulosic materials, accounting for approx. 35% of the total dry weight of higher plants (12). The basic molecular structure of xylan is a linear backbone comprised of β-1,4-linked D-xylopyranose residues which, depending on the origin and method of extraction, may be substituted with branches containing mainly acetyl, arabinosyl and glucuronosyl residues (2, 12). The complete cleavage of the complex structure of xylan molecules requires the combined action of β-xylanase (EC 3.2.1.8) and β-xylosidase (EC 3.2.1.37) with debranching enzymes such as α-arabinofuranosidase (EC 3.2.1.55), acetyl xylan esterase (EC 3.1.1.6) and α-glucuronidase (EC 3.2.1) (2, 22). There is a great interest in the enzymatic hydrolysis of xylan because of possible applications in ruminal digestion, waste treatment, fuel and chemical production, and paper manufacture (2, 12, 27). This work reports the purification and some properties of xylanase II from solid-state cultures of the fungus *Aspergillus fumigatus* Fresenius.

MATERIALS AND METHODS

Organism and Enzyme Production. *A. fumigatus* Fresenius was isolated from a hot fountain in Brasil (Caldas Novas, Goiás) and identified by

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specialists of the Mycology Department from the Universidade Federal de Pernambuco (Brazil). For production of xylanase activity, *A. fumigatus* Fresenius was cultured at 42°C for 7 days in a solid-state medium containing wheat bran (27). After the growth procedure, the contents of the flasks were extracted with 750 ml of 25 mM sodium acetate buffer, pH 5.0 and placed under shaking (120 rpm) at room temperature for 3 h. The resulting crude extract was centrifuged for 30 min at 10,400 g and 5°C, filtered and stored at 5°C for subsequent use as source of xylanase activity.

Enzyme Assays. Xylanase activity was determined by mixing 25 µl of enzyme solution with 50 µl of oat spelt xylan (3.0-50 mg/ml) in 100 mM sodium acetate buffer, pH 5.0 at 50°C for 30 min. The release of reducing sugar was measured using the dinitrosalicylic reagent method (19, 24). Xylanase activity was expressed as µmol reducing sugar formed min^{-1} ml^{-1} enzyme solution, i.e., as IU ml^{-1} and IU mg^{-1} protein. ß-Glucanase, carboxymethyl-cellulase and ß-mannanase assays were performed in the conditions as described above. The activity against filter paper was measured as described by Mandels *et al.* (18). ß-Xylosidase, α-arabinofuranosidase, ß-glucosidase and ß-mannosidase activities were determined as reported elsewhere (24). For the kinetic experiments, soluble and insoluble samples from oat spelt and birchwood xylans were used as substrates in a concentration range of 0.05-8.0 mg/ml. The substrates were prepared as described by Filho *et al.* (11, 13). K_m and V_{max} Values were estimated from Michaelis-Menten equation with a non-linear regression data analysis program (17). The determination of optimum temperature of xylanase II was carried out in the temperature range of 30 to 90°C in 100 mM sodium acetate buffer, pH 5.0. To determine the optimum pH of xylanase II activity at 50°C, the range was from 3.0 to 8.0. McIlvaine type buffer systems were adjusted to the same ionic strength with KCl (7). The temperature stability of xylanase II was determined by pre-incubating the enzyme at 55°C. At various time periods, aliquots were withdrawn and the residual activity was measured under standard conditions.

Protein Concentration. Protein concentration was measured by the method of Petterson (20), using bovine serum albumin as standard.

Enzyme Purification. All purification steps were carried out at 4°C unless otherwise specified. The crude extract was concentrated by ultrafiltration using an Amicon system with a 10 kDa cut-off point membrane (PM 10). Aliquots of the ultrafiltrate were fractionated by gel filtration on Sephadex G-50 (2.3 x 42 cm), pre-equilibrated with 100 mM sodium acetate buffer, pH 5.0. Fractions of 4.0 ml were collected at a flow rate of 12 ml/h. Fractions with xylanase activity were pooled, dialyzed against 10 mM sodium phosphate buffer, pH 7.2, and loaded onto a Econo-Pac CHT-II column (1.3 x 4.1 cm), equilibrated with the same buffer. Fractions of 2.0 ml were collected at a flow rate of 30 ml/h by washing the column with buffer followed by a linear gradient of sodium phosphate buffer (10-400 mM), pH 7.2, and those with xylanase activity were pooled and dialyzed against 20 mM sodium phosphate buffer, pH 7.0. The dialyzed solution was applied to a Econo-Pac High Q column (1.3 x 4.1 cm) pre-equilibrated with 20 mM sodium phosphate buffer, pH 7.0. The column was washed with the same buffer and eluted with a linear gradient of NaCl (0-1 M). Fractions of 2.0 ml were collected at a flow rate of 120 ml/h. Fractions containing xylanase activity were pooled and dialyzed against 20 mM sodium phosphate buffer, pH 6.5. Finally, further enzyme purification was performed in the same column equilibrated with 20 mM sodium phosphate buffer, pH 6.5 at a flow rate of 120 ml/h. The residual protein was eluted with a NaCl linear gradient from 0 to 1 M. Fractions corresponding to xylanase activity were pooled, concentrated by freeze-drying and stored for later use at 4°C.

Electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described by Laemmli (16) using 12% gels. After electrophoresis, protein bands were silver stained by the method of Blum *et al.* (3). The molecular weight of xylanase II was estimated by SDS-PAGE and gel filtration on a fast protein performance liquid chromatography system (FPLC) using MW marker kits from Sigma Chemical Co., USA. The FPLC column (Superose 12) was equilibrated with 20 mM sodium phosphate buffer, pH 7.0. The elution was performed at a flow rate of 30 ml/h.

Hydrolysis Products. The reaction mixture containing 100 µl of enzyme solution (20 µg/ml) and 500 µl of 0.2% xylan in distilled water was incubated for 16 h at 28°C with shaking at 100 rev./min. The reaction mixture from above was stopped by heating in boiling water and centrifuged at 3,000 x g for 5

min. The determination of hydrolysis products was made by high performance anion exchange chromatography coupled with pulsed amperometric detection (Dionex Corp., USA), as described previously (11, 22, 23). The analysis of the hydrolysis products of xylooligomers by xylanase IIa was also performed as described above. However, the hydrolysis of xylooligomers was determined following 1 to 2 h of incubation at 40°C.

Chemicals. Oat spelt xylan and birchwood xylan, were from Sigma Chemical Co., USA. All other chemicals were analytical grade reagents. Deacetylated and acetylated xylans were obtained by dimethylsulfoxide extraction (DMSO) of beechwood and wheat straw holocelluloses, respectively (23). Xylan extracted by HCl from the seaweed *Palmaria palmata* was a gift from Maria G. Tuohy (University College Galway, Ireland). Xylooligosaccharides were prepared as described before (21).

RESULTS AND DISCUSSION

A xylanase was isolated from the xylan-degrading enzyme system of *A. fumigatus* Fresenius, and purified to apparent homogeneity by a combination of ultrafiltration and chromatographic procedures. The purification steps of xylanase II are summarized in Table 1. The ultrafiltration experiment showed that a xylanase activity was found to be present in the ultrafiltrate. For further purification, the ultrafiltrate was subjected to gel filtration chromatography on Sephadex G-50 (Fig. 1). The sample elution resulted in the separation of two peaks

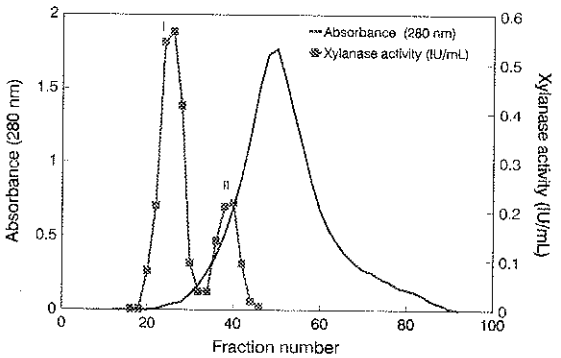

Figure 1. Fractionation on Sephadex G-50 of *A. fumigatus* Fresenius ultrafiltrate. For experimental details see text.

of xylanase activity (I and II). The second peak, designated xylanase II, was used for further purification by hydroxylapatite and anion-exchange chromatographies (results not shown). In both purification procedures, xylanase II was eluted in the pre-gradient wash fractions. The purification step procedures provided an apparently homogeneous preparation of xylanase II, as determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The enzyme migrated as a single 19 kDa band on SDS-PAGE stained with silver nitrate (Fig. 2). However, its apparent size by gel filtration was 8.5 kDa, indicating a physical interaction between the enzyme and chromatography resin (result not shown). This is in agreement with the results obtained for xylan-degrading enzymes from *T. harzianum*, *T. reesei*, *A. oryzae*, *A. fumigatus* VTT-D-71002 and *Bacillus* sp (1, 8, 26). A small amount of carbohydrate was found when the purified

Table 1. Summary of the purification of ß-xylanase II from *A. fumigatus* Fresenius.

Step	Total protein (mg)	Total activity (IU)	Specific activity (IU mg^{-1})	Yield (%)	Purification factor
Crude Extract	196.78	1,234.0	6.27	100	1.0
Ultrafiltration	37.41	210.21	5.62	17.05	0.90
Sephadex G-50	19.23	90.0	4.67	7.30	0.74
Econo-Pac CHT-II	6.13	12.0	2.0	1.0	0.31
Econo-Pac High Q pH 7.0	2.82	6.04	2.14	0.50	0.34
Econo-Pac High Q pH 6.5	0.45	3.21	7.10	0.26	1.32

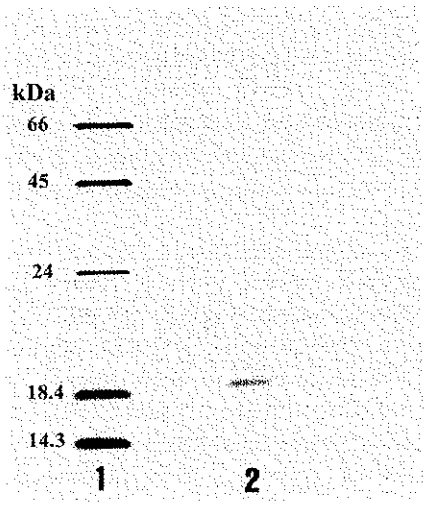

Figure 2. SDS-PAGE (12%) of ß-xylanase II from *A. fumigatus* Fresenius stained with silver nitrate. Lane 1, molecular weight standards (from the top): bovine serum albumin (66 kDa), ovalbumin (45 kDa), trypsinogen (24 kDa), ß-lactoglobulin (18.4 kDa), lysozyme (14.3 kDa); lane 2, xylanase II.

xylanase II from *A. fumigatus* Fresenius was assayed with the phenol-sulfuric acid method (9). The low yield and fold-purification values obtained for xylanase II from *A. fumigatus* were probably underestimated. This phenomenon is often described during purification of xylan-degrading enzymes produced by fungi (10, 13, 14). At least one major and some minor peaks of xylanase activities were also detected in the ultrafiltrate during the purification procedure. Besides, the ultrafiltration procedure retained most of the bulk of xylan-degrading activities in the retentate. Fractionation of the retentate by gel filtration, adsorption and ion-exchange chromatography techniques showed the presence of, at least, nine different xylanase activities. These enzymes may act synergistically with xylanase II to effect the complete hydrolysis of xylan (10).

Xylanase II was optimally active at pH 5.5 and 55°C. This optimum pH value was also described for xylanases from *A. sydowii* MG49 and *A. niger* (15, 25). The purified enzyme showed a half-life of 80 min. when incubated at 55°C and pH 7.0 (Fig. 3). The purified enzyme was not active on CMC, filter paper, para-nitrophenyl ß-D-xylopyranoside, para-nitrophenyl α-L-arabinofuranoside, para-nitrophenyl ß-D-mannopyranoside, para-nitrophenyl ß-D-glucopyranoside, ß-laminarin and ß-mannan. In addition, the enzyme was specific for xylan. The K_m values for soluble xylan from oat spelt were much higher than the insoluble one (Table 2). This might suggests a steric hindrance due to the presence of side-chains groups in soluble xylan. Conversely, the hydrolysis of soluble birchwood xylan by xylanase II was more effective than when the enzyme was incubated with the insoluble xylan, suggesting that the presence of a particular type of substituent (acetyl group) in the vicinity would be a requirement for the action of xylanase II (4). The hypothesis of the substituents are probably situated in regions of the polysaccharide distant from the unsubstituted portions can not be discarded. Debranching activity was also found in some xylanases (2). The K_m values for xylanase II were lower than the values found for xylanases from *F. oxysporum* and *P. capsulatum* (5, 6, 11).

Table 2. Some kinetic properties of the purified ß-xylanase II produced by *A. fumigatus* Fresenius.

Substrate	K_m (mg/ml)	V_{max} (IU/ml)
Soluble oat spelt xylan	5.72	2.34
Insoluble oat spelt xylan	3.01	1.41
Soluble birchwood xylan	2.19	1.55
Insoluble birchwood xylan	5.19	1.89

The predominant hydrolysis products of birchwood and deacetylated xylans by xylanase II ranged from xylobiose to xylohexaose (Table 3). Xylanase IIa was not able to release xylose from xylan molecules, suggesting an endomechanism. Xylan from seaweed (*Palmaria palmata*), a ß-1,3;1,4-linked polymer, was degraded to a mixture of xylobiose and xylotriose, while xylobiose to xylopentaose were released from oat spelt xylan. The high proportion of acetyl groups linked to xylose residues in acetylated xylan was probably a steric obstacle to xylanase II activity (12). The purified xylanase did not show any activity against xylotriose and xylotetraose. The hydrolytic capacity of ß-xylanase increased with increasing chain length of xylooligomers (Table 3). Xylose, xylobiose and xylotetraose were removed from xylopentaose. The enzyme showed transferase activity when incubated with xylohexaose. The purified enzyme was able to produce higher molecular weight transfer products. Transferase activity has been reported for some other xylan-degrading enzymes from *Aspergillus* (1, 5).

In conclusion, the fungus *A. fumigatus* Fresenius produces multiple forms of xylanases. The purified xylanase II is a 19 kDa enzyme with an acidic

Table 3. Hydrolysis products of xylans and xylooligomers by xylanase II from *A. fumigatus* Fresenius. Abbreviations: X1, xylose; X2, xylobiose; X3, xylotriose; X4, xylotetraose; X5, xylopentaose; X6, xylohexaose; ND, not detected.

Substrate	Hydrolysis Products
Oat spelt xylan	X2, X3, X4, X5
Birchwood xylan	X2, X3, X4, X5, X6
Acetylated xylan	ND
Deacetylated xylan	X2, X3, X4, X5, X6
Seaweed xylan	X2, X3
Xilotriose[a]	ND
Xilotriose[b]	ND
Xylotetraose[a]	ND
Xylotetraose[b]	ND
Xylopentaose[a]	X1, X2, X4
Xylopentaose[b]	X1, X2, X4
Xylohexaose[a]	X3, X4, X5
Xylohexaose[b]	X1, X3, X4, X5

[a]One hour incubation; [b]two hours incubation

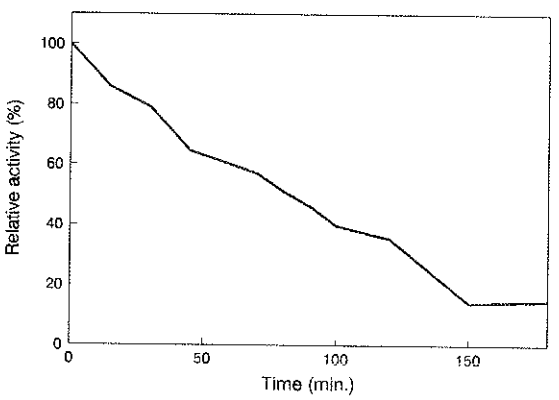

Figure 3. Stability of purified xylanase II from *A. fumigatus* Fresenius at 55°C.

optimum pH and thermostability. It seems to belong to the group of specific xylanases with an endo-acting mechanism.

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RESUMO

Purificação e caracterização de uma xilanase de baixo peso molecular de culturas de estado sólido de *Aspergillus fumigatus* Fresenius

Uma enzima xilanolítica (xilanase II) foi purificada a partir de culturas de estado sólido de *Aspergillus fumigatus* Fresenius. O peso molecular de xilanase II foi estimado em 19 e 8,5 kDa por SDS-PAGE e FPLC, respectivamente. A enzima purificada apresentou maior atividade a 55°C e pH 5,5, além de hidrolisar especificamente xilana. Os valores aparentes de K_m e V_{max} de xilanas solúveis e insolúveis, isoladas de cereal e madeira, mostrou que xilanase IIa foi mais ativa em xilana solúvel de madeira. Estudos sobre produtos de hidrólise de xilanas e xilooligômeros por xilanase II em HPLC revelou que a enzima liberou uma variedade de xilooligômeros (xilobiose-xilohexose) e uma pequena quantidade de xilose a partir de xilooligômeros, apresentando atividade de transferase.

Palavras-chave: xilana, xilanase de baixo peso molecular, purificação da enzima, caracterização da enzima.

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LOW COST PRODUCTION AND PURIFICATION OF POLYCLONAL ANTIBODIES TO STAPHYLOCOCCAL ENTEROTOXIN A

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ABSTRACT

An immunization scheme for production of antiserum to staphylococcal enterotoxin A (SEA) is proposed. The reference method of Robbins and Bergdoll was modified to reduce the number of doses and the amount of toxin used per animal. The best immunization scheme used injections in days 0, 8, 24, 59, 62 and 67. The amount of toxin at each injection was 5, 6, 20, 50, 100 and 200μg, respectively. The total amount of toxin was 381μg, which corresponded to a reduction of 107μg in the amount of toxin for each animal when compared to the reference method. The average antiserum titer using the Optimum Sensitivity Plate - OSP was 1:60 and using ELISA the titer was 1:100.000. The lack of cross-reactivity with other staphylococcal enterotoxins indicated high specificity of the antibody to SEA. The proposed immunization scheme was adequate to produce specific SEA antisera, with high titers and the aditional advantage of reducing the amount of purified SEA required for immunization.

Key words: *Staphylococcus aureus,* enterotoxins, detection, immunization

INTRODUCTION

Staphylococcal food poisoning is a worldwide intoxication caused by the ingestion of staphylococcal enterotoxins (SEs), preformed in food by some *Staphylococcus aureus* strains. Although *S. aureus* can be easily detected in foods, neither its presence necessarily indicates enterotoxin production nor the absence of viable staphylococci assures food safety (4).

The organism looses viability rapidly after the stationary phase, being replaced by harmless saprophytic bacteria. However, the toxins resist both heat treatment and proteolytic enzymes action and they can be detected in precooked, pasteurized and manufactured foods. The direct detection of enterotoxins in foods requires development of practical, rapid and sensitive assays. Currently, the best methods for enterotoxin identification and quantification depend on the availability of specific antibodies for each enterotoxin type. Immunological methods with monoclonal and polyclonal antibodies for the enterotoxin detection at the level of 1 - 2 ng g^{-1} include radioimmunoassay, enzyme-linked immunoassay (ELISA) and reversed passive latex agglutination (5, 7, 15). At least, five diagnostic kits are now available commercially (9, 10, 14).

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Brazilian Public Health Laboratories have reported many outbreaks of food poisoning involving foods contaminated by *S. aureus* (8). However, the cost of imported diagnostic kits creates difficulties to the use of the direct enterotoxin detection techniques in the routine quality control laboratories.

This work reports an immunization scheme for the production of polyclonal anti-staphylococcal enterotoxin A, simplifying the reference procedure of Robbins and Bergdoll (12) and also describes a simple IgG purification method.

MATERIALS AND METHODS

Enterotoxins

Purified enterotoxin A (SEA) for animal immunization was kindly donated by Dr Merlin S. Bergdoll (University of Wisconsin, U.S.A.). Purified enterotoxins B(SEB), C_2(SEC$_2$), D(SED) and E(SEE) for measurement of cross-reactions were acquired from Toxin Technology INC, Madison, Wisconsin, U.S.A.

Preparation of antigen

Complete Freund's adjuvant was used in the initial injections and incomplete Freund's adjuvant for subsequent procedures. Incomplete adjuvant was prepared mixing 8.5 ml of nujol (Vetec Química Fina Ltda.) with 1.5 ml of lanolin (Quimidrol Com. Ind. Imp. Ltda.), while for complete Freund's adjuvant, 10 mg of freeze-dried avirulent *Mycobacterium tuberculosis* strain H 37 Ra was added to the nujol and lanolin mixture. The toxin dose in 1.0 ml of 0.05 M phosphate buffered saline pH 7.4 was added to 1.0 ml of Freund's adjuvant and injected subcutaneously into the three different sites of rabbit shoulder.

Immunization scheme

Anti-SEA was prepared in female New Zealand white rabbits of 2 to 3 kg, using four modifications of the reference procedure (12) (Table 1). One to five rabbits were used for each immunization. The animals were weighed before each injection and every day on the following two days to check for the weight loss (up to 200g). The next injection was postponed, if the weight loss was not regained by the scheduled date.

Determination of antibody titer

The antiserum titer was determined by the Optimum Sensitivity Plate (OSP) method (11), using two-fold dilutions of the test serum and the SEA standard at 2µg and 4µg/ml. Blood was taken from the marginal ear vein eight days after the last immunization dose.

Boostering procedure

The threshold injections were followed with three booster injections on days 63, 66 and 77 after the first injection for the Robbins and Bergdoll's procedure, and on days 59, 62 and 67 for the modified scheme (Table 1). Only rabbits with normal weight and with serum titers of 8 or more received booster injections. Animals with lower than 8 serum titers were discarded. Six weekly bleedings were done starting at week 1 after the last injection.

Antibody purification

The purification of the IgG fraction of the pooled antisera was carried out using a staphylococcal protein A-Sepharose CL-4b column (Pharmacia). 10ml of serum was applied onto the column (5.0 x 1.5 cm) at room temperature, washed twice with 0.2 M PBS, pH 7.4 and the IgG fraction was eluted at 4°C with 15 ml of 0.1 M sodium citrate buffer, pH 2.8. The pH of each fraction was adjusted to 7.0 adding 30µl of 1.0 M TRIS buffer, pH 8.8. Fractions showing the higher absorbances at 280nm were pooled and dialysed overnight at 4°C against 0.02 M PBS, pH 7.4. Protein content was measured according to the method of Bradford (1).

Antibody cross-reactions

Cross-reactivity among staphylococcal toxins was analyzed by OSP (11) and enzyme-linked imunosorbent assay - ELISA (8).

ELISA procedure

Cross-reactions were analyzed adding 100µl of SEA, SEB, SEC$_2$, SED or SEE in PBST to antibody-coated wells of microtitration plates. The toxin concentrations ranged from 0.25 to 8 ng/ml for SEA; 1 to 10µg/ml for SEB, SEC$_2$ and SEE, and 1.0ng to 10µg for SED. The plates were incubated at 37°C for 1 hour, washed five times with PBST, incubated again at 37°C for 1 hour and added of 100µl of antibody-peroxidase conjugate diluted at 1:1000 in PBST. 100µl of 3.3', 5.5' - tetramethilbenzidine solution were added to the washed plates. After 20 minutes, the enzyme reaction was stopped with 40ml of 2 M H_2SO_4 and the absorbance read at 450 nm.

OSP method

Three different *S. aureus* strains from Food Research Institute, Wisconsin, USA (FRI-137; FRI-472; FRI-376), 17 enterotoxigenic *S. aureus* strains isolated from food handlers and 20 non-enterotoxigenic *S. aureus* were tested to measure cross-reactivity. Enterotoxigenic *S. aureus* strains from healthy food handlers producing staphylococcal enterotoxins others than SEA were kindly donated by Prof. Francisco Herrero, from University of Maringá.

RESULTS AND DISCUSSION

The four modifications of the reference scheme (Table 1) intended to reduce the number of injections and the amount of toxin necessary to obtain a titer equivalent to the reference method (12).

Comparing the titers determined by OSP method (11) using 2 and 4 μg of SEA, the lower titer showed the best result, with a sharp immunoprecipitation line.

Toxicity of SEA limits the quantity of toxin to be used as initial dose and only 1 μg of SEA is recommended at the first injection. Five μg may cause animal death. SEA is the most toxic staphylococci enterotoxin and classic immunization procedure allows an initial injection doses of 1μg to SEA and SEE and 10μg to SEB, SEC and SED (12). However, immunization of rabbits with high initial staphylococcal enterotoxin dose, without animal loss, was reported by Miya *et al.* (6).

Initial injection of 5 to 25 μg of SEA did not cause the loss of any of the 13 rabbits tested (Table 1). Only the rabbit of scheme 6, which received 25μg at initial dose (Table 1), presented significant weight loss and slow recovery, although the titer after booster dose was adequate (Table 3). This fact induces improvements in the immunization procedure, with emphasis on low number of injections.

The scheme 1 of Robbins and Bergdoll (12) resulted in the best immunization titer (Table 1), but the same was not observed after boosters (Table 3). Comparatively, our proposed schemes, mainly scheme 2, resulted in higher final titers, which where two to three times the titer of the reference sheme.

An efficient immunological response to SEA depends on the number of injections with high titer related to frequency and gradual increase of toxin concentration (12). Despite of this fact, scheme 2 reduced the needed number of injections, without changing the antiserum titer. The same statement was not valid for schemes 4 and 5, which used two injections for immunization and a higher SEA concentration at the initial injection (Table 1).

Investigations demonstrating action of SEA as superantigen extend other perspectives for immunization pattern and use in immune response stimulation (2, 13).

Table 1. Rabbit immunization schemes for production of antisera to staphylococcal enterotoxin A

	Day												
	0	3	8	21	24	28	59**	62**	63**	66**	67**	71**	
Scheme	SEA dose (μg)												Total
1*(a)	1[a]	2[b]	5[b]	-	10[b]	20[b]	-	-	30[b]	100[b]	-	300[b]	488
2 (b)	5[a]	-	6[b]	-	20[b]	-	50[b]	100[b]	-	-	200[b]	-	381
3 (c)	10[a]	-	-	25[b]	-	-	50[b]	100[b]	-	-	200[b]	-	385
4 (d)	15[a]	-	-	25[b]	-	-	50[b]	100[b]	-	-	200[b]	-	390
5 (e)	25[a]	-	-	25[b]	-	-	50[b]	100[b]	-	-	200[b]	-	400

* Reference procedure of Robbins and Bergdoll (12)
** Booster injections
[a] SEA emulsified in complete Freund's adjuvant
[b] SEA emulsified in incomplete Freund's adjuvant
(a) two rabbits; (b) five rabbits; (c) three rabbits; (d) four rabbits; (e) one rabbit.

Table 2: Antiserum titers after immunization

Scheme	Rabbits	Total SEA (g)	Titer* 2g SEA/ml	Titer* 4g SEA/ml
1	A		32	16
	B	38	32	16
	A		32	12
	B		8	4
2	C	31	8	4
	D		16	8
	E		8	4
	A		4	2
3	B	35	-	-
	C		-	-
	A		32	16
4	B	40	-	-
	C		8	4
	D		-	-
5	A	50	16	8

SEA emulsified in complete (first dose) and incomplete (subsequent doses) Freund's adjuvant.
* As determined by OSP (Optimun Sensitivity Plate)

Table 3: Antiserum titers after booster

Scheme	Rabbits	Total SEA (g)	Titer* 2g SEA/ml	Titer* 4g SEA/ml
1	A	450[a]	48	24
	B		40	20
	A		120	60
	B		60	30
2	C	350[a]	60	30
	D		60	30
	E		40	20
	A		60	30
4	B	350[a]	-	-
	C		-	-
	D		-	-
5	A	350a	48	20

[a]SEA emulsified in incomplete Freund's adjuvant
* As determined by OSP (Optimun Sensitivity Plate)

Animals of scheme 3 were discarded and the booster dose was not given because results of immunization were inadequate (Table 3). Rabbits of scheme 4 were discarded after the booster dose because there was no response in three out of four animals.

Rabbits of scheme 2 received 100 µg less SEA than those of the reference method but reasonable titer was achieved with a reduced number of injections.

Fig. 1 confirms the excellence of scheme 2 for antiserum production, which was equivalent to the method of Robbins and Bergdoll (12).

The cross-reactivity of crude antiserum with

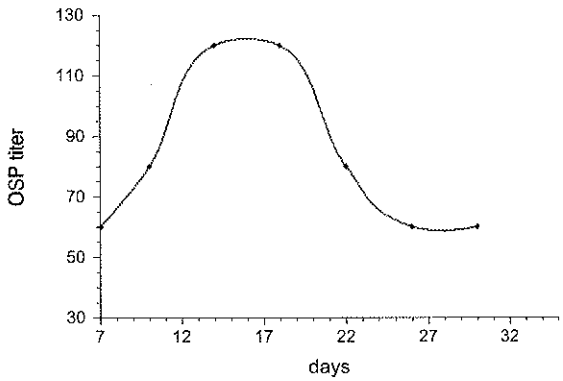

Figura 1. Antibody response in rabbit 2A after booster of 350 µg of SEA

other staphylococcal enterotoxins was analysed by OSP and by ELISA-sandwich method using purified IgG (data not shown). In OSP method, the specificity of crude antiserum was enough to avoid identity or partial identity precipitation against culture supernatant of *S. aureus* strain SFRI-137 (SEC1) 472 (SED) and 326 (SEE). An intense inespecific precipitation line with anti-SEA, observed with SED-producing strain 472, disappeared after absorbing the supernatant, indicating inespecific reaction with protein A. The crude polyclonal anti-SEA did not cross-react with 20 non-enterotoxigenic *S. aureus* strains and 17 other enterotoxin producing strains.

In ELISA-sandwich method, using IgG purified by protein A, the amounts of toxin required for $OD_{450} = 0.5$ were: SEA, 2.5 ng/ml; SED, 6.7 µg/ml; SEC_2, 3.4, µg/ml, SED, >10 µg/ml; SEE, >10 µg/ml. The highest cross-reaction was observed with SEC_2. However, the occurrence of cross-reactions was less than 0.1%.

In conclusion, scheme 2 was adequate to produce specific SEA antisera, with high titers. In addition to the lower amount of toxin required for immunization, the proposed procedure also requires a lower number of injections when compared to the reference method. High titers of antibodies were achieved in all rabbits after booster. The polyclonal antibody was satisfactory as reagent in OSP and the purified IgG was used in ELISA without cross-reactions with other staphylococcal enterotoxins.

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RESUMO

Produção e purificação, a baixo custo, de anticorpos policlonais para enterotoxina estafilocócica A

O objetivo principal deste trabalho foi propor um esquema de imunização para a produção de anticorpos para a enterotoxina estafilocócica A (EEA). O método de referência preconizado por Robbins e Bergdoll foi modificado visando a redução do número de doses e da quantidade de toxina administrada por animal. O melhor esquema de imunização empregou doses de 5, 6, 20, 50, 100 e 200μg de EEA administradas a intervalos de 0, 8, 24, 59, 62 e 67 dias, respectivamente. Esse esquema empregou um total de 381 μg, equivalente à redução de 107 μg de EEA, em relação ao método de referência. O título médio do anti-soro utilizando a técnica de *Optimum Sensitivity Plate* - OSP foi de 1:60 e de ELISA 1:100.000. A ausência de reações cruzadas com outras enterotoxinas estafilocócicas, observada nas técnicas de OSP e ELISA, indicou alta especificidade do anticorpo para EEA, com a vantagem da redução da quantidade de toxina purificada necessária para a imunização.

Palavras-chave: *Staphylococcus aureus*, detecção, enterotoxinas, imunização

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THE GROWTH OF *MICROCOCCUS VARIANS* BY UTILIZING SUGAR CANE BLACKSTRAP MOLASSES AS SUBSTRATE

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ABSTRACT

Comparative studies on the growth of *Micrococcus varians* were carried out in BHI culture medium (control) as well as in a culture medium with 2% diluted sugar cane blackstrap molasses, enriched with 0.1% yeast extract. The experiment was conducted with three samples of the experimental and control media in a 5 liter fermentor with working volume of 3.5 liters, continuous agitation (150 rpm), 35 ± 0.1°C temperature, 0.7 L air. l^{-1} medium. min^{-1}, initial pH 7.0 ± 0.2, 24 hour fermentation period, and approximate inoculum of 6.0 $\log_{10}$ CFU/ml. Samples were collected at 2-hour intervals. *Micrococcus varians* grew in the two culture media studied, which confirms the experimental medium viability for the growth of this species. The final average concentration of biomass was higher in the control medium than in the experimental medium: 0.99 $g.l^{-1}$ and 0.78 $g.l^{-1}$, respectively. The final number of viable cells at the end of fermentation was 20.65 $\log_{10}$ CFU/ml for the control medium (BHI), while in the experimental medium the number of viable cells was 19.43 $\log_{10}$ CFU/ml. The consumption of total sugars was higher for the biomass in the control medium (79.78%), while only 50.53% was consumed for the experimental medium.

Key words: *Micrococcus varians*, fermentation, molasses

INTRODUCTION

The practices concerning food fermentation with the purpose of food preservation have been conducted since ancient times (9). Currently, fermented foods are not produced for preservation only, as there are more affordable techniques for that purpose. Now production aims at the development of aromas and flavors which are peculiar to each product, and the development of the technology utilized (3).

Micrococcus varians is a microorganism commonly found in *starter* culture mixtures utilized in the fermentation of meat products. It is a non-pathogenic microorganism with a nitrate reduction activity, and which is homofermentative, with a scarce production of lactic acid. Among its main characteristics, *Micrococcus varians* provides a pleasant color and flavor to products (11).

In complex medium such as nutrient agar, *Micrococcus varians* appears as spherical cells of 0.5 to 2.0 μm in diameter, gram positive or gram variable, predominantly arranged in tetrahedrons or diplococcus. The *Micrococcus* genera morphology

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does not change according to either the culture medium or the age (1).

The processing industry of fermented meat products such as salami utilizes starter cultures due to the various advantages they provide the process with, reducing the losses in the production process and benefiting the product standardization. However, some industries prefer to utilize chemical acidulants such as gluconodelta lactone, but results are not highly satisfactory with regards to flavor (2).

Starter cultures should be approved as GRAS (Generally Recognized as Safety) and laws vary from country to country. However, just a few countries have already regulated the use of microorganisms when processing cured meat. Brazilian laws only establish that the microorganisms utilized for that purpose should be GRAS (7).

In order for a microbial culture to be utilized as a starter culture it should meet some basic requirements, such as: to be salt-tolerant (6 to 10% brine); to be nitrate-tolerant (80 to 100 $mg.l^{-1}$); to grow within a range of 26 to 43°C and show optimal growing temperature between 32 to 35°C; to be homofermentative; not to be proteolytic; not to be lipolytic; not to produce flavors and aromas which are atypical to the final product; not to be pathogenic and to present thermal degradation at 57-60°C (12). Microbial cultures should not either be toxigenic or produce antibiotics. They should not degrade amino acids into pharmacologically active amines or into sulphidric acid, to produce very little or no hydrogen peroxide, not to produce gas and to produce little or no acetic acid (1).

As in the case of *Micrococcus varians,* starter cultures are usually traded in their lyophilized form and their production occurs in complex media such as the BHI. This results in a high cost for the final product. In the particular case of Brazil, which has a very strong and developed meat processing industry, starters are imported mainly from Europe and the United States.

Sugar cane molasses is a by-product of the sugar industry, and it is referred to by various names. Among these, blackstrap molasses is the most commonly used name for industrial fermentations.

In Brazil, molasses is the cheapest carbon source, in addition to being a good substrate with respect to vitamin and minerals. Sugar cane molasses is also utilized by the pharmaceutical industry for the production of amino acids and antibiotics and by the additive industry, for the production of flavor intensifiers (5).

This study aims at comparing the growing of *Micrococcus varians* in sugar cane molasses with those in the BHI, in order to obtain a starter culture with adequate flavor which can be later used as in its frozen form to processing industries.

MATERIALS AND METHODS

CCT 4492 *Micrococcus varians* was maintained in BHI broth with 2% agar in inclined tubes. The culture was incubated at 35°C for 24 hours and stored at 4°C. In order to prevent the medium from drying out, 3 drops of sterile glycerol were added to the culture surface. The sugar cane molasses was collected in aseptic flasks and frozen at -20°C. The composition of molasses was determined according to the AOAC methods (15): moisture, ashes, nitrogen, reducing sugar as glucose, non-reducing sugar as sucrose, potassium by flame photometric, magnesium and manganese by atomic absorption, phosphorus by spectrophotometry.

The molasses was diluted with distilled water at 50% and centrifuged at 4000 rpm for 25 minutes for removal of insoluble solids. The pH was corrected with NaOH 20 M up to 7.0 ± 0.2 pH. The ideal molasses dilution for this experiment was determined through preliminary tests with a dilution rate varying from 1 to 10%. The best *Micrococcus varians* growth was observed in the medium containing a 2% molasses dilution.

The inoculum was obtained from a cell removed from the maintenance medium, resuspended in 100 ml of BHI broth and incubated at 35°C for 24 hours. Three ml aliquots were placed in sterile flasks to which a sterile glycerol at 2% was added and stored at -20°C. Before each fermentation, an inoculum aliquot was adapted to the proposed culture medium by determining the final concentration by spectrophotometry (8) and the cells viability through a plate counting utilizing BHI agar. A 12 $\log_{10}$ CFU/ml counting was observed.

The inoculum was diluted (1:10) with a saline solution with an approximate concentration of up to 6 $\log_{10}$ CFU/ml. A high concentration of microorganisms in the inoculum provides for a fast exhaustion of the substrate, resulting in a non-recommended reduced exponential phase which damages the production of viable cells.

The experimental medium was constituted of sugar cane molasses diluted at 2.0% and added by

0.1% of yeast extract (w/v). The experiments were conducted in batches, in a *New Brunswick Scientific* fermentor, Bioflo 2000 model, and were repeated 3 times. A BHI broth was utilized as a control medium. The culture was carried out in the following conditions: 3.5 liter medium; 6.0 $\log_{10}$ CFU/ml inoculum; 150 rpm agitation; 35 ± 0.1°C temperature; 0.7 l air.l^{-1}medium.min^{-1} and 24-hour culture period (fermentation).

Samples were aseptically collected in triplicate, at 2-hour intervals for the evaluation of dry weight and pH and for determining total sugars. For determining biomass, 2 ml sample aliquots were collected and centrifuged at 4000 rpm for 15 minutes, discarding the supernatant. Three ml of peptone water at 0.1%, 3 ml of EDTA 1% and two drops of NaOH 20M (8) were added to the precipitated. For an evaluation of the growth of viable cells, standard plate counting with BHI agar was utilized (14), incubated at 35°C for 24 hours. The biomass was estimated by a spectrophotometric reading at 520 nm, (8). Five ml aliquots were utilized for determining dry weight and centrifuged at 4000 rpm for 15 minutes. The pH was monitored during fermentation, at each sample collection. The consumption of sugars was determined by Clegg-Anthrone method (10).

Data were analyzed by analyses of variance. Comparison of treatment means was done using the Tukey's test. A $p < 0.05$ was considered be significant.

RESULTS AND DISCUSSION

The sugar cane molasses utilized in this experiment presented the following composition (w/w): moisture 9.10 g%; ashes 9.98 g%; total nitrogen 0.52 g%; reducing sugar as glucose 14.72 g%; non-reducing sugar as sucrose 33.78 g%.

Few strains of *Micrococcus varians* have specific requirements regarding amino acids and vitamins. Several amino acids may apparently meet the needs of organic nitrogen. However, certain specific strains need methionine. The vitamin requirements for this species include thiamin, niacin and pantothenic acid. These characteristics may explain the good performance of the experimental medium utilized (4).

The consumption of total sugars (TS) was higher in the control medium (BHI) (Fig. 1). In this medium, the average TS consumption was 78.79%, which corresponds to a 2.9 g.l^{-1} consumption, while in the experimental medium it was 51.53% and corresponded to an average consumption of 6.18 g.l^{-1}, during the 24-hour fermentation period. The highest TS average consumption was 2.56 g.l^{-1} (69.5%) and it occurred between the 4th and 10th hour-culture in BHI broth. Within the same period the average consumption of sugar in the experimental medium was 2.44 g.l^{-1} (19.95%). The difference in the consumption of substrate between the fermentation of one medium and the other should be attributed to the media composition and to the manner sugars are biologically available in the two media. In the BHI broth as well as in the experimental medium residual sugar may be attributed to the presence of sugars which are not fermented by *Micrococcus varians*. The high level of residual sugar in the experimental medium (6.05 g.l^{-1}) may also be attributed to nutritional deficiencies.

The peak consumption observed in Fig. 1, at 4 to 10-hour interval, partially differs from the exponential phase of microorganisms growth, as shown in Fig. 2. The initial exponential growth occurred at the interval between 4 to 24 hours for the BHI broth, while in the experimental medium it coincided with the 4 to 10-hour fermentation interval.

The spectrophotometric supervision of cellular growth during fermentation provides an idea of bacterial growth and guidance regarding possible decisions to be made during the experiment. It should be emphasized that the culture is in its stationary

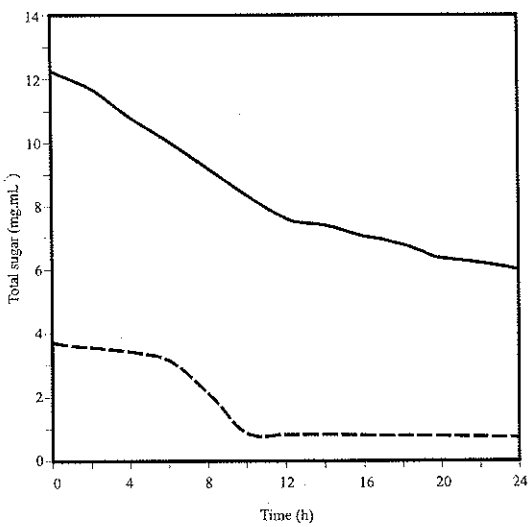

Figure 1 - Consumption of total sugar available for a *Micrococcus varians* culture in BHI broth (- - -) and sugar cane molasses at 2% enriched with 0.1% yeast extract (—).

phase or is starting the declining phase by the end of the fermentation periods. From this stage onwards, spectrophotometric readings give us a false-positive result given that the optic density tends to increase due to an accumulation of suspended cells and not due to bacterial growth. In order to avoid this type of error, a viable cell counting (Fig. 3) was conducted because the greatest interest in developing a starter culture is to obtain the highest number of viable cells possible.

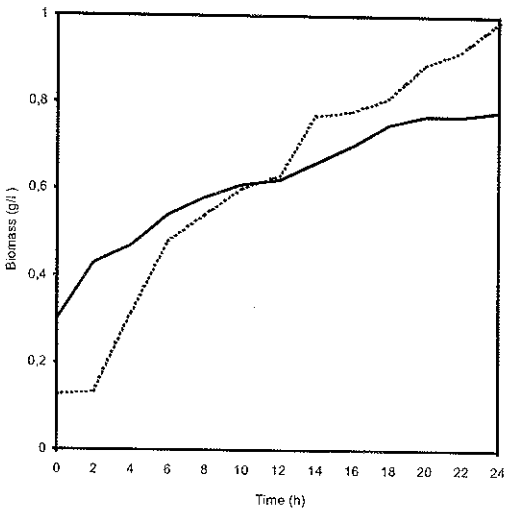

Figure 2 - *Micrococcus varians* biomass concentration in BHI broth (- - -) and sugar cane molasses at 2% enriched with 0.1% yeast extract (—).

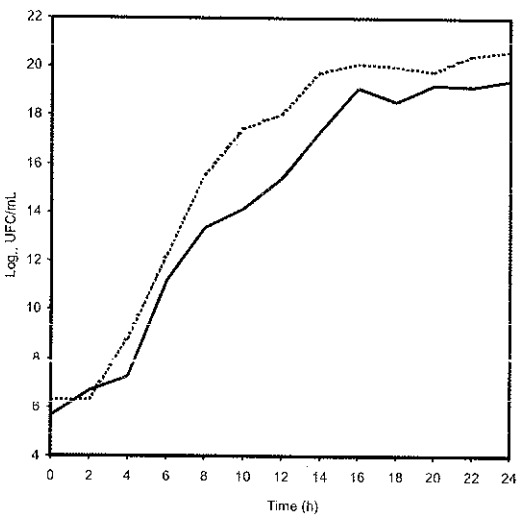

Figure 3 - Viable cells number of *Micrococcus varians* in BHI broth (- - -) and sugar cane molasses at 2% enriched with 0.1% yeast extract (—).

The control medium presented a higher concentration of viable cells (20.65 $\log_{10}$ CFU/ml) than the experimental medium (19.43 $\log_{10}$ CFU/ml). The difference between the results obtained in the control medium and the experimental medium has also occurred probably due to nutritional deficiencies in the experimental medium. However, the sugar cane molasses enriched with yeast extract presented a good growth level which can be attributed to the composition of the yeast extract in which B vitamins are present and act as bacterial stimulants.

The pH values remained within a range close to initial pH (7.0 ± 0.2), which confirms the *Micrococcus varians* characteristic of being a non-acidifier microorganism (6). Small variations may be attributed to the production of both primary and secondary metabolites during the fermentation period, in an interaction with the culture's physicochemical conditions.

The highest final biomass concentration was obtained in the control medium fermentation (BHI), 0.99 $g.l^{-1}$, while a final concentration of 0.78 $g.l^{-1}$ was obtained for the experimental medium, as shown in Fig. 2. Considering that 2% of sugar cane molasses correspond to 0.97 $g.l^{-1}$ of total sugars, we may affirm that 80% of TS was utilized for biomass production in the experimental medium as it was the main carbon source for the medium. In the control medium (BHI), it is difficult to relate the sugar concentration (glucose) with the biomass produced by the great amount of other carbon sources available (peptone proteases).

The highest biomass productivity was obtained in BHI broth, 0.1178 $g.l^{-1}.h$ ($p<0.05$), against 0.02 $g.l^{-1}.h$ obtained in experimental medium.

This experiment sought to quantify N, Mn, Mg, P and K both in the BHI medium and in the experimental medium at the beginning and at the end of the fermentation process with the purpose of detecting nutritional deficiencies in the experimental medium. Results are shown on Table 1.

The consume of the nitrogen total was 1.03 $g.l^{-1}$ in BHI medium, while only 0.027 $g.l^{-1}$ were consumed in the experimental medium. The mineral consumption was very clear in the BHI medium with a consumption of 45.13; 17.04; 50.00; e 10.97% of Mn, Mg, P and K consumed respectively. Mn and Mg were the only minerals which showed sensitivity to the techniques utilized in the experimental medium, resulting in a reduction of 37.37 and 41.98% of these minerals respectively. In regards to P and K in the experimental medium, they were both

Table 1 - Determination of N, Mn, Mg, P and K levels, for *Micrococcus varians* culture in BHI broth and in sugar cane molasses at 2%, enriched with 0.1% yeast extract.

Mineral	BHI		Molasses	
	Initial[a]	Final[b]	Initial[a]	Final[b]
N ($g.l^{-1}$)	1.1191	0.0846	0.0734	0.0464
Mn ($mg.l^{-1}$)	0.667	0.366	0.289	0.181
Mg ($mg.l^{-1}$)	110.500	91.666	0.131	0.076
P (%)	0.008	0.004	0.231	0.231
K (%)	0.082	0.073	0.231	0.231

[a] Beginning of fermentation (initial time)
[b] End of fermentation (24 hours)

quantified at the beginning of the fermentation (0.231 and 0.230 respectively) but only traces of these minerals were found at the end of the process. This means that both minerals were almost entirely consumed.

RESUMO

Crescimento de *Micrococcus varians* em substrato de melaço de cana-de-açúcar

Foram realizados estudos comparativos do crescimento de *Micrococcus varians* em meio de cultura BHI (controle) e em meio constituído de melaço de cana-de-açúcar diluído a 2% enriquecido com 0,1% de extrato de levedura. O experimento foi conduzido com três repetições do meio experimental e controle, em fermentador de 5 litros, com volume de trabalho de 3,5 litros, sob agitação contínua (150 rpm), temperatura de 35° ± 0,1°C, aeração 0,7 L ar. l^{-1} meio. min^{-1}, pH inicial 7,0 ± 0,2, tempo de fermentação de 24 horas e inóculo inicial em torno de $\log_{10}$ UFC/ml. As amostras foram coletadas em intervalos de duas horas. *Micrococcus varians* cresceu nos dois meios de cultura estudados, confirmando a viabilidade do meio experimental pra o cultivo desta espécie. A concentração média final da biomassa foi maior no meio controle do que no experimental, com 0,99 $g.l^{-1}$ e 0,78 $g.l^{-1}$ respectivamente. O número de células viáveis ao final das fermentações foi de 20,65 $\log_{10}$ UFC/ml para o BHI (controle) e 19,43 $\log_{10}$ UFC/ml para o meio experimental. O consumo de açúcares totais no meio controle foi de 79,78%, enquanto no meio experimental foi de apenas 50,53%.

Palavras-chave: *Micrococcus varians*, fermentação, melaço.

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DETECTION AND CHARACTERIZATION OF BACTERIOCIN-PRODUCING *LACTOCOCCUS LACTIS* STRAINS

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ABSTRACT

One hundred sixty seven strains of *Lactococcus lactis* were screened for bacteriocin production by well diffusion assay of GM17 agar. Fourteen (8.4%) produced antimicrobial activity other than organic acids, bacteriophages or hydrogen peroxide. The frequency of bacteriocin production ranged from 2% in *L. lactis* subsp. *cremoris* up to 12% in *L. lactis* subsp. *lactis*. Antimicrobial activities were not observed in any strain of *L. lactis* subsp. *lactis* var. *diacetylactis*. Among thirteen bacteriocin-producing strains and two nisin-producing strains (*L. lactis* subsp. *lactis* ATCC 11454 and *L. lactis* subsp. *lactis* CNRZ 150), eight (53%) were characterized as lactose-positive (Lac^+) and proteinase-negative (Prt^-). The bacteriocin-producing cultures were also characterized on the basis of plasmid content. All strains had 2 to 7 plasmids with molecular weights varying from 0.5 to 28.1 Mdal. Four strains (ITAL 435, ITAL 436, ITAL 437 and ITAL 438) showed identical profiles and the other were quite distinct.

Key words: *L. lactis*, bacteriocins, fermentation, plasmids

INTRODUCTION

L. lactis has been traditionally used as starter in the manufacture of cheese and fermented milk products on account of their function of preservation and contribution to flavor and aroma. Selected strains are used as combined cultures, single or as mixture of single cultures. Preservation of fermented foods is due primarily to the conversion of sugars in organic acids with a concomitant lowering of the pH and removal of large amounts of carbohydrates as nutrient sources. These effects extend the shelf life and safety of the final product (3, 7, 26).

In recent decades, it has become clear that the overall inhibitory action is due to more complex antagonistic systems produced by the starter cultures. Those systems included the production of the hydrogen peroxide, diacetyl, secondary reaction products, and bacteriocins (3). Competition for essential nutrients, the accumulation of D-aminoacids, a lowering of oxidation-reduction potential and coaggregation may also be involved in antagonism (26).

Bacteriocins produced by lactic acid bacteria can be defined as biologically active proteins or protein complexes displaying a bactericidal mode of action

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exclusively towards Gram-positive bacteria and particularly closely related species. They form a heterogeneous group with respect to producing bacterial species, molecular size, antibacterial spectrum, mode of action, stability and physical and chemical properties (6,7).

Bacteriocin production was detected in all genera of lactic acid bacteria and have been well described for the subspecies of *L. lactis*. Kozak *et al.* (15) observed 88% of bacteriocin-producing among 67 nisin-non-producing *L. lactis* subsp. *lactis* strains. In a study with 150 *L. lactis* subsp. *cremoris*, Davey and Richardson (5) found 11 (7%) of strains diplococcin-producing. Geis *et al.* (9) showed the production of bacteriocin in 65 (23.2%) of all 280 strains examined. The frequency was of 1% in *L. lactis* subsp. *lactis* var. *diacetylactis* and 9% and 7.5% in *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris*, respectively. Out of 600 strains isolates from raw buffalo milk by Gupta and Batish (11), 34 (5.7%) inhibited at least one or more indicator strains. The frequency of production ranged from 3.8% in *L. lactis* subsp. *cremoris* to 6.8% and 7.6% in *L. lactis* subsp. *lactis* var. *diacetylactis* and *L. lactis* subsp. *lactis*, respectively. Piard *et al.* (24) found 18% of *L. lactis* bacteriocin-producing of all 50 strains examined. Vanderberg *et al.* (32) reported the production of nisin-like for 8 (14.5%) of all 55 strains examined. Villani *et al.* (33) founded activity bacteriocin-like in 12 (16.7%) of *L. lactis* subsp. *lactis* in a total of 72 strains.

With the emergence of psychrotrophic food-borne microorganisms, the development of new food technologies and the search of consumers for natural food products, the bacteriocins and/or producing microorganisms have been recognized as a potential source of biopreservatives for foods. The objective of this study was to evaluate the production of bacteriocins among 167 strains of *L. lactis* belonging to the Lactic Acid Bacteria Collection of the Instituto de Tecnologia de Alimentos, Campinas, SP, Brazil. Bacteriocin-producing strains were evaluated for fermentation characteristics and plasmid profiles.

MATERIALS AND METHODS

Microorganisms and culture media

The strains of *L. lactis* coming from different sources and tested for antagonistic activity are listed in Table 1. The stock cultures were maintained in frozen storage at -20°C in 14% solids and sterile reconstituted nonfat skim milk powder. Prior to use, the cultures were transferred twice in M17 broth (Oxoid) supplemented with 0.5% glucose (GM17) and incubated at 30°C for 16-18 h. *L. lactis* subsp. *cremoris* ITAL 23, ITAL 257 and ITAL 309 were used as indicator cultures. These cultures were previously selected as the most sensitive representatives in the collection (23). The nisin-producing *L. lactis* subsp. *lactis* ATCC 11454 and *L. lactis* subsp. *lactis* CNRZ 150 were used as positive controls.

Table 1. Origin of lactic acid bacteria strains.

Number of strains	Species	Origin
1	*L. lactis* subsp. *lactis* 11454	ATCC
1	*L. lactis* subsp. *lactis* 150	CNRZ
38	*L. lactis* subsp. *lactis*	Raw buffalo milk
10	*L. lactis* subsp. *lactis*	Cheese whey
10	*L. lactis* subsp. *lactis*	Commercial cultures
12	*L. lactis* subsp. *lactis*	Cheese starters cultures
30	*L. lactis* subsp. *lactis*	Regional cheese
8	*L. lactis* subsp. *lactis*	INRA
2	*L. lactis* subsp. *cremoris*	Raw milk
8	*L. lactis* subsp. *cremoris*	Commercial cultures
1	*L. lactis* subsp. *cremoris*	INRA
38	*L. lactis* subsp. *cremoris*	Cheese starter cultures
10	*L. lactis* subsp. *lactis* var. *diacetylactis*	Commercial cultures

ATCC: American Type Culture Collection, Baltimore, MD, USA; CNRZ and INRA: Centre de Recherches Agronomiques de Jouy-en-Josas, France.

Detection of antagonistic activity

The well diffusion direct assay described by Tagg and McGiven (29) and modified by Benkerroum *et al.* (2) was utilized to detect the bacteriocin-producing strains. The GM17 broth was supplemented with sodium β-glycerophosphate (2%) and catalase (Sigma Chemical Co., Dorset, England) at final concentration of 100U. Twenty mililitres of GM17 agar inoculated with 1% (10^5-10^6 cfu ml^{-1}) of stationary-phase sensitive indicator cultures were poured in a sterile Petri dish and allowed to harden. Holes were punched out of the agar, by using a cork bore (4 mm of diameter). The base of each hole was sealed with a drop (50µl) of GM17 soft agar (0.75% agar) and then filled with 50µl overnight test strains. The inoculated plates were incubated at 30°C for 18-24 h and checked for the presence of inhibition zones as a result of antimicrobial activity. In addition, two other techniques for inoculation were tested. The test

producer cultures were inoculated as a single streak and as a spot onto the surface of GM17 agar, previously inoculated with the sensitive indicator cultures. The plates were incubated at 30°C for 18-24 h and were checked for inhibition zones around the spot and/or the streak.

Detection of lisogenic strains

To detect the presence of lisogenic strains the reverse side technique was used. GM17 agar plates were streaked with test producer cultures and incubated at 30°C for 24 h. The agar was detached from edges of the Petri dish with a sterile spatula and flipped on the lid. The back surface was flooded with 4.5ml of GM17 soft agar (0.75% agar) containing 500µl of a 1:10 v/v dilution of the stationary-phase indicator cultures *(L. lactis* subsp. *cremoris* ITAL 23, ITAL 257 and ITAL 309). The plates were incubated at 30°C for 24-48 h and examined for inhibition zones (30).

Fermentation characteristics of bacteriocin-producing strains

Bacteriocin-producing strains were evaluated for the ability of lactose fermentation and proteinase activity. The strains were inoculated (1%) in 11% solids and sterile reconstituted nonfat skim milk powder (RSM 11%), milk RSM 11% supplemented with milk protein hydrolysate (0.25%), milk RSM 11% supplemented with glucose (1%) and milk RSM 11% supplemented with both. The series were incubated at 21°C for 16-48 h and examined for the presence of coagulation and pH (21, 25).

Plasmid profiles of the bacteriocin-producing strains

Plasmid profile was obtained in order to observe homology between the bacteriocin-producing strains. Cells were grown in GM17 broth at 30°C for 6 h. Plasmid extraction and agarose gel electrophoresis followed the method of Anderson and McKay (1) and Maniatis *et al.* (19). Plasmid profile of *L. lactis* subsp. *lactis* ATCC 11454, previously reported (10), was used as a standard.

RESULTS AND DISCUSSION

Screening for antibacterial activity

The assays for the presence of bacteriocins were designed to minimize the effect of other interfering inhibitors. For example, to exclude the possibility of an antagonistic effect on indicator strain due to hydrogen peroxide, catalase was added to GM17 agar (4, 11, 24, 30). Likewise, to exclude the inhibition by low pH due to the production of organic acids, the solid media was supplemented with sodium β-glicerophosphate (5, 14, 27, 30). Among 167 strains of *L. lactis* screened for antibacterial activity against three indicator strains (*L. lactis* subsp. *cremoris* ITAL 23, ITAL 257 e ITAL 309), fourteen (8.4%) inhibited the growth of all indicator cultures. The average of the inhibition zone diameters for each strain tested is shown in Table 2.

Table 2. Inhibitory activity of 14 strains of *L. lactis* against selected indicator strains

Microorganisms	Indicator cultures		
	ITAL 23	ITAL 257	ITAL 309
L. lactis subsp. *cremoris* ITAL 402	++	++	+
L. lactis subsp. *lactis* ITAL 104	+++	++	++
L. lactis subsp. *lactis* ITAL 179	+++	+++	++
L. lactis subsp. *lactis* ITAL 185	+++	++	++
L. lactis subsp. *lactis* ITAL 187	+++	+++	+
L. lactis subsp. *lactis* ITAL 383	++	++	+
L. lactis subsp. *lactis* ITAL 387	+++	++	++
L. lactis subsp. *lactis* ITAL 403	+++	++	++
L. lactis subsp. *lactis* ITAL 404	+++	++	+
L. lactis subsp. *lactis* ITAL 408	++	++	+
L. lactis subsp. *lactis* ITAL 435	+++	+++	++
L. lactis subsp. *lactis* ITAL 436	+++	+++	++
L. lactis subsp. *lactis* ITAL 437	+++	+++	++
L. lactis subsp. *lactis* ITAL 438	+++	+++	++

+: Weak inhibition (zone of 4mm of diameter); ++ Moderate inhibition (zone of 5-9mm); +++: High inhibition (zone of 10-15mm)

The comparison among the methods of simultaneous antagonism assay (inoculation in spot, streak and wells) showed that the inoculation in wells lead to the formation of larger and more consistent inhibition zones. Indeed, these methods allowed the standardization of the inoculum, making possible the comparison between diameters of the inhibition zones (29). Benkerroum *et al.* (2) had concluded that this method is suitable and designated "lyse zones" as opposed to the spot-on-the-law method. The lyse zones could be seen after 3 h of incubation by wells diffusion method, while 48 h were necessary to see definitive inhibition zones by the spot test assay or streak inoculation. However, Lewis *et al* (17) reported that the spot

method was more reproducible, rapid and easy to score than flip-plate or well diffusion methods. Spelhaug and Harlander (27) also found the spot method superior to flip-plate method assay in a study of three bacteriocin-producing strains against a large panel of food-borne microorganisms. The antagonism deferred method for inoculation in points was more suitable when compared with the streak and wells methods due to its reproducibility, rapidity and simplicity (17).

Detection of lisogenic strains

Lisogenic strains and bacteriocin-producing strains show similar spots. The differentiation between them is difficult, which interferes with the result evaluation (20, 30, 34). An important differential characteristic is that bacteriocins, unlike bacteriophages, do not carry the genetic determinants necessary for self-replication within susceptible organisms. Only the bacteriophages can propagate in sensitive strains (20, 22, 30).

Based on the capacity of bacteriocins to diffuse in agar, the reverse technique, which eliminated the contact between the producers and sensitive strains, was used for lyse differentiation (30). The formation of the diffusion zones was observed after incubation of the Petri dishes, previously streaked with the producer strains and subsequent addition of the soft agar seeded with the sensitive strains, in the reverse side. This indicated that the substance diffuses into the agar (30). Alternatively, the observation of the characteristics of the inhibition zones helped in the differentiation of the nature of the inhibitory substance. Increasing the dilution factor resulted in reduction of the diameter of the inhibition zones until its total disappearance, indicating the presence of bacteriocins. In high dilutions the formation of the lysis plaque, characteristic of the presence of bacteriophages or bacteriocin activity, was not observed, whereas if present, phages would still be detected at much higher dilution (16, 20).

Of the 167 strains tested, 14 (8.4%) showed bacteriocin activity against all to the indicator cultures (Table 3). Thirteen (92.9%) strains are *L. lactis* subsp. *lactis* and only one (7.1%) strain is *L. lactis* subsp. *cremoris*. The frequency of bacteriocin production ranged from 2% in *L. lactis* subsp. *cremoris* to 12% in *L. lactis*. None of the *L. lactis* subsp. *lactis* biovar. *diacetylactis* strains tested in the study was able to produce bacteriocins.

Table 3. Occurrence of bacteriocin-producing *L. lactis*.

Species	Number of strains		%
	Tested*	Sensitive	
L. lactis subsp. *cremoris*	49	1	2
L. lactis subsp. *lactis*	108	13	12
L. lactis subsp. *lactis* var. *diacetylactis*	10	0	0
Total	167	14	8.4

* By the well-diffusion assay

The screening for the detection of bacteriocin-producing strains is highly dependent on several factors: testing method, composition of the culture medium and nature of the indicator culture. The choice of the indicator (bacteriocin-sensitive strain) used in the screening is very important and may greatly influence the results (22). Therefore, the confrontation of the results achieved with those of others authors is very difficult. Nevertheless, the occurrence of bacteriocin-producing lactococci in this study is similar to that found in the literature: 8% Vandenberg *et al.* (32), 5.7% Gupta and Batish (11) and 7.3% Davey and Richardson (5). In counterpart, these results are higher than those obtained by Zezza *et al.* (35) 1.5% or Uhlman *et al.* (31) 1.6% and lower than those from Villanni *et al.* (33) 16.7%, Piard *et al.* (24) 18%, Geis *et al.* (9) 25% and Kozak *et al.* (15) 88%.

Table 4 shows the origin of the bacteriocin-producing strains of *L. lactis* selected in this study. Among 13 *L. lactis* subsp. *lactis* strains, 6 (46.1%) were isolated from raw milk, 3 (23.1%) from regional cheese and 4 (30.8%) from industrial lactic starters. *L. lactis* subsp. *cremoris* was isolated from industrial lactic starters.

Table 4. Origin of *L. lactis* bacteriocin-producing strains.

Origin	Species (%)	
	L. lactis subsp. *cremoris*	*L. lactis* subsp. *lactis*
Raw milk	0.0	46.1
Regional cheese	0.0	23.1
Cheese starter cultures	2.0	30.8

Fermentation characteristics of bacteriocin-producing strains

The fermentation characteristics of bacteriocin-producing strains were evaluated after 24 h at 21°C in 11% RSM milk and the same medium

supplemented with 1.0% glucose, 0.25% milk protein hydrolysate or both. The nisin-producing strains *L. lactis* subsp. *lactis* ATCC 11454 and *L. lactis* subsp. *lactis* CNRZ 150 were also included in this test. Seven cultures were classified as fast strains (ITAL 104, ITAL 179, ITAL 187, ITAL 403, ITAL 404, ITAL 408 and CNRZ 150). They coagulated milk within 16 h at 21°C. Eight strains required 16 h or more to coagulate milk, and were classified as slow (ITAL 185, ITAL 383, ITAL 387, ITAL 435, ITAL 436, ITAL 437, ITAL 438 and ATCC 11454). Slow variants may be defective in lactose metabolism (Lac^-) and/or proteinase activity (Prt^+). These characteristics are carried on plasmids in some strain (13).

In glucose-supplemented milk, the slow cultures remained defective in proteolytic enzyme activity but did lower the pH, indicating that glucose was being fermented. When the milk was supplied with an exogenous nitrogen source, the cultures showed slow fermentation of glucose as evidenced by the decrease in pH. To determine the characteristic Prt^-, the cultures were grown in milk supplemented with glucose or glucose plus an exogenous nitrogen source. The cultures were Prt^- since that had little, if any, proteolytic activity. This was confirmed by low pH that did occur if the glucose was supplemented with a nitrogen source. This indicated that the inability to produce acid from glucose was due to the inability to acquire needed nitrogenous compounds (12, 21, 25).

Eight (53%) out of 15 strains were characterized as lactose-positive and proteinase-negative (Lac^+Prt^-) (Table 5). Vandenberg *et al.* (32) showed that 21.6% of lactic acid bacteria isolated from different sources lacked any sign of extracellular proteolytic activity. This confirmed the inability of bacteriocin-producing lactococci to grow and to produce acid at rates suitable for cheese manufacture. The failure acid production by lactococci when grown in milk is due to the lack of the proteinase enzyme system (8, 18, 25). Strains lacking this enzyme system are designated as proteinase-negative, and they grow in milk only until soluble nitrogenous compounds are depleted (25). The reasons for the lack of proteinase activity among nisin-producing lactococci are the loss of the proteinase and incompatibility of the genes for proteinase activity with the genetic elements encoding bacteriocin production (25, 28).

Plasmid profiles of the lactococci bacteriocin-producing

Table 6 shows the molecular weight of the plasmids found in bacteriocin-producing strains selected in this study. The *L. lactis* subsp. *lactis* ATCC 1454 contain six plasmids with molecular weights of 31.8, 29.1, 21.9, 19.4, 3.6 and 1.49 Mdal (10). Agarose gel eletrophoresis of isolated DNA samples revealed two to seven plasmids in these cultures. Their molecular weights ranged from 0.5 to 28.1 Mdal when compared with standard plasmids (Table 6). No plasmids larger than 100 Mdal were observed. The strains *L. lactis* subsp. *lactis* ITAL 435, ITAL 436, ITAL 437 and ITAL 438 showed similar plasmid profiles. These cultures were isolated

Table 5. Number and molecular weight of plasmids found in bacteriocin-producing *L. lactis* subsp. *lactis* strains.

Bacteriocin-producing strains	Plasmids	
	Number	Molecular weight (Mdal)
L. lactis subsp. *lactis* ITAL 104	5	26.4; 25.9; 14.2; 9.4; 0.7
L. lactis subsp. *lactis* ITAL 179	4	26.8; 25.9; 9.8; 0.5
L. lactis subsp. *lactis* ITAL 185	5	26.4; 23.7; 14.6; 8.9; 3.8
L. lactis subsp. *lactis* ITAL 187	4	25.5; 24.6; 23.7; 9.8
L. lactis subsp. *lactis* ITAL 383	2	27.7; 25.9
L. lactis subsp. *lactis* ITAL 387	5	28.1; 26.8; 23.7; 13.8; 2.9
L. lactis subsp. *lactis* ITAL 403	7	27.7; 26.8; 25.5; 23.7; 20.7; 9.4; 1.2
L. lactis subsp. *lactis* ITAL 404	4	26.8; 25.9; 9.4; 1.2
L. lactis subsp. *lactis* ITAL 408	4	28.1; 27.2; 13.8; 5.1
L. lactis subsp. *lactis* ITAL 435	4	28.1; 26.8; 23.7; 22.4
L. lactis subsp. *lactis* ITAL 436	4	28.1; 26.8; 23.7; 22.4
L. lactis subsp. *lactis* ITAL 437	4	28.1; 26.8; 23.7; 22.4
L. lactis subsp. *lactis* ITAL 438	4	28.1; 26.8; 23.7; 22.4

from raw milk and may be derived from the same strain.

Table 6. Fermentation characteristics of bacteriocin-producing lactococci at 21°C for 24 h in 11% RSM, supplemented or not with 1.0% glucose, 0.25% milk protein hydrolysate or both.

Bacteriocin-producing strains	Fermentation characteristics*
L. lactis subsp. *lactis* ITAL 104	Lac^+Prt^-
L. lactis subsp. *lactis* ITAL 179	Lac^+Prt^+
L. lactis subsp. *lactis* ITAL 185	Lac^+Prt^-
L. lactis subsp. *lactis* ITAL 187	Lac^+Prt^+
L. lactis subsp. *lactis* ITAL 383	Lac^+Prt^-
L. lactis subsp. *lactis* ITAL 387	Lac^+Prt^-
L. lactis subsp. *lactis* ITAL 403	Lac^+Prt^+
L. lactis subsp. *lactis* ITAL 404	Lac^+Prt^+
L. lactis subsp. *lactis* ITAL 408	Lac^+Prt^+
L. lactis subsp. *lactis* ITAL 435	Lac^+Prt^-
L. lactis subsp. *lactis* ITAL 436	Lac^+Prt^-
L. lactis subsp. *lactis* ITAL 437	Lac^+Prt^-
L. lactis subsp. *lactis* ITAL 438	Lac^+Prt^-
L. lactis subsp. *lactis* CNRZ 150	Lac^+Prt^+
L. lactis subsp. *lactis* ATCC 11454	Lac^+Prt^-

* Lac^+Prt^-: Lactose-positive and proteinase-negative
** Lac^+Prt^+: Lactose-positive and proteinase-positive

On the basis of plasmid profiles the bacteriocin-producing strains were classified in three categories according to Gupta and Batish (11). Group I (low plasmid group) comprised one strain (ITAL 383), with one to three plasmids. Group II (moderate plasmid group) comprised eight strains (ITAL 104, ITAL 179, ITAL 185; ITAL 187, ITAL 387, ITAL 404, ITAL 408; ITAL 435, ITAL 436, ITAL 437 and ITAL 438), with four to five plasmids. Group III (high plasmid group) comprised one strain (ITAL 403), with six or more plasmids.

Our results showed that antagonistic substances produced by the tested strains are bacteriocins and suggest that bacteriocin-producing lactococci are present in low number in the environment These substances were heat resistant and their proteinaceous nature was demonstrated by sensibility to proteolytic enzyms (23). They also demonstrate the inability of bacteriocin-producing lactococci to grow and to produce acid at rates suitable for cheese manufacture. Due to these factors, the use of bacteriocin-producing starter have not been fully successful in dairy products

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RESUMO

Detecção e caracterização de *Lactococcus lactis* produtores de bacteriocinas

Um total de 167 linhagens de *L. lactis* foi selecionado para os testes de produção de bacteriocinas pelo método de difusão em poços em agar GM17. Desse total, 14 (8.4%) produziram substâncias inibidoras que não foram associadas com ácidos orgânicos, peróxido de hidrogênio e bacteriófagos. A frequência de produção de bacteriocinas variou de 2% em *L. lactis* subsp. *cremoris* a 12% em *L. lactis* subsp. *lactis*. Nenhuma das linhagens de *L. lactis* subsp. *lactis* var. *diacetylactis* produziu substâncias inibidoras. De 13 linhagens produtoras de bacteriocinas e duas de nisina *(L. lactis* subsp. *lactis* ATCC 11454 e *L. lactis* subsp. *lactis* CNRZ 150), 8 (53%) foram caracterizadas como lactose-positivas (Lac^+) e proteinase-negativas (Prt^-). As linhagens produtoras de bacteriocinas também foram caracterizadas no seu conteúdo de plasmídios. Elas apresentaram de 2 a 7 plasmídios, com pesos moleculares aproximados de 0.5 a 28.1 Mdal. Quatro linhagens (ITAL 435, ITAL 436, ITAL 437 e ITAL 438) mostraram idênticos perfis de plasmídios. Os perfis das linhagens restantes foram distintos.

Palavras-chave: *L. lactis*, bacteriocinas, fermentação, plasmídios

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other applications were reported, including the use of TTC in microbiology (4, 10, 12, 17, 23). Huddleson and Baltzer (7) observed that colonies of different microbial species or varieties of the same specie may develop several shades of red when plated in medium with TTC. Gershenfeld and Weber Jr. (3) reported that coagulase-positive *Staphylococcus* sp. form colonies with an orange center when TTC is added to the medium, while coagulase negative *Staphylococcus* sp. colonies are pink. Turner *et al.* (22) observed that *Streptococcus cremoris* could be differenciated from *Streptococcus lactis* with the use of TTC, because it is able to reduce the dye while the other is not. Similarly, Rioux *et al.* (19) reported that *C. albicans* could be distinguished from other yeasts, because they form pink colonies in medium with TTC, while other yeast colonies are white.

Some factors, like pH, temperature, light and concentration of the dye, also interfere in TTC reduction. The reduction of TTC is more intense at high pH (9).The concentration of TTC added to the culture medium is very important because high levels may have a deleterious effect (21). Consequently, the concentration of TTC used in culture media should be low enough to avoid inhibition of growth, but high enough to allow color development (8,15).

The variation in the capacity to reduce TTC by many microorganisms may be beneficial for their differentiation, but, in techniques where TTC is used to help the counting of colonies, this variation may be pose a problem. The colonies of microorganisms that are unable to reduce TTC are not red, and consequently, may not be counted, leading to errors.

The dry rehydratable film system for microbiological analysis of food is largerly used in many countries, including Brazil. It has several advantages, like simplicity and accuracy and is cost-effective (5,6,20). However, the application of this system for counts of aerobic bacteria in Brazilian pasteurized milk has been hampered by counts that are frequently lower than those obtained by the conventional plating procedure (21) in this particular type of food (Franco, B.D.G.M. personnal communication). This phenomenon seems to be restricted to pasteurized milk produced in certain regions of Brazil. The objective of this study was to survey the frequency of microorganisms that are unable to reduce TTC in pasteurized milk produced in one of these regions, and to determine how these microorganisms would interfere in the accuracy of the dry rehydratable film system.

MATERIALS AND METHODS

Determination of minimal inhibitory concentration of TTC in pasteurized milk: Five pasteurized milk samples, purchased from local stores in the city of Londrina, Paraná, BR, were submitted to decimal dilutions in 0.85% saline (10^{-1}, 10^{-2} and 10^{-3}), according to Lanara (1) and 1 ml of each dilution of each sample was pour-plated, in duplicates, in Plate Count Agar – PCA (Difco) containing 0.005%, 0.01%, 0.015%, 0.025% or 0.05% TTC (Sigma) and also in PCA without TTC. Plates were incubated at 35°C +/- 1°C for 24 hours and 48 hours, and the number of CFU/ml of pasteurized milk was determined according to the counting procedures recommended by Swanson *et al.* (21). All colonies were counted, regardless their color or size.

Frequency of TTC non-reducing microorganisms in pasteurized milk: 34 samples of pasteurized milk, produced by dairies in the region of the city of Londrina, Paraná, BR, were purchased from local stores, and transported under refrigeration to the Laboratory of Inspection of Animal Products, Department of Preventive Veterinary Medicine, Londrina State University. All samples were submitted to decimal dilutions in 0.85% saline, and 1 ml of each dilution was pour-plated, in duplicates, in PCA containing 0.015% TTC and also in PCA without TTC. The desired concentration of TTC in PCA was achieved using a 0.5% "mother-solution", kept in amber flasks covered with *aluminum* foil and maintained under refrigeration until the moment of use. PCA plates were incubated at 35°C. Colony Forming Units (CFUs) were counted after 24 hours and 48 hours of incubation, The number of CFU/ml in each sample was determined according to Swanson *et al.* (21). Colonies were classified according to color: colorless, white, cream or yellow were considered TTC non-reducers and pink or red were considered TTC reducers. Colonies that changed color between the two countings were also recorded.

Preliminary identification of TTC non-reducing colonies: After 48 hours of incubation, hundreds of colonies considered as non reducers were streaked on PCA with 0.015% TTC and on Blood Agar. Plating was repeated until pure cultures were obtained. From these, 233 were selected for Gram staining.

RESULTS AND DISCUSSION

Besides facilitating the counting of colonies, TTC is also a powerful tool to distinguish colonies from food particles, which don't react with the dye. Use of TTC is highly recommended for milk testing, because the opacity of the plates, specially those containing the less diluted samples, makes the counting unaccurate (16). Many microbiologists use TTC added to the media as an over-lay after incubation rather than added to PCA prior to plating, because TTC may be deleterious to many microorganisms (4). In this study, TTC was used as a supplement of PCA because further testing of colonies grown on the agar was needed. Futhermore, in the dry rehydratable film procedure, TTC is also part of the film (5,6,20).

The best performance of TTC for counting of colonies in PCA was achieved by the concentration of 0.015% and very little inhibition of microbial growth was observed when compared to counts in PCA with no dye. TTC reducing colonies were easily distinguished from those that didn't reduce the dye. There isn't a consensus on the ideal concentration of TTC for colony counting. According to Swanson *et al.* (21), the concentration of TTC in the agar should be 0.005%. May *et al.* (13) reported that 0.05% TTC was not inhibitory for Gram negative bacteria, including *E.coli*. For Gram positives, like *B.subtilis* and *S.pyogenes*, the minimal inhibitory concentration (MIC) was 0.0032%. Ohara (16) observed that 0.1% and 2% TTC should be used for bacteria and yeasts countings, respectively.

From a total of 50,574 colonies counted in PCA with 0.015% TTC, 19,665 (38.88%) were not able to reduce the dye in 48 hours. After incubation of 24 hours, among 28,717 colonies, 8,983 (31.28%) did not reduce TTC. From the 21,857 colonies that grew in the second incubation of 24 hours, 10,682 (48.87%) were non-reducers (Table 1). Between the two countings, 571 (6.36%) non-reducer colonies changed to pink.

Table 1. Frequency of TTC reducing and non-reducing microorganisms in pasteurized milk, determined by pour-plating in Plate Count Agar supplemented with 0.015% TTC, incubated at 35°C during 24h and 48h.

	TTC reducing		TTC non-reducing	
	nº	%	nº	%
24h counting	19,734	68.72	8,983	31.28
48h counting	30,909	61.12	19,665	38.88

Comparing the number of colonies obtained in PCA with and without TTC, after 48 hours, a 6.16% inhibition was detected. The number of colonies in PCA was 53.896 CFUs, while in PCA with 0.015%, the number was 50.574 CFUs. The size of the colonies in the medium with TTC was a little smaller when compared to the same sample plated in PCA without TTC. Among the colonies submitted to Gram stain, 110 (47.21%) were Gram positive coccobacilli, 80 (34.33%) were Gram positive bacilli, 33 (14.16%) were Gram positive cocci, 4 (1.72%) were yeasts, 3 (1.29%) were Gram negative bacilli, 2 (0.86%) were actinomyces and 1 (0.43%) was a Gram negative coccobacillus. These data show that more than 95% of the TTC non-reducer colonies were Gram positive bacteria and only less than 2% were Gram negative (Table 2).

Table 2. Characteristics of TTC non-reducing microorganisms isolated from pasteurized milk.

colonies	Number	Percentage
Gram positive coccobacilli	110	47.21%
Gram positive bacilli	80	34.33%
Gram positive cocci	33	14.16%
Yeasts	4	1.72%
Gram negative bacilli	3	1.29%
Actinomyces	2	0.86%
Gram negative coccobacilli	1	0.43%
Total	233	100%

Gram positive microorganisms, like *Micrococcus*, coryneforms and some heat resistant bacilli are often present in raw milk and are part of remaining microflora in pasteurized milk. In counterpart, Gram negative microorganisms are much less common in raw milk. This suggests that the TTC non-reducing Gram positive bacteria detected in the pasteurized milk samples are those that survived to the heat treatment. Their incapacity to reduce TTC before the pasteurization was done could not not tested, but sucessive platings of many of these colonies in non-selective Blood Agar plates didn't make them them able to reduce TTC. This suggests that a possible heat-injury that affected the capacity of microorganisms to reduce TTC was irreversible.

RESUMO

Freqüência de bactérias não-redutoras de cloreto de 2,3,5-trifeniltetrazólio (TTC) em leite pasteurizado

Cloreto de 2,3,5-trifeniltetrazólio (TTC) é um corante muito utilizado para contagem de colônias de microrganismos em meios de cultura sólidos, sendo um componente-chave do sistema de filme seco rehidratável usado para a análise microbiológica de alimentos. Esse corante é incolor na forma oxidada e vermelho quando reduzido por microrganismos, devido a formação de formazano. Nesse trabalho, TTC foi adicionado a Agar Padrão para Contagem (PCA) para enumeração de microrganismos em trinta e quatro amostras de leite pasteurizado, com o objetivo de se determinar a freqüência de microrganismos incapazes de reduzir o TTC. As amostras de leite foram submetidas a diluições decimais e a plaqueamento em profundidade em PCA com 0,015% de TTC. As colônias foram enumeradas após 24h e 48h de incubação a 35°C. De um total de 50.574 colônias, 19.665 (38,88%) não reduziram TTC em 48h. Observou-se que 571 (6,36%) colônias incolores em 24 ficaram vermelhas em 48h. Entre as colônias não redutoras de TTC, 233 foram purificadas e submetidas à coloração Gram, verificando-se que 229 (98,71%) eram cocos ou bacilos Gram positivos. Os resultados indicam que há uma elevada porcentagem de bactérias não redutoras do TTC em leite pasteurizado, e, portanto, não são detectadas quando são utilizados métodos laboratoriais baseados na formação de colônias vermelhas.

Palavras-chave: cloreto de 2,3,5-trifeniltetrazólio, TTC, leite pasteurizado, contagem de microrganismos

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GENETIC VARIABILITY OF HIV-1 ISOLATES FROM MINAS GERAIS, BRAZIL

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SHORT COMMUNICATION

ABSTRACT

We report results of nucleotide sequencing and phylogenetic analysis of the *env* gene of 11 HIV-1 isolates, in Belo Horizonte, Brazil. Ten isolates belonged to HIV-1 subtype B and one was a probable B/F mosaic. This putative B/F recombinant is similar but not identical in its nucleotide sequence to other B/F mosaics described in Brazil.

Key words: HIV-1, genome, PCR, AIDS, hemophiliacs.

Human immunodeficiency virus establishes persistent infections in humans, in most cases leading to AIDS. HIV-1 has been genetically classified into major (M) and outlier (O) groups. The M group is further divided into nine subtypes (A-I), based on sequence diversity, where members of the same subtype differ in general by less than 10%, and those of different subtypes by 30% or more, if *env* gene is analyzed (2,4,6). Variation of HIV-1 can result in a spectrum of viruses exhibiting differences in cell tropism, replication, transmission rates and cytopathogenicity. At present there is considerable effort in genotyping viruses recovered from infected individuals to gain insights as to the types of viruses being transmitted in different geographical regions (6). Belo Horizonte is the third largest city in Brazil, with over 3 million inhabitants in the metropolitan area and the fourth in absolute number of AIDS cases in Brazil, as of March 1997, with over 2,400 cumulative cases (7). The objective of this work was to examine the genetic variation and the phylogenetic analysis the *env* gene of HIV-1 isolates of seropositive patients with history of use of clotting factor concentrates (n=6), multiple transfusions (n=1), heterosexual transmission (n=3) and homosexual contact (n=1) in Belo Horizonte, Brazil.

After informed consent, samples of peripheral blood of 11 HIV-1 seropositive patients were collected in EDTA. PBMCs (peripheral blood mononuclear cells) were separated using a Ficoll-Hypaque® gradient (Pharmacia, Sweden). Crude lysates were obtained and PCR was performed using primers encompassing the C2-C3 region of the *env* gene of HIV-1 (nested reaction). Primers were based on isolate HXB2 genome (GenBank accession number KO3455), and were provided by the World

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Health Organization (WHO). The first round reactions of the *env* gene were performed using primers ED3/ED14, corresponding to positions 5956-5985 and 7960-7931, which amplified a 2.0 Kb fragment. The nested reactions were done with primers ED31/ ED33, corresponding to positions 6816-6844 and 7359-7380, producing a 0.5 Kb fragment (region C2-C3). Nucleotide sequencing (Sequenase®, USB, USA) was performed directly on DNA amplified by PCR of the PBMC lysates, using the primers ED31, ED33 and ES7 (C2-V3 region). Nucleotide sequences were obtained from both strands and each was done at least twice, using the dideoxi termination method (9). Sequences were aligned using Clustal W. Pairwise distance methods, using the Jukes and Cantor correction and bootstrap analysis, were performed for phylogenetic inference, with the package Treecon 3.1 for Windows (10).

HIV-1 *env* sequences obtained from Belo Horizonte isolates clustered with isolates of subtype B in the phylogenetic analysis performed (Fig. 1). These results are in concordance with *env* heteroduplex mobility assays (HMA) (1) results of the same samples, except for an isolate from an asymptomatic blood donor (BHGM19), whose HMA results showed F subtype mobility, but whose C2V3 *env* sequences clustered consistently with subtype B. This discordant branching has been reported before in an isolate from São Paulo, Brazil (93BR019), which was a mosaic of subtypes B and F (8).

In nine of eleven *env* amino acid sequences, the tetrameric subdomain at the crown of V3 loop (Table 1) had the sequence GPGR, including the B/F isolate (BHGM19, blood donor, heterosexual transmission), whose crown had the sequence HI**GPGR**AF, identical to previously described and independently collected F subtype isolates from other cities in Brazil (3,5). One isolate (JS, hemophiliac patient) had the GPGQ sequence, which is common in subtypes A,

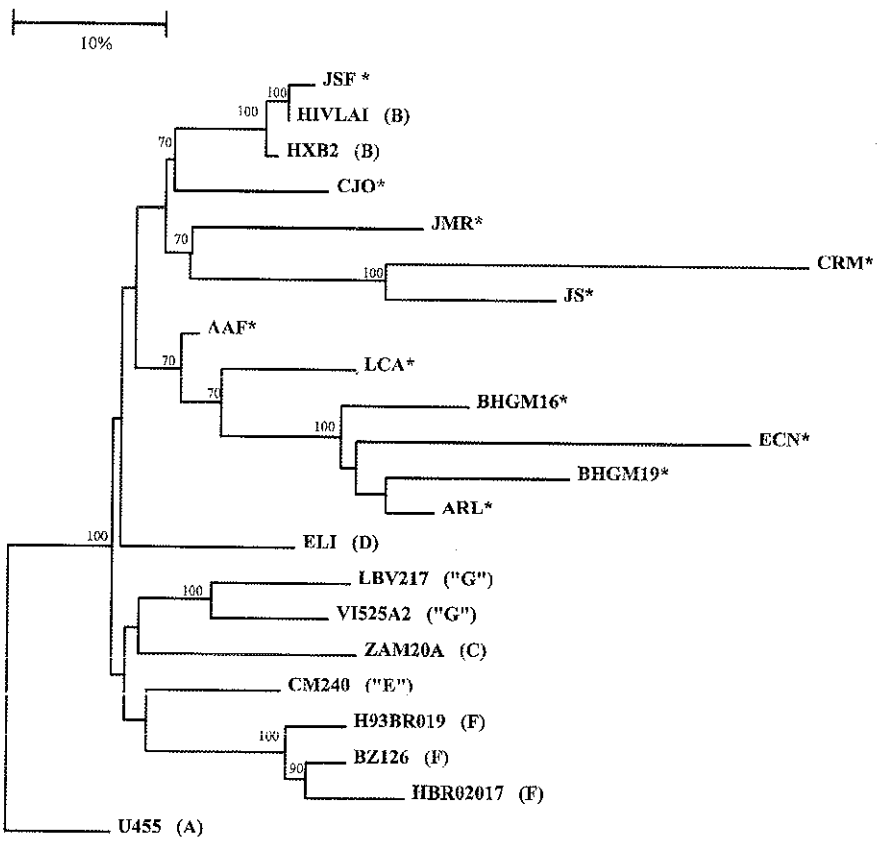

Figure 1 - Phylogenetic relationships of the sequences of the C2V3 region of HIV-1 *env* gene of isolates from Minas Gerais (asterisks), compared to sequences representative of subtypes A-G. Isolate U455 was chosen arbitrarily to root the tree. Quotes indicate "mosaic" subtypes. Values at the nodes represent bootstraps analysis (shown only values above 75%).

C and E isolates and one (JMR, heterosexual transmission) had the sequence GPGK, rarely reported in subtype B. The five cysteine residues of the C2-V3 region were conserved in all isolates, including the ones at the base of V3 loop (C1 and C35).

Table 1 - Aminoacid sequences of the V3 crown of HIV-1 *env* gene and risk factor in 11 seropositive patients, Belo Horizonte, Minas Gerais, 1997.

Patient	V3 Loop Crown Sequences	Probable mode of transmission
ECN	GPGR	Hemophiliac patient[1]
CJO	GPGR	Hemophiliac patient[1]
JSF	GPGR	Homosexual transmission
CRM	GPGR	Hemophiliac patient[1]
AAF	GPGR	Transfusion[2]
ARL	GPGR	Hemophiliac patient[1]
GM16	GWGR	Heterosexual transmission
GM19	GPGR	Heterosexual transmission
JS	GPGQ	Hemophiliac patient[1]
JMR	GPGK	Heterosexual transmission
LCA	GPGR	Hemophiliac patient[1]

[1] Use of clotting factor concentrates
[2] Use of packed red blood cells

All the isolates in this study, with one exception, belonged to HIV-1 subtype B, which is the most prevalent in the United States and Europe. The exception was a probable B/F mosaic. This putative B/F recombinant from Belo Horizonte (BHGM19) is similar, but not identical in its nucleotide sequence to other B/F mosaics described in Brazil (3,5,8). Sequencing of additional regions of these viruses may help to understand the recombination events that took place to generate these chimeric genomes.

GeneBank accession numbers: AF025918 to AF025926; U46210; U46122

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RESUMO

Variabilidade genética de isolados de HIV-1 em Minas Gerais, Brasil

Relatamos resultados do estudo de seqüência de nucleotídeos e análise filogenética do gene *env* 11 isolados HIV-1 em Belo Horizonte, Brasil. Dez isolados pertenciam ao subtipo B e um era provavelmente um mosaico B/F. Este possível recombinante B/F é similar, mas não idêntico, em sua seqüência de nucleotídeos, aos demais mosaicos B/F descritos no Brasil.

Palavras-chave: HIV-1, genoma, PCR, AIDS, hemofílicos.

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DISCRIMINATION OF MEMBERS OF THE *MYCOBACTERIUM AVIUM* COMPLEX BY POLYMERASE CHAIN REACTION

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SHORT COMMUNICATION

ABSTRACT

Mycobacterium avium complex (MAC) species cannot be discriminated by the usual methods of biochemical identification of mycobacteria. This study showed that amplification by PCR of DT1 and DT6, two single copy sequences identified in the genome of *M. avium* serotype 2, the insertion sequence IS*1245*, found to be consistently present in *M. avium* strains and the heat-shock protein gene *hsp65*, followed by restriction polymorphism analysis, are rapid and accurate tests for the differentiation of the species *M. avium*, *M. intracellulare*, and *M. scrofulaceum*.

Key words: amplification, PCR, MAC, *Mycobacterium*, identification

Infections caused by *Mycobacterium avium* complex (MAC) were infrequent in the pre-HIV era, appearing in patients with preexisting lung conditions (6). The importance of these mycobacteria, particularly of *M. avium*, increased in the last years due to its role as opportunist and cause of disseminated infections in patients with AIDS (9,18). In a study with 125 bone marrow aspirates from AIDS patients in São Paulo, Brazil, conducted between 1990 and 1992, MAC was isolated from 23 samples (18.4%) and *Mycobacterium tuberculosis* from 9 (7.2%). Between 1985 and 1990, only 11 MAC positive cultures were isolated among 60,000 cultures from HIV-negative patients in São Paulo (2). These findings suggest that MAC is an important opportunist in Brazil, specially among AIDS patients.

Further evaluation of the possible reservoirs for MAC infection in humans are needed, and the development of molecular methods for the determination of strain relatedness will certainly improve the epidemiological studies of *M. avium*-related infections.

The precise diferentiation of MAC species is a fundamental step in epidemiological studies. MAC consists of a group of 28 serovars including two closely related species, *M. avium* and *M. intracellulare* and this designation may also include other less defined groups. These bacteria have also been referred as *M.avium-M.intracellulare* complex. The usual methods for identifying *Mycobacterium*

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species based on bacteriological characteristics and biochemical tests may not be accurate for the phenotypic differentiation of the these species and most laboratories report strains as members of MAC. Although the term MAC is reserved for groups of clinical isolates that normally do not include *M. scrofulaceum*, similar plasmids can be found in strains referred to as *M. avium*, *M. intracellulare* and *M. scrofulaceum*. This group is known as MAIS or as *M. avium- M. intracellulare-M. scrofulaceum* complex (4).

Differentiation can be achieved by serotype determination (7,15), but this methodology is applied in few reference laboratories. The commercial AccuProbe Culture Identification Test (Gen-Probe Inc., San Diego, Ca) is a sensitive and specific method and is now used by many clinical laboratories. Nevertheless, besides being an expensive test, it has been reported that many isolates do not react with these probes (5). The 16S rRNA sequencing has been particularly helpful in clarifying the complex taxonomical status of the members of the *M.avium-M.intracellulare* complex. (12,13). Unfortunately, this methodology remains relatively cumbersome, with application limited to reference laboratories.

PCR could be a useful alternative for the identification of MAC species in the clinical laboratory and also for epidemiological and taxonomical studies. Several methods for the differentiation of members of MAC have been reported. DT1 and DT6 are single copy sequences identified in the genome of *M. avium* serotype 2 (17). Positive amplification of DT6 with primers AV6/AV7 has been obtained with *M.avium* strains, and DT1 has been amplified with primers IN38/IN41 in *M. intracellulare* strains. The insertion sequence IS*1245* was found to be consistently present in *M. avium* strains and has been used for analysis of strain relatedness (3,8,11). The procedure called polymorphism restriction analysis (PRA), based on the enzymatic amplification of the *hsp65* gene followed by digestion with *Bst*EII and *Hae*III was developed by Telenti *et al.* (16). The authors identified 29 mycobacterial species and subspecies, including members of MAC. In this study we compared these three different PCR techniques in eighteen isolates, identified as MAC by biochemical methods (10) (Table 2).

A loopfull of mycobacteria grown on solid medium (Lowenstein-Jensen, Stonebrink, or Petragnani) was suspended in 0.4 ml of TE (Tris-HCl pH 7.4 10mM, EDTA 1mM) with 1% Triton X-100 and was submitted to three cycles of freezing and thawing. Five to ten microliters of the lysates were used for the PCR reactions.

DT1/DT6 PCR was performed as described by Thierry *et al.* (17), IS*1245* amplification was described by Guerrero *et al.* (8), and the PRA reaction was performed as described by Telenti *et al.* (16) Primers and PCR conditions are described in Table 1. The positive control for the PCR was purified DNA of *M. avium* serotype 2 (ATCC 25291). Positive and negative controls were included in all assays. PCR amplicons and digestion products were electrophoresed through 1% agarose (Gibco) or 2% NuSieve (FMC Bioproducts)/2% agarose gels, respectively. The gels were stained with ethidium bromide (Sigma) and photographed on a UV transilluminator. PCR results were compared and a genetic identification was obtained.

Positive amplification with the DT6 primers was obtained from 13 out of 18 strains: ten human and animal isolates identified as MAC, two reference *M. avium* strains (number 3 and 12) and the strain serotype VIII (number 16) identified previously as *M. intracellulare*. With the DT1 primers, amplification was positive in 4 out of 18 strains: one animal isolate biochemically identified as MAC (number 11), two isolates representing the same reference *M. intracellulare* strain, before and after passage through animal (mouse) (number 13 and 14) and one reference *M. avium/intracellulare* strain (number 18) (Fig. 1). One strain biochemically identified as *M. intracellulare* (number 15) and *M. scrofulaceum* (number 17) did not amplify with either pair of primers. Therefore, the genetic identification of these two strains could not be obtained solely by this approach. Amplification with both pairs of primers was obtained from a MAC isolate from a zoo bird (number 11) and from the positive control, ATCC 25291. According to the group that sequenced the DT1 and DT6 fragments, strains amplifying with both pairs of primers could represent *M. avium* serotype 2 or 3 (17). Comparing the results of DT1 and DT6 amplifications, species identification could be obtained in 16 out of 18 strains (Table 2).

Amplification with the IS*1245* primers was positive in all clinical isolates identified biochemically as MAC and the reference *M.avium* strains (D4, 20485 and ATCC25291) (Fig. 1). DNA from strain serotype VIII, previously identified as

Table 1 – Primers and PCR conditions used in this work.

PRIMERS	PCR MIX	PCR
DT6 **AV6**:5'-ATGGCCGGGAGACGATCTATGCCGGCGTAC-3' **AV7**:5'-CGTTCGATCGCAGTTTGTGCAGCGCGTACA-3' **DT1** **IN38**:5'-GAACGCCCGTTGGCTGGCCATTCACGAAGGAG-3' **IN41**:5'-GCGCAACACGGTCGGACAGGCCTTCCTCGA-3'	1X Buffer (Gibco), 2mM $MgCl_2$, 200µM DNTPs, 100µg/ml BSA, 100pmols of each primer, 2U Taq polymerase	Denaturation 94°C 5min., 25 cycles at 94°C 1min., 60°C 1 min., 72°C 1 min. Final extension 72°C 7 min.
IS*1245* **P1**: 5'-GCCGCCGAAACGATCTAC-3' **P2**: 5'-AGGTGGCGTCGAGGAAGC-3'	1X Buffer (Gibco), 1,5mM $MgCl_2$, 200µM DNTPs, 10% glycerol, 0,5µM of each primer, 1U Taq polymerase	Denaturation 94°C 5min., 30 cycles at 94°C 1min., 60°C 1 min., 72°C 1 min. Final extension 72°C 7 min.
PRA **Tb11**: 5'-ACCAACGATGGTGTGTCCAT-3' **Tb12**: 5'-CTTGTCGAACCGCATACCCT-3'	1X Buffer (Gibco), 1,5mM $MgCl_2$, 200µM DNTPs, 10% glycerol, 0,5µM of each primer, 1U Taq polymerase	Denaturation 94°C 5min., 45 cycles at 94°C 1min., 65°C 1 min., 72°C 1 min. Final extension 72°C 7 min.

Table 2 – Isolates and strains, PCR results and biochemical identification. + and – indicate positive and negative amplification by PCR, respectively. PRA identification was obtained after *Bst*EII and *Hae*III digestion of the *hsp65* amplicons.

Number	Strain/Isolate	Biochemical Identification	DT1	DT6	IS1245	PRA
1	Human	MAC	-	+	+	*M. avium*
2	Human	MAC	-	+	+	*M. avium*
3	D4*	MAC	-	+	+	*M. avium*
4	Bovine lymph node	MAC	-	+	+	*M. avium*
5	Swine lymph node	MAC	-	+	+	*M. avium*
6	Bovine lung	MAC	-	+	+	*M. avium*
7	Bovine lung	MAC	-	+	+	*M. avium*
8	zoo bird cecum	MAC	-	+	+	*M. avium*
9	zoo bird feces	MAC	-	+	+	*M. avium*
10	Swine lymph node	MAC	-	+	+	*M. avium*
11	Crane intestine	MAC	+	+	+	*M. avium*
12	20485	*M. avium*	-	+	+	*M. avium*
13	CC1400/mouse	*M. intracellulare*	+	-	-	*M. intracellulare*
14	CC1400/mouse	*M. intracellulare*	+	-	-	*M. intracellulare*
15	IB collection**	*M. intracellulare*	-	-	-	*M. intracellulare*
16	Serotype VIII	*M. intracellulare*	-	+	+	*M. avium*
17	IB collection**	*M. scrofulaceum*	-	-	-	*M. scrofulaceum*
18	ATCC13950	*M. avium-intracellulare*	+	-	-	*M. intracellulare*
+	ATCC25291	*M. avium*	+	+	+	*M. avium*

* D4 = avian PPD strain
** IB = Instituto Biológico, São Paulo

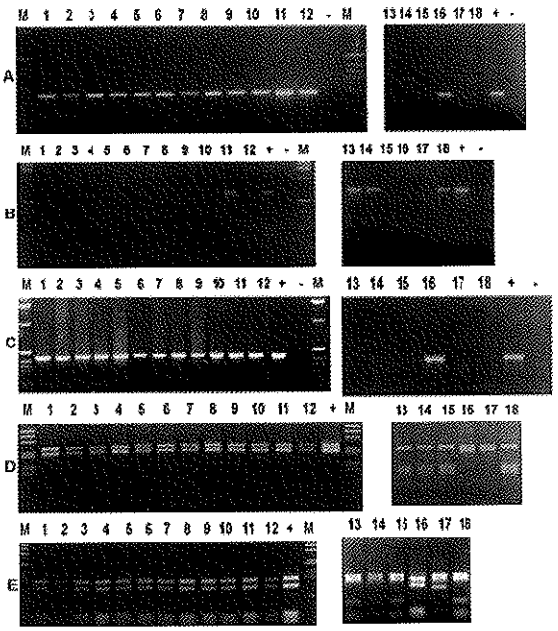

Figure 1: Agarose gel stained with ethidium bromide showing results of amplification of fragments DT6 (A), DT1 (B), IS*1245* (C) and *hsp65* digested with *Bst*EII (D) and *Hae*III (E). 1 – 18: isolates described in Table 2, M: 1kb DNA ladder (Gibco/BRL), + positive control, *M. avium* ATCC 25291, - negative control

M. intracellulare (number 16), amplified with these primers. It also amplified with the DT6 primers, as has been shown above. Serotype VIII was identified as *M. intracellulare* before the DNA-DNA hybridization studies were performed (7,15). After that it has been identified as *M. avium* (1,14). The results obtained in this study agree with this last identification.

All strains were amplified by the *hsp65* gene primers. The bands obtained after *Bst*EII and *Hae*III enzymatic digestions were analysed using the Molecular Analyst Software (BioRad). The patterns were specific for *M. avium, M. intracellulare* and *M. scrofulaceum* and species discrimination could be easily performed by visual inspection (Fig. 1).

The combined analysis of the three PCR protocols and comparison to the biochemical identification showed that PCR is a useful method for the identification of species belonging to MAC. Better results were obtained by combining different amplification methods or by the PRA method. PCR is simple, rapid and easily performed in multiple samples. It can be a useful tool in epidemiological medical and veterinarian studies. This identification approach could not only give important insights for the control of infections caused by these bacteria, but also would help to clarify the complex taxonomical status of their members.

RESUMO

Identificação molecular de membros do complexo *Mycobacterium avium*

A identificação bioquímica de micobactérias não permite a discriminação das espécies do complexo *Mycobacterium avium* (MAC). Este estudo mostrou que a amplificação por PCR de DT1 e DT6, duas sequências de cópia única identificadas no genoma de *M. avium* sorotipo 2, da sequência de inserção IS*1245,* encontrada consistentemente em cepas de *M. avium* e de um fragmento do gene da proteína de choque térmico *hsp65,* seguida da análise do polimorfismo de restrição, são testes rápidos e acurados para a diferenciação das espécies *M. avium*, *M. intracellulare* e *M. scrofulaceum.*

Palavras-chave: amplificação, PCR, MAC, *Mycobacterium,* identificação

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UPTAKE OF IRON FROM DIFFERENT COMPOUNDS BY ENTEROINVASIVE *ESCHERICHIA COLI*

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SHORT COMMUNICATION

ABSTRACT

This work aimed the study of acquisition of iron from different compounds by EIEC strains. Seven serotypes were studied (O28ac.H$^-$, O29:H$^-$, O136:H$^-$, O144:H$^-$, O152:H$^-$, O164:H$^-$ and O167:H7$^-$), in a total of 31 strains. All serotypes were able to use distinct iron sources: hemin, hematin, hemoglobin, ovotransferin, lactoferrin, transferrin and ferritin.

Key words: EIEC, iron, iron uptake

The process of infectious disease depends largely upon the efficiency with which the microorganisms gain access to the host, colonize, cause pathological alterations and disseminate to a new host (5). The process of colonization involves two steps – adherence and multiplication. The process of multiplication requires acquisition of growth-essential nutrients, including iron from the host (14). Iron is an essential element for living organisms. This ion is a very versatile biocatalyst (12) and this characteristic is responsible for its involvement in so many essential processes for the cells, such as breathing and ribonucleotide synthesis. Despite its importance, iron is not readily available in aquatic or terrestrial environments and in animal hosts (8).

The amount of free iron available in the human host is extremely low (10^{-18}M) which is insufficient for bacterial growth (4). The majority of iron is found intracellularly as mioglobin, ferritin, hemosiderin and hemoglobin (12). The trace quantities of extracellular iron is bound to the glycoproteins transferrin, in the blood, and lactoferrin, in secretions and mucosal surfaces (1, 19). The ability of pathogenic microorganisms of obtaining iron from host is essential for the production of disease (5).

More commonly, enterobacteria synthesize low-molecular-weight iron-binding compounds, siderophores, and their associated outer membrane proteins receptors. There are two predominant chemical classes of siderophores in *E. coli*, enterobactin (fenolate) and aerobactin (hidroxamate). Siderophores have extremely high affinity for iron and are able to remove iron from glycoproteins like transferrin and lactoferrin (10).

Unlike siderophore-based systems, the utilization of host iron-binding proteins directly as source of iron

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requires direct contact between these proteins and the bacterial cells. Several pathogens can utilize this strategy which requires also outer membrane proteins receptors. The ability to utilize these proteins is often not the only strategy an organism has at its disposal for acquiring iron. For example, vibrios synthesize siderophores in addition to utilizing heme (19).

Several studies have been done to know which iron host compounds can support the growth of microorganisms. *S. flexneri* can use transferrin, lactoferrin and hemin as iron sources (18). *Yersinia pestis* and *Y. enterocolitica* can use hemin as iron source (17). Enteropathogenic *E. coli* (EPEC) uses hemin and hemoglobin as iron sources (9).

E. coli encloses several clones which together have evolved the ability to cause a broad spectrum of human diseases. EIEC are diarrheagenic *E. coli* strains which are closely related to *Shigella* sp. (15) However, very little is known about the iron uptake systems of enteroinvasive *E. coli* (EIEC) and the host sources of iron for these bacteria as well.

In the present study, a plate assay employing Luria-Bertani (LB) agar depleted of iron has been used and also with different iron sources to verify which one could support the growth of these bacteria.

A total of 31 EIEC strains, isolated from diarrheagenic patients, belonging to 7 serotypes, were tested. These strains were previously identified by biochemical and serological methods and by Serény test (13). The EIEC strains were grown in Minimal Medium broth (MM) (9) by shaking (170 rpm) at 37°C for 6 hours. The iron chelator, α, α′-dypirydil, was added to melted LB agar (triptone 10 g, yeast extract 5 g, NaCl 10 g, NaOH 1N 12.5 ml, agar 1.5%, H_2O 1 L) in final concentrations of 400μM. The compounds transferrin, ovotransferrin, human and bovine lactoferrins, hemin, hematin and hemoglobin were added as iron sources in concentration of 25μg/ml (9). Bacterial suspension containing 10^5 CFU was inoculated as spots on the surface of LB agar supplemented with α, α′-dypirydil and the iron source. The plates were incubated at 37°C overnight for bacterial growth. The bacterial growth in LB agar and LB agar supplied with α, α′-dypirydil only were used as positive and negative controls, respectively. The iron sources and α, α′-dypirydil were purchased by Sigma Co (St. Louis, MO USA). The other reagents were purchased from DIFCO Laboratories (Detroit, Michigan USA).

The tests for enterochelin and aerobactin were done by Dr. Shelley Payne (Department of Microbiology-Univ. of Texas, Austin-USA), according to Arnow (2) and Csaky (6), respectively.

It was observed that the 7 serotypes could take iron up from distinct sources. Positive or negative results were based on visualization of bacterial growth on the media surface (Fig. 1). Heme group (hemin, hematin and hemoglobin) and/or glycoprotein group (ovotransferrin, lactoferrins and transferrin) were used by 26 strains (84%), while ferritin was used by 16 strains (52%). The strains did not show a profile of iron utilization, once there was variation in the iron source used by different serotypes, as well as by different strains of a same serotype (Table 1). The iron sources utilization was not species specific because EIEC strains could take iron up from ovotransferrin and bovine lactoferrin. The intracellular localization of ferritin can contribute to the decreasing capacity to take iron up from this compound by the siderophores. Besides, the iron must be reduced to be removed from ferritin, because without reducers, the siderophores are either ineffective for releasing iron from ferritin or they can remove it very slowly (1).

All EIEC strains produced enterobactin and some strains aerobactin (Table 1). The siderophores systems are used to obtain iron from glycoprotein group and ferritin (3; 17), however, some strains did not use one or more of these compounds as iron sources. This suggests that there can be a flaw in

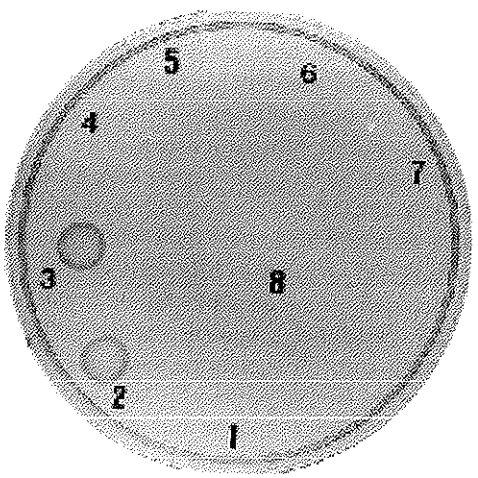

Figure 1 - Positive and negative growth of the different EIEC strains on Luria-Bertani agar with α, α' - dypiridil (300 μM) and hemin (25 μg/ml)
1 and 2 - strains 20 and 22 (152:H⁻); 3 - strain 24 (O164:H⁻), 4 - strain 11 (O29:H⁻); 5 and 7 - strains 25 and 26 (O164:H⁻); 6 - strain 31 (O167:H⁻); 8 - strain 2 (O28:H⁻).

some steps of this process, because for the microorganisms to take iron up from siderophores, the complex iron-siderophore has to be recognized by receptors in the outer membrane and the iron has to be released by specific enzymes action (16;19).

Bacteria present in mucosal surfaces can take iron up from heme or hemoglobin released from dead epithelium cells (7). In the present work, most EIEC strains used the heme group (hemin, hematin and hemoglobin) as iron source. Stugard *et al.* (21) reported the presence of an outer membrane receptor for hemin (~100kDa) in *Shigella flexneri* and EIEC strains. Thus far it can be realized that this receptor is likely to be the mechanism used by these bacteria to take iron up from hemin and, maybe, from other heme compounds, once in *S. flexneri* the hemin utilization as iron source was shown to be independent of the siderophore system (11).

Being the iron transport protein present in secretions and mucosal surfaces, such as the

Table 1. Iron sources utilized by enteroinvasive *Escherichia coli*

		Siderophores		Glycoproteins[1]	Ferritin	Heme[2]
Strains	Serotypes	Aerobactin	Enterobactin			
1	O28:H-	-	+	+	-	+
2	O28:H-	-	+	+	+	+
3	O28:H-	-	+	+	-	-
4	O28:H-	-	+	+	-	+
5	O28:H-	ND[3]	+	+	+	+
6	O28:H-	ND	+	+	+	+
7	O28:H-	ND	+	+	+	+
8	O28:H-	ND	+	-	-	-
9	O28:H-	ND	+	+	+	+
10	O29:H-	-	+	-	-	-
11	O29:H-	-	+	+	-	+
12	O29:H-	-	+	+	-	+
13	O29:H-	ND	+	-	-	+
14	O29:H-	ND	+	+	+	+
15	O136:H-	+	+	+	+	+
16	O136:H-	+	+	+	+	+
17	O144:H-	-	+	+	+	+
18	O144:H-	-	+	+	-	+
19	O144:H-	-	+	+	+	+
20	O152:H-	-	+	+	-	+
21	O152:H-	-	+	+	+	-
22	O152:H-	-	+	+	+	+
23	O164:H-	+	+	+	-	+
24	O164:H-	-	+	+	+	+
25	O164:H-	-	+	-	-	+
26	O164:H-	+	+	+	+	-
27	O164:H-	+	+	+	-	+
28	O167:H-	-	+	-	+	+
29	O167:H-	-	+	+	+	+
30	O167:H-	-	+	+	-	+
31	O167:H-	-	+	+	-	+

[1] - Glycoproteins: transferrin, human and bovine lactoferrin and ovotranferrin
[2] - Heme: hematin, hemoglobin and hemin
[3] - ND - Not done

gastrointestinal tract, the lactoferrin can be an important iron source for enteropathogens because of its localization. The iron uptake from lactoferrin can be processed either by syderophores system or by specific receptors in the outer membrane of some pathogens (17). A putative lactoferrin receptor in EIEC strains outer membrane is being investigated.

The results obtained in this work indicate that there was variation in the iron sources used by different serotypes, as well as by different strains of a same serotype. This fact might suggest that EIEC strains have a high capacity of adaptation, using the iron uptake system that requires the lowest energetic consumption. The use of several iron sources suggests that EIEC strains can use more than one mechanism for it. Therefore, the infections processes of these bacteria can be more easily achieved due to their capacity of using distinct iron sources.

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RESUMO

Obtenção de ferro a partir de diferentes compostos por *Escherichia coli* enteroinvasora

Foi investigada a aquisição de ferro por cepas de EIEC a partir de diferentes compostos. Sete sorotipos foram estudados (O28ac:H$^-$, O29:H$^-$, O136:H$^-$, O144:H$^-$, O152:H$^-$, O164:H$^-$ and O167:H7), totalizando 31 cepas. Foi observado que todos os sorotipos puderam usar fontes de ferro distintas: hemina, hematina, hemoglobina, ovotransferrina, transferrina, lactoferrina e ferritina.

Palavras-chave: EIEC, ferro, captação de ferro

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SPONTANEOUS KANAMYCIN-RESISTANT *ESCHERICHIA COLI* MUTANT WITH ALTERED PERIPLASMIC OLIGOPEPTIDE PERMEASE PROTEIN (OPPA) AND IMPERMEABILITY TO AMINOGLYCOSIDES

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ABSTRACT

A spontaneous kanamycin-resistant *Escherichia coli* mutant, showing cross resistance to five other aminoglycosides and absence of the OppA protein was isolated. [^{3}H]-dihydrostreptomycin uptake is reduced in this mutant, implying that the oligopeptide transport system is involved in accumulation of aminoglycosides, although apparently not related with aminoglycoside permeability alteration due to bacterial adaptation to osmotic changes.

Key words: aminoglycosides-resistance, *Escherichia coli*, OppA.

A new class of mutants that show resistance to several aminoglycoside antibiotics has been selected by exposing *Escherichia coli* and other Gram-negative bacteria to kanamycin in high osmolarity media (12, 13). Kashiwagi *et al.* (6) suggested that the oligopeptide carrier system was involved with slow accumulation phase (EDP-I, ref. 3) of those antibiotics because the introduction of additional copies of the *opp*A gene increases *Escherichia coli* susceptibility to aminoglycosides. In fact, some *E. coli* kanamycin resistant mutants show decrease or deficiency of the oligopeptide permease OppA, a periplasmic protein, as found by SDS-PAGE and Western Blotting with the specific antibody (15). Those mutants revert to the kanamycin sensitive phenotype when transformed with the wild-type *opp*A gene. Two distinct mutants were detected: one carries a nonsense mutation that abolishes translation of OppA and the other one shows a reduction of about one third of the OppA protein translation, although transcriptional levels are about normal (K. Kashiwagi, personal communication). The reduction of OppA synthesis was correlated with reduction of ornithine synthesis and subsequently of polyamine contents, which is important for stimulation of OppA synthesis at the translational level (5, 7). We show here that an *E. coli* mutant lacking OppA is resistant at the same time to different aminoglycosides due to a reduced accumulation of the drug, thus confirming the participation of the oligopeptide transport system

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in the uptake of several aminoglycosides. We also show that, even lacking OppA, this mutant still responds to the osmolarity effect.

Strain and culturing conditions. The *Escherichia coli* strain J53 *pro*A *met* (4) was used. All media components were from Difco Labs. and hydrated with distilled water. Media used: Nutrient Broth (NB) and Nutrient Agar (NA), low osmolarity medium; and high osmolarity L2 medium (2% tryptone, 1% yeast extract, 1% NaCl and 2% bacto-agar when required). Media osmolarity was measured in an Osmette-Precision Systems Inc. osmometer.

Susceptibility and resistance evaluation. Bacterial overnight cultures were used after 1000 fold dilution to inoculate plates with increasing concentration of antibiotic. After incubation for 24 hours at 37°C, the minimal inhibitory concentration or the level of resistance were estimated from the resulting growth. The following antibiotics were used: amikacin and kanamycin from Bristol Laboratories, streptomycin (Squibb & Sons Ltd.) gentamicin (Schering Corp.), neomycin (Lafi Lab.), tobramycin (Eli Lilly & Co.), dihydrostreptomycin (Fontoura) and [^{3}H]-dihydrostreptomycin (Amersham).

[^{3}H]-dihydrostreptomycin uptake measurement. Exponentially growing cells (OD_{620}=0.5) were diluted 10 times in the same medium used for growth plus 4,000cpm/μg of dihydrostreptomycin (at a final concentration of 50μg/ml), and incubated at 37°C for 30 minutes. Duplicated 1ml samples were taken at 0, 5, 10, 15, 20 and 30 minutes after culture dilution and kept on ice until filtration on Millipore 0.22μm teflon coated filters (GYWP01300), previously saturated with 1ml of culture medium plus 1mg of streptomycin. After sample filtration, filters were washed with 2ml of culture medium and 3ml of 3% (w/v) NaCl and dried at room temperature. Radioactivity was measured using scintillation liquid in a Beckman LS 5000TD scintillation counter. Dihydrostreptomycin uptake was calculated by subtracting background radioactivity and dividing by cpm value obtained by measuring 1ng of dihydrostreptomycin.

Periplasmic proteins extraction (Ames *et al.*, 1984, modified). Cells from 3ml of overnight culture were collected by centrifugation at 3,000rpm for 10 minutes at room temperature, resuspended on residual medium, mixed with 40μl of chloroform and incubated for 25 minutes at room temperature. 200μl of 10mM Tris-HCL pH 8.0 was added and the suspension was centrifuged at 3,000rpm for 25 minutes at 4°C. Supernatant was carefully recovered (periplasmic proteins fraction). Protein concentration was measured as in Lowry *et al.*, 1951.

SDS-PAGE and Immunoblotting. 11% SDS-PAGE was performed as in Lugtenberg *et al* (1975) with modifications: running gel was made by mixing 6.25ml of 44% acrilamide/0.8% bis-acrilamide, 0.5ml of 10% SDS, 0.63ml of 1% ammonium persulfate, 12ml of 0.75M Tris-HCl pH8.8 and 5.12ml of deionized water. Stacking gel was made by mixing 0.5ml of 44% acrilamide/0.8% bis-acrilamide, 50μl of 10% SDS, 0.12ml of 1% ammonium persulfate, 2.5ml of 0.25M Tris-HCl pH6.8 and 1.83ml of deionized water. Polimeration was started by addition of TEMED to 0.02%. After running the gel, proteins were silver stained as in Morrissey (1981) or transferred to nitrocellulose filter in a MultiphorII apparatus (Pharmacia). Western blotting was performed as described in Maniatis *et al.* (1989) using specific anti-OppA rabbit serum kindly offered by D. D. Santos (Centro de Biotecnologia, UFRGS) diluted 1: 1,000, anti-rabbit IgG peroxidase conjugated diluted 1: 2,500 and diaminobenzidine as chromogenic substrate.

When the aminoglycoside sensitive *Escherichia coli* J53 is plated on the high osmolarity medium L2 (448.6 mOsm) containing 20μg/ml of kanamycin, resistant colonies arise at frequencies around $5x10^{-6}$. One high level kanamycin-resistant clone (A31) was chosen to have its permeability to aminoglycosides examined, in comparison with the original sensitive strain J53. This clone is resistant to 200μg/ml of kanamycin, streptomycin, tobramycin, gentamicin, amikacin and neomycin when L2 medium is used, and lacks a periplasmic protein (8) that was identified as OppA by Western Blotting (Fig. 1). Given that previous data pointed out to the importance of the medium osmolarity on aminoglycoside susceptibility (8, 9), we evaluated the [^{3}H]-dihydrostreptomycin uptake on NB (63.1 mOsm) and NB plus 0.5M sorbitol (531.1 mOsm). The resistant mutant showed remarkably reduced antibiotic accumulation (Fig. 2) even in low osmolarity conditions, where the susceptibility is higher (13), thus confirming the mutant impermeability hypothesis as the resistance mechanism. The osmolarity effect on bacterial susceptibility to aminoglycosides has been shown to be present on sensitive bacteria (14) as well as on resistant strains (16), even if the resistance is not related with OppA alteration, but is due to other

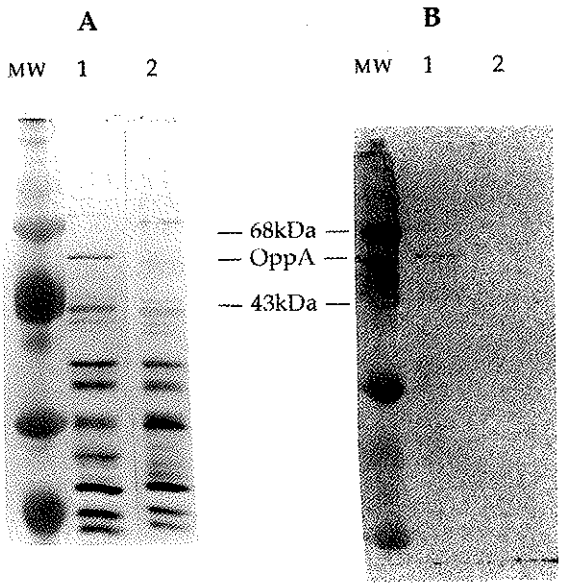

Figure 1. OppA absence on kanamycin resistant mutant. (**A**) shows a 11% SDS-PAGE of periplasmic proteins and (**B**) shows a Western blot using anti-OppA, from the aminoglycoside sensitive strain J53 (lanes 1) and the resistant clone A31 (lanes 2).

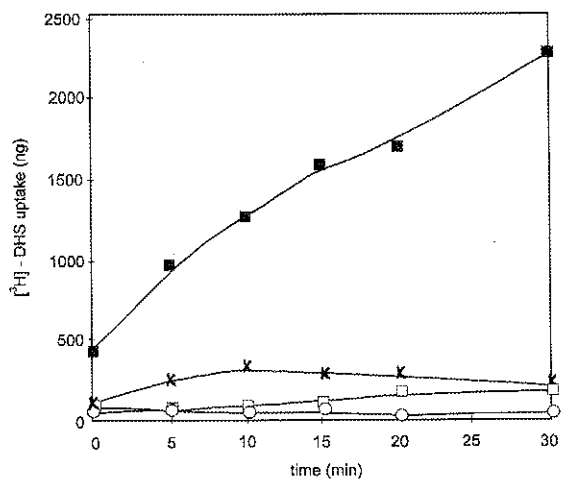

Figure 2. [^{3}H]-dihydrostreptomycin ([^{3}H]-DHS) uptake by aminoglycoside resistant A31 cells in low osmolarity NB (x) and high osmolarity NB + 0.5M sorbitol (o); and by sensitive J53 cells in NB (filled squares) and NB + 0.5M sorbitol (open squares).

mechanisms such as enzymatic inactivation of the drug (1). Our results show that the high osmolarity protective effect is indeed due to a reduction of the bacterial permeability to aminoglycosides. This may be a side effect of a general reduction of permeability for high osmolarity adaptation and rather than a specific one for aminoglycosides. On the other hand, the fact that a spontaneous aminoglycoside resistant mutant, lacking OppA, has reduced permeability to these antibiotics, confirming that the oligopeptide carrier system plays a role on aminoglycoside transport, may represent an important information for antimicrobial therapy, since mutants lacking OppA have recently been isolated from clinical samples (1).

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RESUMO

Mutante espontâneo de *Escherichia coli* resistente à canamicina com expressão de oligopeptídeo permease periplasmática (OppA) alterada e impermeabilidade aos aminoglicosídeos

Um mutante espontâneo de *Escherichia coli* foi selecionado com canamicina e mostrou resistência cruzada a cinco outros aminoglicosídeos e ausência da proteína OppA. A incorporação de diidroestreptomicina tritiada mostrou-se reduzida nesse mutante, implicando que o sistema de transporte de oligopeptídeos está envolvido na acumulação de aminoglicosídeos, embora aparentemente não esteja relacionado com a alteração de permeabilidade aos aminoglicosídeos decorrente da adaptação bacteriana a mudanças osmóticas.

Palavras-chave: aminoglicosídeos, *Escherichia coli*, OppA.

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PRODUCTION OF AMYLASES BY *ASPERGILLUS TAMARII*

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ABSTRACT

A strain of *Aspergillus tamarii*, a filamentous fungus isolated from soil, was able to produce both α-amylase and glucoamylase activities in mineral media supplemented with 1% (w/v) starch or maltose as the carbon source. Static cultivation led to significantly higher yields than those obtained using shaking culture. The production of amylases was tolerant to a wide range of initial culture pH values (from 4 to 10) and temperature (from 25 to 42°C). Two amylases, one α-amylase and one glucoamylase, were separated by ion exchange chromatography. Both partially purified enzymes had optimal activities at pH values between 4.5 and 6.0 and were stable under acid conditions (pH 4.0-7.0). The enzymes exhibited optimal activities at temperatures between 50° and 60° C and were stable for more than ten hours at 55°C.

Key words: α-amylase, *Aspergillus tamarii,* glucoamylase.

INTRODUCTION

A variety of industries (e.g. food, chemical, detergent, textile) employ microbial amylolytic enzymes to convert starch into different sugar solutions (2,13,15,18). Several types of enzymes are involved in the degradation of starch, mainly α-amylase (1,4 α-glucan glucanohydrolase, EC 3.2.1.1), β-amylase (1,4 α-glucan maltohydrolase, EC 3.2.1.2), and glucoamylase (1,4 α-glucan glucohydrolase, EC 3.2.1.3) (2,8). These enzymes are common in fungi, and *Aspergillus* sp and *Rhizopus* sp are often used as sources of industrial amylases (3). Although genetic manipulation by classical mutation techniques and recombinant DNA technology are frequently used to increase the expression levels of a large number of microbial enzymes, including amylases, in well-known microorganisms, traditional screening procedures make possible to find new attractive wild microorganisms able to produce useful enzymes. Some environments such as fertile soil, have great variety of microbial populations. Hundred of different species of fungi inhabit the soil, especially near the soil surface where aerobic conditions prevail (2). Such fungi are active in degrading a wide variety of biological materials present in the soil. They thrive on such material by secreting extracellular enzymes able to degrading large polymeric plant molecules such as cellulose, hemicellulose, starch and pectin, with subsequent assimilation of the liberated nutrients. Considering that a strain of *Aspergillus tamarii* isolated by soil during a screening programme for xylanase producing microorganisms

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(7) showed great growth rate in starch as the only carbon source, the purpose of this work was to investigate the ability of *Aspergillus tamarii* to produce amylases as well as the effect of pH and temperature in the enzyme activity.

MATERIALS AND METHODS

Microorganism and culture conditions: *Aspergillus tamarii* was isolated from soil during a screening programme for xylanase-producing microorganisms (7). It was deposited in the Fundação Tropical de Pesquisas e Tecnologia André Tosello Collection, Campinas, Brazil. In the lab, it was routinely maintained on potato dextrose agar at 4°C. The cultures were developed on 250 ml Erlenmeyer flasks containing 50 ml of Vogel salts (10), and various carbon sources (1%, w/v) at 30°C on a rotary shaker at 120 rpm or under static condition. Flasks were harvested at periodic intervals, the contents filtered through tared filter paper and the mycelia dried to constant weigh at 60°C. The filtrates were assayed for α-amylase and glucoamylase activities, and protein. Results were expressed as the mean of at least three independent measurements. Residual maltose and starch concentrations in the culture filtrates were estimated using anthrone reagent (6).

Enzyme assays: Two different methods were used to quantify the glucoamylase and α-amylase activities in the culture filtrates. Firstly, total amylase activity was estimated by analysis of reducing sugars released during hydrolysis of 0.5% (w/v) starch in 0.05 M phosphate buffer pH 6.0 at 40°C by the dinitrosalicylic acid method (9). Secondly, glucoamylase activity (EC 3.2.1.3) was estimated by determining the D-glucose content released during hydrolysis of starch by the peroxidase-glucose oxidase assay (1). Finally, α-amylase activity (EC 3.2.1.1) was estimated by deducting the reducing sugars from the values of D-glucose determined enzymatically. One unit of amylase activity was defined as the amount of enzyme that releases 1 μmol of reducing sugar as D-glucose per min under the assay conditions. The results are presented as specific activity (U/mg extracellular protein). Extracellular proteins were estimated as described by Bradford (4) using bovine serum albumin as the standard.

Separation of the α-amylase and glucoamylase activities of the culture filtrates: The culture filtrates were concentrated by precipitation with 3 volumes of acetone at 4°C, the pellet dissolved in 0.01M phosphate buffer, pH 6.8, and charged on to a DEAE-cellulose column (1.5 x 20 cm) equilibrated with the same buffer. The column was washed with 10 bed volumes of buffer and the protein adsorbed in the column was eluted with a linear gradient of NaCl (0-0.50 M) in the same buffer at 15 ml/h. Each fraction (2.5 ml) obtained during washing and elution was assayed for amylase activity and absorbance at 280 nm.

Chromatography of hydrolysis products: The pattern of soluble starch hydrolysis by amylolytic enzymes from *A. tamarii* was examined using paper chromatography. Amylases I and II from *A. tamarii* were incubated with 1.0 ml of solution containing 1% of starch in 0.05 M phosphate buffer, pH 6.0. After 30 and 60 min, the reactions were stopped by boiling and stored at 4°C. Samples (15 μl) were spotted onto Whatman N° 1 chromatographic paper and descending chromatography carried out at room temperature with a benzene:n-butanol:piridine:water (1:5:3:3) solvent system. The chromatograms were stained with silver nitrate (17).

Chemicals: Starch, amylopectin, amylose, glycogen, maltose, and glucose were obtained from Sigma Chemical Corp. (St. Louis, Mo). All other reagents were of analytical grade.

RESULTS AND DISCUSSION

The ability of *A. tamarii* to grow and to produce amylases on different carbon sources was studied. Significant growth of *A. tamarii* was observed on monosaccharides, disaccharides and polysaccharides 6 days-cultures (Table 1). The existence of low level constitutive production of glucoamylase and α-amylase was detected in sucrose, cellobiose, glucose and raffinose cultures, but active synthesis of both enzymes took place only during growth on maltose, starch, amylose, amylopectin and glycogen. Although higher biomass levels were obtained with shaking cultures, a four-fold increase in both glucoamylase and α-amylase activities was observed under static conditions. For this reason, static conditions were used in all following experiments.

A typical time course of extracellular α-amylase and glucoamylase production is shown in Fig. 1A-1B. Maximal activities were attained on day 4 and 6,

Table 1. Effect of the carbon source on growth and amylase production by *Aspergillus tamarii*.

Carbon source (1%, w/v)	Static conditions		Shaking conditions	
	biomass (mg)	total amylase (U/mg)	biomass (mg)	total amylase (U/mg)
none	28± 5	15± 2	45± 5	8± 1
sucrose	260±30	24± 3	317±40	10± 2
cellobiose	250±21	25± 3	320±28	12± 2
glucose	355±30	10± 2	440±50	7± 1
raffinose	215±23	68± 8	320±40	20± 2
maltose	284±30	155±16	410±37	38± 5
glycogen	310±27	180±21	480±51	47± 6
starch	290±32	176±15	440±48	42± 4
amylopectin	289±35	170±20	490±53	40± 5
amylose	380±42	144±16	400±37	32± 2

The cultures were grown in 250 ml Erlenmeyer flasks containing 50 ml minimal medium supplemented with various carbon sources at 1% (w/v) under static or shaking conditions for 6 days at 30°C. The mycelia (biomass) were separated by filtration and dried to constant weigh at 60°C. One unit of total amylase (glucoamylase plus α-amylase) was defined as the amount of enzymes that releases 1 µmol of reducing sugar as D-glucose per min. under the assay conditions. The results are presented as specific activity (U/mg extracellular protein). The results express the media±SD of three different experiments.

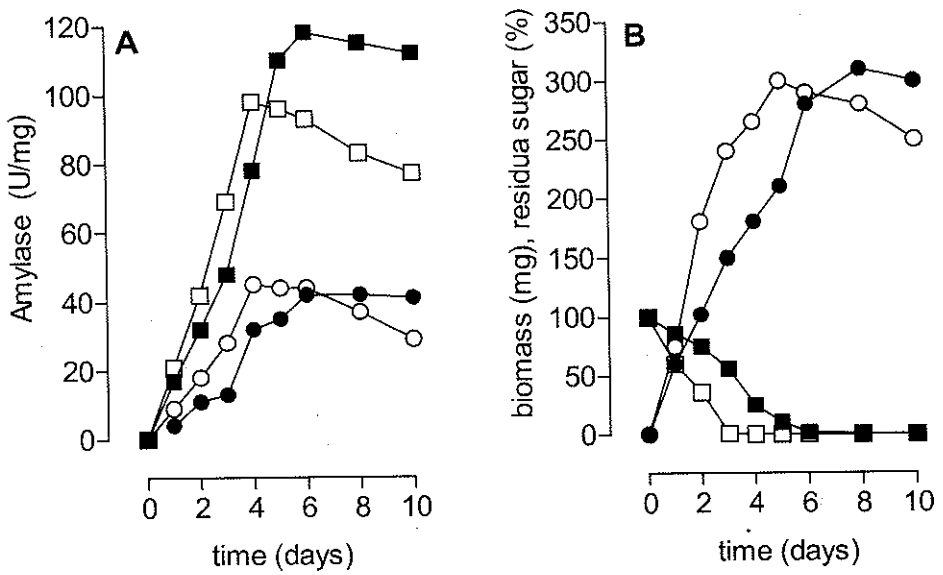

Figure. 1. Time course of amylase and biomass production by *Aspergillus tamarii*.
A: (■) α-amylase activity on starch culture; (□) α-amylase activity on maltose culture: (●) glucoamylase activity on starch culture; (○) glucoamylase activity on maltose culture; B: (●) biomass production on starch culture; (○)biomass production on maltose culture; (■) residual starch; (□) residual maltose.

respectively for the maltose and starch cultures, at the same time of the peak of maximal biomass production. A decrease in enzyme production was observed once the stationary phase of the culture had began. Similar results were obtained in several other microorganisms (12,14,16). Less than 5% of the initial concentration of maltose or starch was present in the filtrate cultures after 4 days of incubation (Fig. 1B). No significant amounts of glucose were detected in the culture filtrates, suggesting that this sugar was rapidly consumed by the fungus (data not shown).

Studies on the effect of the initial pH and temperature on the enzyme production and growth were carried out within the pH 3 to 12 and temperature range 28 to 45°C. At 30° C, growth and enzyme production were inhibited when the initial pH of the medium was above 10 or below 4 (Table 2). The optimal temperature for growth and enzyme production was 30°C (Table 3). Temperatures between 30° and 42°C had little influence on growth and amylase production. However, the fungus did not grow at temperatures above 42°C.

Table 2. Effect of initial pH on amylase production by *A. tamarii*

Initial pH	final pH	biomass (mg)	glucoamylase (U/mg)	α-amylase (U/mg)
3	4.35	ND	ND	ND
4	5.38	248±20	128±	30±3
5	6.28	279±32	129±	31±4
6	6.90	295±28	136±	35±3
7	7.10	256±29	130±	32±5
8	7.18	254±21	131±	32±3
9	7.47	213±26	125±	29±3
10	7.68	208±29	121±	28±4
11	9.20	ND	ND	ND

The cultures were grown in 250 ml Erlenmeyer flasks containing 50 ml minimal medium supplemented with 1% (w/v) starch under static conditions for 6 days at 30°C at different initial pH. After autoclaving the medium, pH was adjusted using sterilized 10% (w/v) Na_2CO_3 solution or 5N HCl. The mycelia (biomass) were separated by filtration and dried to constant weigh at 60°C. One unit of amylase (glucoamylase and α-amylase) was defined as the amount of enzymes that releases 1 µmol of reducing sugar as D-glucose per min. under the assay conditions. The results are presented as specific activity (U/mg extracellular protein). The results express the media±SD of three different experiments. ND= not determined.

The filtrates of starch and maltose cultures were dialyzed against water, the proteins concentrated by precipitation with acetone, and then loaded on to a DEAE-cellulose column at pH 6.8. In each case, the amylase activity was eluted in two peaks, designated as amylase I and II (Fig. 2). Approximately 70% of the total amylase activity was due to amylase II, and 30% was due to amylase I. The fractions with activity were pooled and concentrated by lyophilization. The enzymes were incubated with starch as substrate and, samples withdrawn periodically from the reaction mixture were analysed by descending paper chromatography (Fig. 3). A mixture of sugars, larger oligosaccharides, traces of glucose and mainly maltose, was obtained from starch when the hydrolysis was carried out using the amylase II,

Table 3. Effect of temperature on amylase production by *A. tamarii*

temperature (°C)	biomass (mg)	glucoamylase (U/mg)	α-amylase (U/mg)
25	210±18	129±15	30±4
28	280±24	128±11	31±3
30	295±28	136±12	35±3
32	300±31	130±16	32±5
35	292±30	129±10	32±4
37	289±31	120±15	30±3
39	276±30	125±15	28±2
40	280±25	122±12	25±4
42	230±21	119±10	20±3
45	ND	ND	ND

The cultures were grown in 250 ml Erlenmeyer flasks containing 50 ml minimal medium supplemented with 1% (w/v) starch under static conditions for 6 days at different temperatures. The mycelia (biomass) were separated by filtration and dried to constant weigh at 60°C. One unit of amylase (glucoamylase plus α-amylase) was defined as the amount of enzymes that releases 1 µmol of reducing sugar as D-glucose per min. under the assay conditions. The results are presented as specific activity (U/mg extracellular protein). The results express the media±SD of three different experiments. ND= not determined

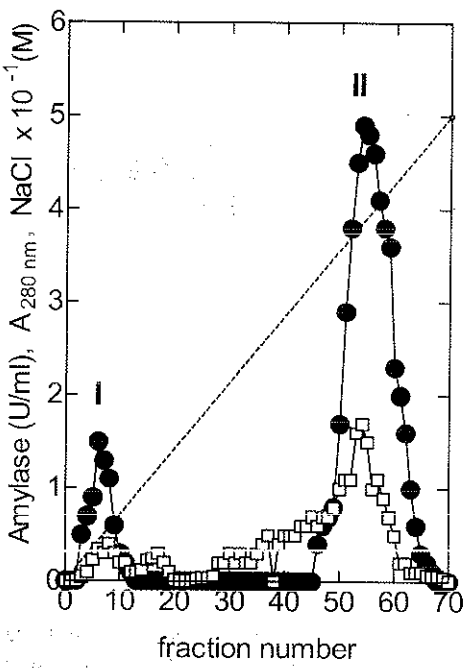

Figure 2. Distribution of protein and total amylase activities after DEAE-cellulose chromatography. (●) amylase activity; (□) absorbance at 280 nm; (---) NaCl concentration.

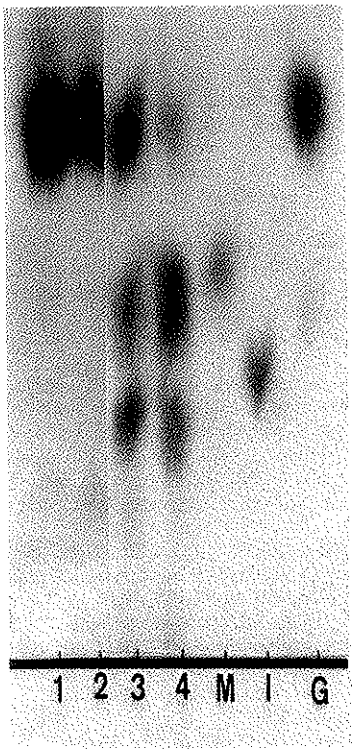

Figure 3. Paper chromatography of hydrolysis products from starch by amylase I and II. 1 and 2: starch plus amylase I (glucoamylase) after 30 and 60 min. of incubation, respectively as described in MATERIALS AND METHODS; 3 and 4: starch plus amyalse II (α-amylase) after 30 and 60 min. of incubation, respectively. M, I, and G are maltose, isomaltose and glucose standards, respectively.

suggesting that this fraction contained an α-amylase. Amylase I released only glucose from starch, indicating that this fraction contained a glucoamylase.

Some properties of amylases were studied. The effect of pH on the glucoamylase and α-amylase was studied using starch as the substrate in McIlvaine buffers (Fig. 4). The initial rate of starch hydrolysis at 40°C was maximal at pH 4.5-5.0, but substantial activities were detected at pH values ranging from 4.0 to 7.0 (Fig. 4A). At 40°C and pH 4.0 to 7.2, the enzymes were stable for 2 days (data not shown). These data are in agreement with the general behaviour reported for other fungal amylases (5,11) The recommended pH range for hydrolysis of starch with commercially available fungal amylases is between 4.0 and 4.5 (3) and the higher pH range observed may have the advantage of requering less adjustment of pH between sequential α-amylase (liquefaction) and glucoamylase (saccharification) treatments. The enzymes were highly active over a broad temperature range of 40-70°C. At pH 6.0, the optimal activity was observed at a temperature of 50-55°C for both enzymes (Fig. 4B) Thermal inactivation of the partialy purified amylases was investigated by incubating the enzymes at 55°C and 65°C in 0.05 M sodium phosphate buffer, pH 6.0, without substrate (Fig. 4C). The enzymes were stable at 55°C for more than 10 hours, and at 65°C exhibited an half-life of 30 min. (amylase I) and 45 min. (amylase II).

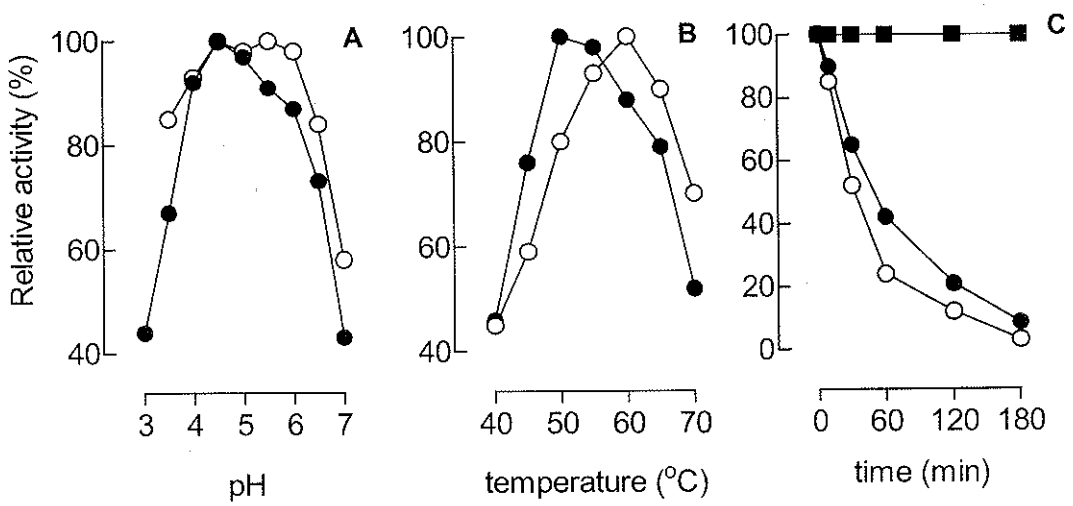

Figure 4. Effect of pH (A) and temperature (B) on the activity and effect of temperature on the stability (C) of amylases from *Aspergillus tamarii*. A and B: (●) glucoamylase activity; (○) α-amylase activity; C: (■) residual glucoamylase and α-amylase activities at 55°C; (●) residual α-amylase activitiy at 65°C; (○) residual glucoamylase activity at 65°C.

Some characteristics of *A. tamarii* described in this paper, such as easy cultivation, production of two types of amylases (glucoamylase and α-amylase) on cultures at different pH and temperature conditions, and stability of the enzymes at an acidic range of pH, make this fungus a potential source of amylase for future biotechnological applications. Purification of these amylases is in progress in our laboratory.

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RESUMO

Produção de amilases por *Aspergillus tamarii*

Uma cepa de *Aspergillus tamarii*, um fungo filamentoso isolado do solo, foi hábil em produzir α-amilase e glucoamilase em meio mineral suplementado com amido ou maltose a 1% (p/v) como fonte de carbono. A produção de amilases em culturas estacionárias foi significativamente maior do que em culturas sob agitação. Uma ampla faixa de pH inicial (de 4 a 10) e temperatura (de 25 a 42°C) pode ser utilizada nos cultivos para a produção das amilases. Duas amilases, uma α-amilase e uma glucoamilase foram separadas por cromatografia de troca iônica. As enzimas parcialmente purificadas exibiram máxima atividade na faixa de pH entre 4,5-6,0 apresentando grande estabilidade sob condições ácidas (pH 4 a 7). Máxima atividade foi obtida nas temperaturas entre 50 e 60°C, e as enzimas foram estáveis por mais de dez horas à 55°C.

Palavras-chave: α-amilase, *Aspergillus tamarii*, glucoamilase.

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THERAPEUTIC PROPERTIES OF WHEY USED AS FERMENTED DRINK

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ABSTRACT

Bioconversion of whey for preparation of beverage was standardized by utilizing yoghurt cultures. The product, wheyghurt drink, made with 4% yoghurt cultures inoculated in deproteinized whey (4.8% lactose, 0.66% ash, 0.46% fat and 0.40% protein adjusted to pH 6.4) and incubated at 42°C for 8h had all the technological requisite and dietetic criteria required in the product. The factors affecting the antibacterial activity of wheyghurt drink against *Escherichia coli*, *Staphylococcus aureus*, *Shigella dysenteriae* and *Bacillus cereus* were determined. There was a significant variation ($P<0.05$) in the antibacterial activity of wheyghurt drink with different levels of inoculum (1,2,4, and 8%) and concentration of sugar at 37, 42 and 45°C. Incubation at 42°C with 4% culture in whey exhibited highest inhibitory activity. The product stored up to 5 days under refrigeration was of acceptable organoleptic quality and requisite amount of microbial population (10^8 cfu/ml) to be potentially beneficial.

Key words: whey, yoghurt, antibacterial activity

INTRODUCTION

The bioconversion of whey is an interesting process from the view point of human nutrition, especially for therapeutic purposes, in regard to economy, and with advantage for reducing environment pollution. Ancient Greeks as well as Hippocrates, in 460 B.C., prescribed cheese whey for the assortment of human ailments. Use of *Lactobacillus delbrueckii* subsp., *bulgaricus* and *Streptococcus thermophilus* in the manufacturing of yoghurt have been extensively studied throughout the world. Regular intake of this product looks effective both in prevention and treatment of various illness in man viz. gastrointestinal disorders (14), hypercholesterolemia (10), antitumoral (3, 14), reduced protein allergencity, treatment of vaginal discharge, a cure for osteoporosis etc. (10). Although yoghurt bacteria can grow well in whey (5, 23, 27) use of these organisms in the preparation of whey drink is still limited.

The present communication includes a report on the preparation of wheyghurt drink, a fermented whey beverage prepared by using *L. delbrueckii* subsp. *bulgaricus* W and *S. thermophilus* H as culture organisms, assessment of its antibacterial activity as well as its acceptability and survival of the culture organisms in the gastrointestinal segments of wheyghurt drink fed rats.

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MATERIALS AND METHODS

Preparation of whey

Whey was prepared by heating pooled cow milk to 82°C and 2% citric acid solution was added at the rate of 2gm. Per kg of milk. Complete coagulation was effected within one minute and the whey filtered muslim cloth is popularly known as *chhana* whey in India where the coagulum *chhana* is used as a base material for traditional sweetmeats. Whey obtained was adjusted to pH 5.5 using 10% $NaHCO_3$ solution and was heated at 100°C for 10 minute with 0.4% $CaCl_2$ and kept undisturbed overnight at room temperature and filtered to obtain deproteinized whey (20). The product was then polished aseptically through washed diatomaceous earth built up as one half inch cake on a No. 54 Whatman filter paper placed in Buckner funnel (19). The average composition of whey was 4.8% lactose, 0.60% ash, 0.46% fat and 0.4% protein.

Source and Maintenance of Cultures

Lactobacillus delbrueckii subsp. *bulgaricus* W and *Streptococcus thermophilus* H along with the test cultures of pathogenic organisms viz. *Bacillus cereus, Escherichia coli, Shigella dysenteriae and Staphylococcus aureus* were obtained from the National Collection of Dairy Organisms, National Dairy Research Institute, Karnal, India. *Lactobacillus delbrueckii* subsp. *bulgaricus* W. and *Streptococuss thermophilus* H were maintained in sterile deproteinized whey peptone broth (8), with the following composition: peptone, 1gm; sodium chloride, 0.5gm and whey 100ml. pH of the media was maintained at 7.0. This whey medium was transferred to standard corning screw capped tubes (15x125 mm) by filling upto neck and were sterilized by steaming for 30 min on three consecutive days. The stock cultures were activated by three successive transfers at 48 h interval. The pathogenic cultures were maintained on nutrient agar slants (oxoid) and were activated by three successive transfers at 24 h intervals in nutrient broth.

Preparation of Wheyghurt Drink

A Schematic diagram conceptualizing the process employed for the production of wheyghurt drink using *L. delbrueckii* subsp. *bulgaricus* W and S. *thermophilus* H cultures for direct consumption is showed in Fig. 1. The effect of some factors such as i) size of inoculum, viz. 1, 2, 4 and 8%. ii) incubation temperature viz. 37, 42 and 45°C iii) concentration of sucrose viz. 0, 6, 8, 10, 12 and 16% and iv) storage at refrigeration temperature (5°C) for 1, 2, 5, 10 and 15 days – on the antibacterial activity of the drink were also examined.

Analysis

Wheyghurt drink was analyzed for titratable acidity (6), volatile acidity (15), lactic acid (4) and β-D-galactosidase activity (13). The antibacterial activity of the product was estimated by the modified cup agar assay technique (7). Culture filtrates (or cell free extracts) were collected by centrifugation at 3000 rpm for 15-20 min. These were passed through Seitz filter separately. Wells of 5 mm diameter were made on solidified nutrient agar (inoculated with the pathogenic test organisms) in each plate, and 50 µl of the cell-free extract introduced transferred to wells. The plates were incubated without inverting at 37°C for 18-24 h and the diameters of inhibition zones were statistically evaluated by analysis of variance (29).

Samples of wheyghurt drink were subjected to sensory evaluation by a panel of 7 judges 9-point hedonic scale (2) and analysed statistically by 2-way classification (29).

For survival of wheyghurt drink organisms in the intestine of rats 10 weanling male albino rats ≥ 21 and ≤ 28 days old were used. Each animal was fed with 15 g of rat feed synthetic ration containing 20% casein, 50% sucrose, 24% hydrogenated vegetable oil, 2% cod liver oil, 4% USP salt mixture, one multivitamin capsule (500 mg Pfizer) per kg. diet and 20 ml wheyghurt drink as preliminary diet for 7 days immediately prior to lights being extinguished. After 16 h, food cups and any remaining food were removed from the cages, the rats fasting for 8 h before being fed again. One the 8th and final day of the experiment, the animals except for one which served as a fasted control were provided with only 20 to 24 g of specific test meal (wheyghurt drink) and given 30 min to consume it. At intervals of 60, 120 and 180 min. after the meal animals were anesthetized with ether, weighed and its abdomen opened and contents of the stomach, duodenum, and jejunum were sampled after injecting and mixing 1.0 c.c. sterile saline (0.85% NaCl) into the clamped-off segments and aspirating with a sterile 5 c.c. syringe and a 22 gauge needle. Serial ten-fold dilutions of the aspirated contents were then prepared with sterile saline, and pour plated in duplicate on Elliker Agar (12).

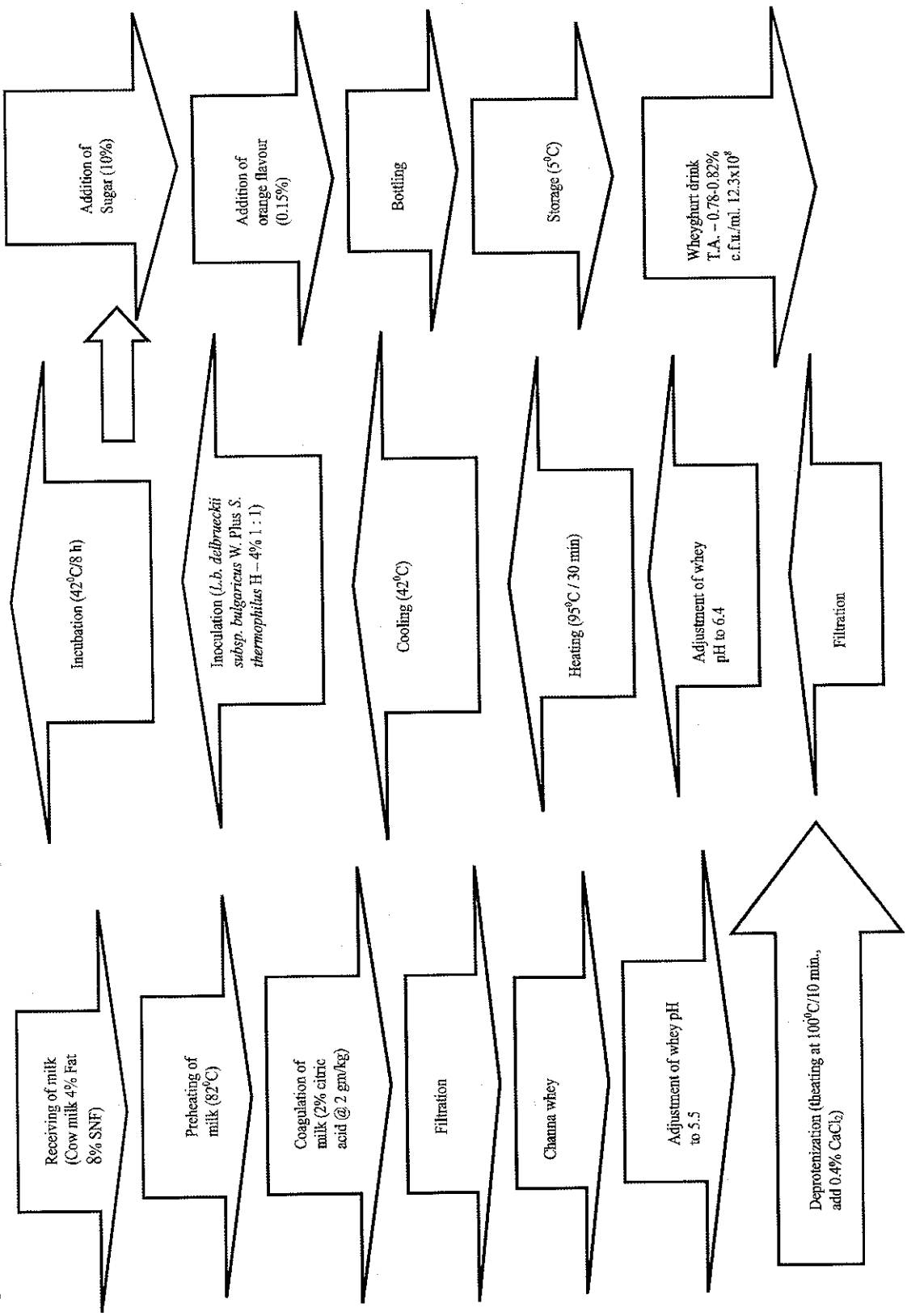

Figure 1. Schematic diagram for the manufacture of wheyghurt drink

RESULTS AND DISCUSSION

Characteristics of the Product

The procedure shown in Fig.1 was adopted for the preparation of wheyghurt drink using 4% mixed culture of *L. delbrueckii* subsp. *bulgaricus* W and *S. thermophilus* H in the ratio of 1:1. The final product had a titratable acedity of 0.78 – 0.82%, 2.0 to 2.4 ml. of volatile acidity, 204-207 µg/ml lactic acid, β-D-galactosidase activity of 2.30 µmol of lactose hydrolysed/gm/h., mild acidic flavour, antibacterial activity against all the four test organisms viz. *E. coli, S. aureus, Shigella dysenteriae* and *B. cereus* (inhibitory zone 8 to 10 mm) and a viable count of 12.3 x 10^8 c.f.u./ml. Rasic and Kurmann, 1979, recommended acidity level of 0.78 to 0.85% for yoghurt preparation (25). Considering that the minimum acidity of 0.7% is specified for yoghurt by the International Dairy Federation (1969), the product showed a desirable acidity level (16). Tramer, 1973 (31); Rasic and Kurmann, 1979 (25) and Singh, 1983 (28) recommended an inoculum of 1-3% for the preparation of yoghurt, but in the present study 4% inoculum was used due to low total solid content in whey. The use of high inoculum ensures a normal course of lactic acid fermentation and restrict unfavourable growth conditions as residual antibiotic, lack of growth substances etc. (22). Viable lactic acid bacteria population in the range of 10^8 to 10^9 cell/ml. of the fermented product causes successful seeding in intestine during consumption (17, 21, 30) and the product prepared according to the schematic chart (Fig. 1) satisfied the condition.

Effect of the Levels of Inoculum

The effect of 1, 2, 4 and 8% inoculum of *L. delbrueckii* subsp. *bulgaricus* W and *S. thermophilus* H (1: 1) on the antibacterial acitivity against four test organisms is depicted in Table 1. There was a significant variation ($P<0.05$) in the antibacterial activity due to change in level of inoculum. A 4% inoculum showed maximum antibacterial activity against *S. aureus* (10 mm.) and *B. cereus* (8 mm.), although antibacterial activity against these two organisms decreased at inoculum level of 8% (9 mm. for *S. aureus* and 7 mm. for *B. cereus*). Pette and Lolkema (24) reported that higher inoculum level increases the *Lactobacillus* content of yoghurt. Single strain culture of *L. delbreueckii* subsp. *bulgaricus* W showed lower antibacterial activity against *S. aureus* and *B. cereus* in comparison to *S. thermophilus* H in *channa* whey. The product exhibited similar antibacterial activity against the other two test organisms viz. *E. coli* and *Shigella dysenteriae* at all inoculum level.

Effect of Incubation Temperature

The data on the effect of different incubation temperature viz. 37°C, 42°C and 45°C on the antibacterial activity of wheyghurt drink is presented in Table 2. At 45°C weak (6 mm. inhibition zone against *E.coli, S. aureus* and *Shigella dysenteriae*) or no antibacterial activity (against *B. cereus*) was visible, in despite of maximum titratable acidity (0.82% against 0.74% and 0.80%, respectively, at 37 and 42°C) was reported at this temperature. The data indicated that production of antibacterial substances was not related to titratable acidity (9, 26). Maximum antibacterial activity of the product was obtained at 42°C, probably due to increased total cell count of 12.5 x 10^8 c.f.u./ml. promoted by temperature, leading to increase in the production of antibacterial substances.

Table 1. Effect of level of inoculum on the antibacterial activity of wheyghurt drink

Culture Combination: *Lactobacillus delbrueckii subsp. bulgaricus* W and *Streptococus thermophilus* H (1: 1)

Percent Inoculum	Titratable Acidity (LA %)	Total cell count (c.f.u./ml)	Dia. of zone of Inhibition (mm.)*			
			E. coli	*S. aureus*	*Shigella dysenteriae*	*B. cereus*
1	0.64	2.89 x 10^7	8.5	9.0	8.5	7.5
2	0.74	3.47 x 10^7	9.0	8.5	9.0	7.0
4	0.80	12.30 x 10^8	9.0	10.0	9.0	8.0
8	0.90	21.0 x 10^8	9.0	9.0	9.0	7.0

* Included dimateter of well (5 mm.) (amount of supernatant in well 0.05 ml).

Effect of Sugar Concentration

When sweetened wheyghurt drink was prepared using different concentrations of sugar (0, 6, 8, 10, 12 and 16%) it was observed that as the level of sugar addition increased there was very slight change in the titratable acidity, total viable count and antibacterial activity of the product upto 10% level of sucrose (Table 3) but at 12% sucrose level the changes were significant ($P<0.05$). Addition of 16% sucrose exhibited no antibacterial activity against any of the four test organisms with a low acidity (0.68%) and viable count (3.2 x 10^8 c.f.u./ml). Tramer (31) also reported that during preparation of yoghurt addition of sugar should not allow total solids to exceed 22% to avoid severe inhibition of yoghurt starters. However, it was observed that wheyghurt drink with 10% level of sucrose was excellent in taste with optimum titratable acidicy (0.78%) and recommended viable count (12.1 x 10^8 c.f.u./ml).

Effect of Storage at Refrigeration Temperature

Refrigerated storage (5°C) of wheyghurt drink for 15 days indicated that the storage time increased beyond 5 days caused decrease in the antibacterial activity against the four organisms tested and with sharp decline after 10 days (Table 4). The total viable count decreased from 12.5 x 10^8 c.f.u./ml. to 54 x 10^6 c.f.u./ml. after 15 days of storage. The product was very sour in taste after 10 days of storage and was not liked by the consumers (sensory score 4.90). Kumar *et al.*, (18) also reported a highly acidic product from fermentation of whey with yoghurt culture. Average sensory evaluation of wheyghurt drink by a panel of seven judges showed that maximum average sensory score of 6.50 in nine point hedonic scale was obtained after 24 h. of storage. The product was acceptable on the basis of mouthfeel, overall appearance and optimum level of acidity up to 5th day of storage (sensory score 6.00).

Survival of Wheyghurt Drink Microflora in the Rat Intestine

The total viable cell counts of the gastrointestinal segments (stomach, jejunum and duodenum) of wheyghurt drink fed rats at intervals of 60, 120 and 180 min. after meal are presented in Table 4. The count remained elevated until 2 to 3 h after ingestion of wheyghurt drink thereby demonstrating significant survival and potential metabolic activity in the upper gastrointestinal tract of the animals. Highest count

Table 2. Effect of incubation temperature on antibacterial activity of wheyghurt drinks

Incubation Temperature (0°C)	Acidity (LA %)	Total cell count (c.f.u./ml)	Dia. of zone of Inhibition (mm.)*			
			E. coli	*S. aureus*	*Shigella dysenteriae*	*B. cereus*
37	0.74	11.2 x 10^8	9.5	9.5	8.5	7.5
42	0.80	12.5 x 10^8	9.0	10.0	9.0	8.0
45	0.82	6.8 x 10^8	6.0	6.0	6.0	-

* Included well dimateter of well (5 mm.) (amount of supernatant in well 0.05 ml).

Table 3. Effect of concentration of sugar on antibacterial activity of wheyghurt

Concentration of Sugar (Percent)	Acidity (LA %)	Total cell count (c.f.u./ml)	Dia. of zone of Inhibition (mm.)*			
			E. coli	*S. aureus*	*Shigella dysenteriae*	*B. cereus*
0	0.80	12.5 x 10^8	9.0	10.0	9.0	8.0
6	0.80	12.5 x 10^8	9.0	10.0	9.0	8.0
8	0.80	12.3 x 10^8	9.0	9.5	9.0	8.0
10	0.78	12.1 x 10^8	9.0	9.5	9.0	8.0
12	0.74	10.8 x 10^8	8.0	9.0	8.0	7.5
16	0.68	3.2 x 10^8	-	-	-	-

* Well diameter included (5 mm.)
- :No inhibition observed.

Table 4. Effect of storage at refrigeration temperature (5°C) on bacterial growth, antibacterial activity and sensory score of wheyghurt drink

No. of Days of Storage	Total cell count (c.f.u./ml)	Dia. of zone of Inhibition (mm.)*				Average Sensory score.
		E. coli	*S. aureus*	*Shigella*	*B. cereus dysenteriae*	
1	12.5×10^8	9.0	10.0	9.0	8.0	6.50
2	11.2×10^8	9.0	10.5	9.0	8.5	6.35
5	9.6×10^8	8.5	9.0	8.5	8.0	6.00
10	32×10^7	7.0	8.0	7.5	7.0	4.90
15	54×10^6	6.0	8.0	7.0	7.0	3.00

* Well diameter included (5 mm.)

Table 5. Viable cell counts of gastrointestinal segments of rat given special dietary treatment.

(Treatment = Rat feed (sucrose) + 20 ml. Wheyghurt Drink as preliminary meal and Wheyghurt Drink as test meal)

Gastrointestinal Segements	Log Counts of Viable Cells per ml.		
	60 min.	120 min	180 min
Stomach	7.83	6.60	3.41
Jejunum	4.90	7.45	5.20
Duodenum	4.20	5.17	5.58

was observed in the stomach whereas lowest count was observed in the duodenum. This may be due to the effect of bile salt in the duodenum which altered permeability of the bacterial cells and thereby resisted the growth of the organisms. Acott and Labuza (1) have shown that yoghurt microflora were capable of surviving simulated gastric digestion where Goodenough and Kleyn (13) have demonstrated gastrointestinal survival of yoghurt organisms in vivo up to 3 h. after feeding.

CONCLUSION

Wheyghurt drink made with yoghurt cultures showed potential therapeutic properties, and optimum sensory qualities with a shelf life of 5 days. The yoghurt microflora survived in the gastrointestinal tract, and the mass effect combined with the antagonistic activity against undesirable organisms represents an important factor for the utilization of fermented whey drink preparation with both dietetic and technological properties.

RESUMO

Propriedades terapêuticas de soro de leite usado como bebida fermentada

A bioconversão de soro de leite para preparação de bebida foi padronizada utilizando culturas de iogurte. O produto feito com culturas de iogurte a 4%, inoculadas em soro desproteinizado (lactose 4,8%; cinzas, 0,66%; gordura 0,46% e proteína 0,40%, pH 6,4), incubado a 42°C por 8h, apresentou todos os requisitos tecnológicos e critérios dietéticos requeridos para o produto. Os fatores que afetam a atividade antibacteriana do produto contra *Escherichia coli*, *Staphylococcus aureus*, *Shigella dysenteriae* e *Bacillus cereus* foram determinados. Houve uma variação significativa na atividade antibacteriana do produto contendo diferentes níveis de inóculo (1, 2, 4 e 8%) e concentração de açúcar a 37, 42 e 45°C. Incubação a 42°C com cultura a 4% no soro apresentou a maior atividade inibitória. O produto armazenado até 5 dias em refrigeração apresentou características organolépticas aceitáveis e microrganismos em quantidade adequada (10^8 ufc/ml) para ser considerado benéfico.

Palavras-chave: soro de leite, iogurte, atividade antibacteriana

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ENHANCEMENT OF GAMMA-LINOLENIC ACID PRODUCTION BY THE FUNGUS *MUCOR* SP LB-54 BY GROWTH TEMPERATURE

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ABSTRACT

As a relatively prolific producer of GLA, the strain of *Mucor* sp LB-54 was selected for a study at different growth temperatures in shaker flask culture. The strain used in our experiment was capable to accumulate a relatively high amount of intracellular lipid, 20.73 % of dry cell weight, and GLA content of 15 % of total fatty acids after 5 days of incubation at 28°C. As the growth temperature was decreased from 28 to 12°C the percentage of GLA increased from 15 to 24 % of total fatty acids. In order to optimize the culture conditions for rapid biomass production and lipid production with a high proportion of GLA, the fungus was grown at two temperature combinations associated supplies of carbon source (glucose) in the culture medium. Maximal production of GLA (74 mg/l) was obtained from the *Mucor* sp LB-54 strain after 5 days of incubation at 28°C in basal medium following glucose addition (7 % w/v) and incubation for an additional 3 days at 12°C. The identity of GLA found in the strain of *Mucor* sp LB-54 was confirmed by the coupled gas chromatography-mass spectrometry

Key words: Gamma-linolenic acid, *Mucor* sp, unsaturated fatty acids

INTRODUCTION

Gamma-linolenic acid (GLA, 6,9,12-octadecatrienoic acid) is an important intermediate in the biosynthesis of biologically active prostaglandin from linolenic acid. GLA has been reported to be effective for the prevention or curing of cardiovascular diseases (10), hypercholesterolemia (11), menstrual disorders (16), for applications in curing certain skin-related (19), as well as a variety of other diseases (3).

At the present time, GLA is commercially produced from the seeds of evening primrose (*Oenothera biennis*) and boragem (*Borago oficinallis*). However, the productivity of GLA from the seed oil is extremely low, since both a long period and a huge area for harvesting seed are required (8). To overcome these problems, microorganisms have been investigated as an alternative GLA source and some suitable strains have been proposed. *Mortierella ramanniana* (9), *Mucor* sp (21), *Cunninghamella japonica* (7), and *Entomophthora exitalis* (13) were reported as perspective GLA producers. Currently, only Japan is producing GLA commercially, using the fungus *Mortierella* (17).

There is sufficient information in the literature

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on the fatty acid composition of fungi to warrant a statement that Phycomycetes are characterized in their ability to synthesize GLA, whereas the members of Ascomycetes and Basidiomycetes, with a few exceptions, produce alfa linolenic acid (20). Most of these investigations were carried out to determine the fatty acid composition of different fungi and to compare them with other groups of organisms to obtain information on their phylogenetic relationships.

In the preceding paper (5) we reported a strain of *Mucor* sp screened in our laboratory would be a promising producer of GLA. Therefore, further works on the optimization of fermentation process are needed in order to increase the productivity of GLA. The temperature is the principal regulation factor of the degree of unsaturated in the lipids of this organism (4). In many species of fungi there is a pronounced influence of growth temperature on the biosynthesis of unsaturated fatty acids. In general, organisms grown under low temperature conditions possess a relatively high degree of unsaturation in their lipids, presumably as a part of the adaptive response to the cold environment (15).

This paper deals with the effects of growth temperature for the fungal growth, lipid and GLA contents in cellular lipid of *Mucor* sp LB-54 with an emphasis on GLA productivity.

MATERIALS AND METHODS

Strain

The fungal strain used in this study was designated *Mucor* sp LB 54 by Biochemistry Laboratory at State University of Campinas, Brazil (5). The pure cultures of fungi were stored on PDA slants and kept at 4°C until used.

Medium and culture conditions

The basal medium for fungal growth and GLA production is the same as in the previous paper containing glucose, 20g/l and yeast extract, 10g/l (5). 50ml Erlenmeyer flask containing 25ml of culture medium was inoculated with spore suspension at the final concentration of 7.10^7 spores per millilitre of cultivation medium. The initial pH of the medium was adjusted to 7.0 and liquid cultures were grown on a rotative shaker at 120 strokes per min for 1- 15 days. To study the effects of growth temperature on GLA production, mycelia were incubated at each temperature ranging from 5 to 40°C.

Measurement of dry cell weight and total lipid

The mycelia fungi were separated by centrifugation (15,000 rpm for 15 min at 10°C) and the harvested fungi were washed twice with destined water. The dry cell weight was determined by drying the cells with acetone and in a vacuum oven to constant weight at 40°C. Before extraction of the lipids, the biomass was pulverized. The lipids were extracted from the dried biomass with chloroform/ methanol/water (2).

Analysis of fatty acid composition

The lipids were saponified with 0.5M NaOH and esterified with methanol-BF_3 (1). The fatty acids methyl esters (FAME) were analyzed by gas chromatography in a Chrompack CG instrument equipped with flame ionisation detectors (FID). The separations were carried out on a 50m × 0.25mm fused silica WCOT CP-Sil 88 capillary column (Chrompack, Holland) using temperature programme of 180-220°C, 5°C/min; hydrogen was used as carrier gas. FAME were identified by comparing retention times with those of authentic standards (Sigma Chemical Co.) and determined by relative percentage. GLA was confirmed by the coupled gas chromatography-mass spectrometry (Hewlett-Packard 5890).

RESULTS AND DISCUSSION

In Fig. 1 the data obtained for the determination of growth of *Mucor* sp LB-54 at temperature ranging from 5 to 40°C are summarized. The results show that *Mucor* sp LB-54 is a slightly mesophilic fungus and that the minimum, optimum and maximum temperatures for growth are 8, 28 and 38°C respectively. It was also noted that the cultures grown at 28°C achieved maximum growth within 5 days, whereas at temperatures above or below this optimum, it took 10 days or more.

For further investigation on the biomass and lipid content, growth temperatures of 12, 28 and 38°C were selected since growth was negligible below 10°C and above 38°C. The culture was incubated for 5 days and then the production of GLA was studied. The data in Table 1 show the strain of *Mucor* sp LB-54 produced relatively high cell weight and total lipids when grown at 28°C. The yield of lipids was 20.7 % of dry cell weight of the culture, which mean the maximal concentration of 44 mg of GLA per liter of culture medium. Although the lipids of culture grown at 12°C showed the highest GLA

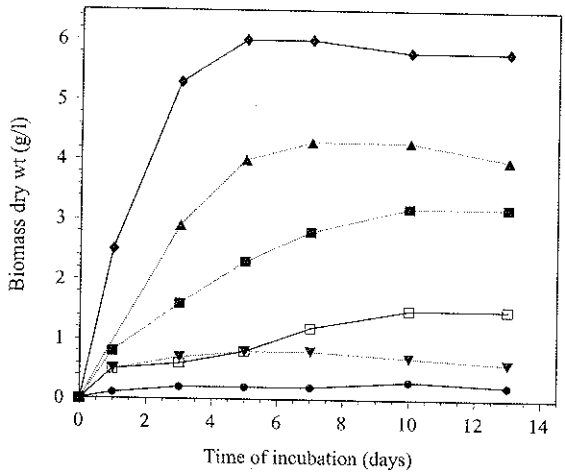

Figure 1. Growth of *Mucor* sp LB-54 on basal medium as a function of time at temperatures ranging from 5 to 45°C. The incubation temperatures (°C) are: 5 = ●—●, 8 = □—□, 12 = ■---■, 28 = ◆—◆, 38 = ▲---▲, 45 = ▼---▼. Each point is the mean of three repetitions.

content, about 24 % of total fatty acids, that seem not suitable for the production of GLA because of low lipid yield (15.8 % of dry cell weight).

The degree of unsaturation in the fatty acid composition is known to be influenced by temperature, i. e. when the growth temperature is lowered the proportion of unsaturated acids tend to increase. An earlier study with fungus has shown that there was increased production of GLA at low growth temperature with a corresponding increase in the degree of unsaturation of total lipids (6).

The temperature did not only influence growth and lipid production; it also affected the cell morphology. At 38°C big fluffy pellets were formed (10 mm in diameter) during the first 24 h, and then the pellets were transformed into mycelia that tended to clump. The mycelium formation was avoided by running the cultures at 28°C and 12°C, where stable pellets were formed; the pellets decreased in diameter with decreasing temperature (0.5 mm and 0.3 mm, respectively). This minute size of pellets would give a great advantage in the large scale and high-density cultivation of fungi when the mass transfers problems of filamentous fungal growth.

The Fig. 2 shows the time course of the main fatty acids contents in the total lipid fraction of *Mucor* sp LB-54 grown at 12, 28 and 35°C. The major fatty acids common to three growth temperatures and incubation periods were palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2) and GLA (18:3). A small part, about 2 – 4 % of the lipid fraction consisted of other fatty acids, myristic acid (C14:0), palmitoleic acid (C16:1) and stearic acid (18:0). The analysis of fatty acids revealed an insignificant content of fatty acids with an odd number of carbon atoms (< 1 %) and the complete absence of the alfa –isomer (alfa linolenic acid). It is also evident that at the lower temperature there was an increase in the amount of linoleic acid and GLA with a corresponding decrease palmitic acid and oleic acid. A temperature decrease to 12°C resulted in an increase in percent composition of GLA for 24 % of total fatty acids. The percent composition of other fatty acids did not vary appreciably under the influence of different temperatures.

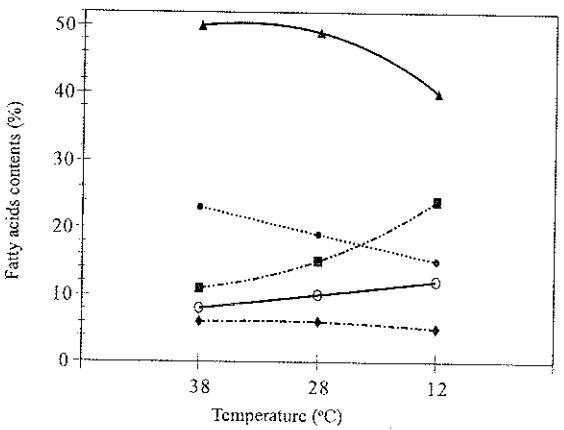

Figure 2. Changes the main fatty acids contents in lipid produced by *Mucor* sp LB-54 as a function of growth temperature. The fatty acids contents were expressed relative percentage of total fatty acids. Palmitic acid (16:0) = ●----●, Stearic acid (18:0) = ◆----◆, Oleic acid (18:1) = ▲---▲, Linoleic acid (18:2) = o—o, γ-Linolenic acid (18:3) = ■----■. Each point is the mean of three repetitions.

Table 1. Effect of growth temperature on total lipid, biomass contents and production of GLA by *Mucor* sp LB-54.

Temperature (°C)	Biomass (g/l)	Lipid (g/l)	Total lipid/dry weight (%w/w)	GLA/total fattyacids (%w/w)	GLA (mg/l)
12	2.47	0.39	15.84	24.02	23.40
28	5.83	1.21	20.73	14.68	43.97
38	4.29	0.49	11.42	11.66	22.90

The results of this study show that the lower growth temperatures simulate the biosynthesis of highly unsaturated fatty acids, a phenomenon that has already been observed for some mesophilic and psychrophilic *Mucor* species (22). The effects of temperature on the degree of lipid unsaturation may be exerted through the influence of temperature on oxygen tension of the media. Oxygen is a necessary cofactor in enzymatic desaturation, resulting in lower levels of unsaturated fatty acids as temperature increases (15). The conversion of saturated into unsaturated fatty is known to be regulated by desaturase enzymes which require oxygen as a cofactor together with acetyl coenzyme A (acetyl CoA), acyl carrier protein (ACP), reduced nicotinamide adenine dinucleotide ($NADH_2$) and reduced nicotinamide adenine dinucleotide phosphate ($NADPH_2$) (13). Accumulation of unsaturated fatty acids at low temperature could to be a resources used for the increased biosynthesis of GLA by *Mucor* sp LB-54.

To confirm the GLA peak obtained from the cellular lipids of *Mucor* sp LB-54, a mass spectrometric analysis was applied. Fig. 3 shows a molecular ion peak at m/e 292 and intense fragment ion peak at m/e 93, 79, 67 and 41. Each peak is in good accord with the corresponding one of the authentic standard.

In order to combine the beneficial effects of rapid biomass production and a high production of GLA by *Mucor* sp LB-54, we tested the effect at two temperature combinations associated additional of glucose. Table 2 shows the results of the five culture conditions in shaker culture on total lipid, biomass contents and GLA production of *Mucor* sp LB-54.

When the culture was started at 28°C and the temperature was changed to 12°C, the proportions of GLA of total fatty acids increased (15.90 %), moreover, occurs the depletion tendency of lipid with culture time (Condition 2). This agree with the others reported (14, 23) indicating that the accumulated lipids are used as a carbon and energy supply by fungi when glucose to become exhaustion.

On the other hand, cultures with glucose added was possible to achieve both a high final lipid content and a reasonably good production of GLA by *Mucor* sp LB-54. Optimal production of GLA was obtained by incubating first for 5 days at 28°C in a basal

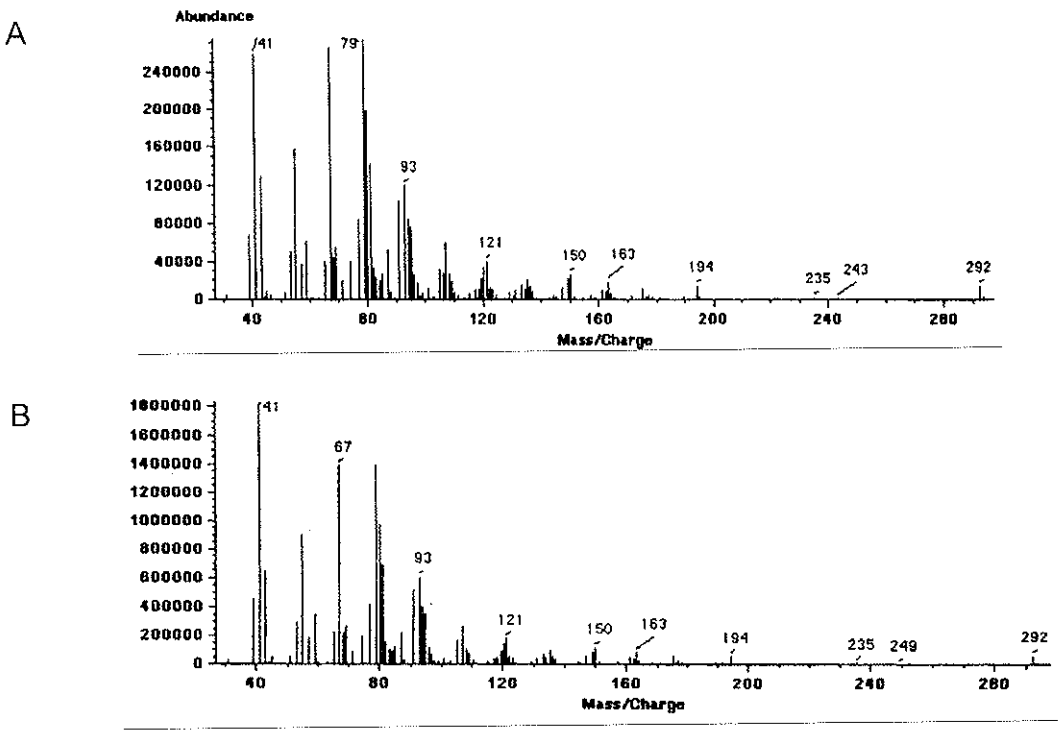

Figure 3. Mass spectra of authentic standards of GLA (A) and C18:3 acid methyl ester from cellular lipids of strain *Mucor* sp LB-54 (B).

Table 2. Effect of growth temperature and glucose addition on total lipid, biomass contents and production of GLA by *Mucor* sp LB-54

Incubation conditions	Biomass (g/l)	Lipid (g/l)	Total lipid/ dry weight (%w/w)	GLA/ total fatty acids (%w/w)	GLA (mg/l)
1	5.83	1.21	20.73	14.68	43.97
2	6.03	0.96	15.71	15.90	37.34
3	5.47	1.28	23.47	17.17	54.69
4	6.27	1.57	25.07	19.40	74.10
5	6.33	1.22	19.15	18.35	55.33

1: 5 days at 28°C in basal medium
2: 5 days at 28°C in basal medium following incubation for 3 days at 12°C (not addition glucose)
3: 5 days at 28°C in basal medium following glucose addition (3% w/v) and incubation for 3 days at 12°C.
4: 5 days at 28°C in basal medium following glucose addition (7% w/v) and incubation for 3 days at 12°C.
5: 5 days at 28°C in basal medium following glucose addition (10% w/v) and incubation for 3 days at 12°C.

medium and then supplementing with extra glucose (7 % w/v) followed by additional incubation for 3 days at 12°C (Condition 3). Using a relatively high initial growth temperature for biomass production, and glucose feeding followed by a temperature shift to 12°C, GLA production by *Mucor* sp LB-54 was enhanced to 74 mg of GLA per liter of culture medium, corresponding to 1.7 fold enrichment of GLA. This is mainly due GLA content of 19.40 % of the total fatty acids associated the increased to the content of lipid 25.07 % in dry cell which is higher than the baseline cultures (Condition 1).

Certain species of *Mucor* are capable of producing relatively large quantities of GLA. For example, *Mucor circinelloides* grown at 30°C with acetic acid as carbon source produced 90 - 120mg GLA l^{-1} (18) and strain of *Mucor* sp KCTC 8405P isolated in Korea cultured on 3% glucose and 0.1% $(NH_4)_2SO_4$ produced about 14 % (w/w) of GLA in total lipids (12).

With this strategy, we were able to stimulate GLA production by of *Mucor* sp LB-54 from the baseline level of about 44 mg l^{-1} to 74 mg l^{-1}. The data presented in this paper show the significant influence which incubation temperature and supply glucose exerts on the GLA production by *Mucor* sp LB-54. Presumably, it is not the temperature, as such, that affects the unsaturated fatty acids but the solubility of O_2, which increases at decreasing temperatures (13). These results suggest that *Mucor* sp LB-54 may have potential for commercial development for the production of GLA by fermentation techniques.

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RESUMO

Aumento da produção de ácido gama linolênico por fungo *Mucor* sp LB-54 de acordo com a temperatura de cultivo

A linhagem de *Mucor* sp LB-54, considerada uma potencial produtora de ácido gama-linolênico (GLA), foi selecionada para o estudo de diferentes temperaturas de cultivo em agitador rotativo. A linhagem usada neste experimento era capaz de acumular uma quantidade alta de lipídeos intracelulares, 20,73 % do peso seco de biomassa e conteúdo de GLA de 15 % dentre os ácidos graxos totais de sua constituição após 5 dias de incubação à 28°C. Quando a temperatura de cultivo foi diminuída de 28°C para 12°C, o conteúdo de GLA aumentou de 15 para 24% dentre os ácidos graxos totais de sua constituição. Com o objetivo de otimizar as condições de cultivo para a produção rápida de biomassa e produção de lipídeos contendo conteúdo alto de GLA, o fungo foi cultivado em duas combinações de temperaturas associadas com a suplementação de fonte de carbono (glicose). A produção máxima de GLA (74mg/l) pela linhagem de *Mucor* sp LB-54 foi obtida após 5 dias de incubação à 28°C em meio base, seguida da adição de glicose (7% p/v) no meio de cultura e uma posterior incubação por mais 3 dias a 12°C. A identidade do GLA foi confirmada pelo sistema acoplado cromatógrafo à gás – espectrômetro de massa.

Palavras-chave: ácido gama-linolémico, *Mucor* sp, ácidos graxos insaturados

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