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Short communication

Isolation and characterization of a symbiotic cellulolytic mixed bacterial culture

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Summary. By using batch-culture enrichment techniques a mixed culture of two bacterial species identified as *Cellulomonas flavigena* and *Xanthomonas sp* was isolated. The capacity of both bacteria to grow as pure cultures in a mineral medium with alkaline pretreated sugar cane bagasse or cellobiose was tested. *C. flavigena* as pure culture was able to grow on both substrates only when yeast extract or biotin and thiamine were added to the culture medium, while *Xanthomonas sp.* could not grow on sugar cane bagasse, but assimilated cellobiose if yeast extract was supplied. However, both bacteria in mixed culture grew very well on both substrates and did not require any growth factor. It was concluded that the interaction was favourable to both species. The mixed culture had the capacity to degrade a number of different agricultural wastes and to use them as the sole carbon and energy source for the production mainly of biomass. More than 80% of pineapple bagasse, without chemical pretreatment, was used up by the microbial system.

Introduction

Many research groups all over the world, are working on the hydrolysis of cellulosic wastes to produce SCP, ethanol, glucose, biogas, liquid fuels, etc. (Moo-Young and Campbell, 1981). Some attempts have been made to use mixed cultures in order to increase the biomass productivity and enzyme production of the cellulose fermentations (Peitersen 1975; Callihan and Dunlap 1971; Kristensen 1978; Perotti de Galvez and Molina 1981; Rolz and Humphrey 1982). However, most of these mixed cultures were produced in the laboratory by mixing two strains that were isolated as pure cultures. Natural populations, i.e. mixed cultures isolated as such, could be advantageous, because the microorganisms must already have been naturally selected and must interact to survive. In other words, these are metabolically coupled systems (Harrison and Wreng 1977) and therefore have better stability characteristics than systems in which the patners do not have complementary metabolisms. Besides their good stability characteristics, mixed cultures are a way of overcoming feed back regulation and catabolic repression, since the products of one commensal are substrates for the others commensals.

When enrichment techniques are used to isolate microorganism, either in batch or continuous culture, mixed populations are frequently obtained; these techniques could therefore be useful for isolating metabolically coupled microbial systems.

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Materials and Methods

Culture media. For the isolation and screening experiments the mineral medium used was the mineral solution described by Han and Srinivasan (1968); to this, 0.5 or 1% filter paper (Whatman # 1), carboxy-methylcelullose (CMC) (technical grade), cellobiose (Calbiochem) or cellulosic residues were added as carbon sources and yeast extract was omitted. In all the other experiments amonium sulphate was substituted by ammonium phosphate. For solid media 2% agar (Difco)was added and CMC was used as carbon source.

Culture conditions. The cultivations were car

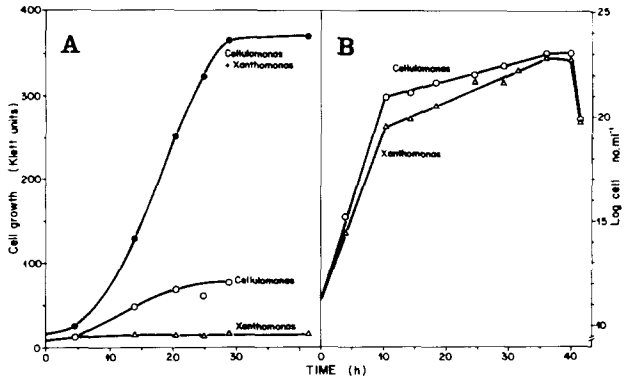

Fig. 1A and B; A Growth of *Cellulomonas flavigena* and *Xanthomonas sp.* as pure cultures or in mixed culture on sugar cane bagasse; B Time course of *Cellulomonas flavigena* and *Xanthomonas sp.* growing in mixed culture on sugar cane bagasse

with those of the first screening experiments that pointed out the higher cellulolytic capacity of the culture R-1b and show that the protein productivity of the mixed culture is higher too. The mixed culture was selected for further experiments. The screening method developed by Poincelot and Day (1971) for cellulolytic fungi could be successfully applied to bacteria The only constraint found was the oxygen transfer rate but it was overcome by using shaken flasks.

Interaction between the two strains that Integrated the Mixed Culture

The two strains integrating the mixed culture were characterized as *Cellulomonas flavigena* and *Xanthomonas sp.* (de la Torre 1981). No species was assigned to the *Xanthomonas* isolate because the species of this genus can only be distinguished with certainty by plant-host reactions and the phytopathogenicity is lost when the bacteria are kept in artificial media (Dye 1962). Some experiments were carried out in order to compare the behavior or the mixed culture with that of the pure cultures. *C. flavigena* and *Xanthomonas sp.* as pure or in mixed culture were grown in the mineral medium (ammonium sulphate was substituyed by ammonium diphosphate) and 1% of pretreated sugar cane bagasse at 37°C. The inocula were grown in brain-heart infusion. *Xanthomonas sp.* was not able to grow in this medium, while the maximum growth reached by *C. flavigena* was only 19.1% of that one of the mixed culture and its specific growth rate was 1.3 times lover (Fig. 1A). When an aliquot of the culture of *C. flavigena* was transferred to the same medium, no growth was obtained. It was concluded that in the experiment shown on Fig. 1A the growth of *C. flavigena* was due to the growth factors contained in the inoculum. In another experiments it was found that the *Cellulomonas* isolate requires biotin and thiamine.

The *Xanthomonas* strain could grow in the mineral medium with cellobiose as only carbon source, but it was necessary to add 0.1% yeast extract. Further experiments are carried out to stablish which are the growth factors required by this strain.

Whereas both strains as pure cultures required growth factors, the mixed culture did not require any growth factor and could grow very well in the mineral medium with cellobiose or pretreated sugar cane bagasse as only carbon and energy source. Even in the mineral medium with cellobiose and 0.1% yeast extract, the specific growth rate of the mixed culture was higher than those of the pure cultures of *C. flavigena* or *Xanthomonas*, the specific growth rates were 0.28, 0.21 and 0.19 per hour. In order to determine the growth kinetics of the individual strains when *C. flavigena* and *Xanthomonas* grow in mixed culture, they were grown in the mineral medium with 1% pretreated sugar cane bagasse and the growth was followed by viable count (Fig. 1B). Both strains grew simultaneously with similar specific growth rates. Two exponential growth phases were observed. A two-phase growth kinetics for a *Cellulomonas* strain growing on sugar cane bagasse has already been reported by Enriquez et al. (1981). They determined that during the first exponential phase the most accessible cellulose fractions were used and during the second,the fractions with a

ried out in shaken flasks with a working volume of 20% of the total volumen. The incubation temperature was 28° or 37°C and the initial pH was adjusted to 7.3 with phosphoric acid. Inocula were usually prepared in the culture medium to be tested, and an inoculum size of 10% of the working volume was utilized. In experiments in which different cultures or media were compared, the optical density of the inocula was adjusted to the same value.

Isolation. Soil samples were mixed with small pieces of filter paper and the humidity was adjusted to 60%-70% of its saturation capacity. The mixtures were incubated at 28°C for one week then decimal dilutions ($1:10^4$ to $1:10^7$) were made and aliquots were seeded in Erlenmeyer flasks with the mineral solution and filter paper. The flasks were incubated on a rotatory shaker at 28°C. When growth was visually detected, and aliquot was aseptically transferred to the same culture medium. After six transfers, samples of the cultures were streaked on CMC plates, nutritive-agar plates (Bioxon) and CMC-slants. The cellulolytic capacity of the isolated cultures was tested by inoculating them on the surface of a filter paper strip standing in a test tube with the mineral medium. The test was considered negative if after one week of incubation at 28°C, the paper was not partially degraded as indicated by spread after slight agitation.

Culture maintenance. The cultures were maintained on CMC-slants at 4°C with monthly transfers.

Screening. The screening was carried out as described by Poincelot and Day (1971) utilizing shaken flasks instead of test tubes in order to have higher oxygen transfer rates. During the assays, the microorganisms were growing in the mineral medium with 1% of dyed filter paper as the carbon source. As the paper was degraded, the dye (Remazol brillian blue, Calbiochem) was released to the broth. The absorbance of the previously centrifuged samples was read in a Coleman spectrophotometer Perkin-Elmer 35 at 595 nm.

Alkaline pretreatment. The lignocellulosic residues were pretreated with sodium hydroxide in order to increase their digestibility. The pretreatment conditions were those used by Han and Srinivasan (1968).

Microbial growth. The samples were filtered through filter paper (Whatman # 1) to eliminate residual cellulose. The filtered cell suspension was used to determine insoluble proteins by Lowry's method (Lowry et al. 1951), dry weight or turbidity (Klett colorimeter with red filter). The cell mass adsorbed to the residual substrate was considered negligible.

Viable count. Aliquots withdrawn from the culture were inmediately diluted and 0.1 ml of the appropriate dilutions were spread with a glass spatula on the surface of brain-heart plates (Bioxon). The plates were incubated at 37°C for 48 h. *Cellulomonas flavigena* colonies were pale yellow and *Xanthomonas sp.* colonies had an intense yellow colour.

Residual substrate. Samples were filtered through preweighted filter paper (Whatman # 1). The residue was washed three times with distilled water and dried to constant weight in an oven at 100°C.

Results and Discussion

Isolation and Screening

Twenty two cultures of prototrophic bacteria were isolated. Since most of them degraded poorly the dyed filter paper used as carbon source, it was concluded that the great majority of the isolated cultures had a low cellulolytic capacity and only the two cultures with the hightes capacity were selected for further experiments. The strain named p-9c was a culture of Gram-negative rods and R-1b was a mixed culture of Gram-positive and Gram-negative rods In order to find out which of the selected cultures had the higher biomass productivity, they were grown in shaken flasks with the mineral medium and 0.5% of pretreated sugar cane bagasse. Both cultures had a protein yield of 0.18 g protein/g consumed substrate, gave a maximal protein concentration of 0.35 g/l and hydrolised 38% of the initial sugar cane bagasse, but had different growth rates. The specific growth rate of R-1b was twice that of p-9c and therefore the protein productivity of the former was higher.

The effect of several nitrogen sources on the fermentation parameters on sugar cane bagasse was assessed with both cultures. The ammonium sulphate of the original medium was substituted by ammonium diphosphate or ammonium nitrate. The media containing ammonium sulphate or ammonium diphosphate were adjusted to equivalent nitrogen concentration (0.42 g/l) and that with ammonium nitrate had 0.7 g/l. These changes had almost no effect on the fermentation parameters of the strain p-9c, but for R-1b the total protein produced was 2.4 times higher with ammonium diphosphate. In addition, the protein yield was increased from 0.18 (g protein/g consumed substrate) to 0.31 and the consumed substrate from 32.6% to 58% of the initial substrate. These results agreed well

Table 1. Utilization of different cellulosic residues[a] by the mixed culture

Substrate	Biomass g/l	Degraded substrate %
Sugar cane bagasse (whole)	1.3	48.7
Sugar cane bagasse (pith)	1.6	65.6
Pineapple bagasse	1.9	84.3
Wheat straw	1.6	65.9
Corn stalks	1.7	66.8
Rice husks	0.8	32.4
Cofee husks	0.4	15.6
Coconut shells	0.5	18.8
Guayule bagasse	0.3	19.1

[a]The substrates underwent alkaline pretreatment with exception of the pineapple bagasse. The substrate concentration was 0.5% and the biomass was determined after 48 h of incubation at 37°C

higher cristallinity degree were hydrolysed. We found that the mixed culture had a high xylanase activity (de la Torre 1981), therefore it might be that during the first growth phase the hemicellulose fractions are used by the mixed culture. Studies are carried out to determine precisely which fractions of the substrate are used up at each phase.

The results obtained indicate that the interaction is necessary for the survival of both bacteria growing on cellulosic substrates without exogenous growth factors and has positive effects on both populations. Therefore, the mechanism of the interaction could be characterized as mutualism according to the definition of Fredrickson (1977) and the mixed culture is a coupled metabolic system (Harrison 1978). On cellobiose plus yeast extract, the interaction was not obligatory for the survival of the strains but it was beneficial to both partners; consequently, the interaction mechanisms might be protocooperation (Fredrickson 1977). This implies that the interaction system between *Cellulomonas flavigena* and *Xanthomonas sp.* could be modified by changing the composition of the culture media. It is known that the nature of the interaction between two or more microbial populations depends on the culture conditions. Tseng et al (1981)demonstrated that in the *Proteus vulgaris - Saccharomyces cerevisiae* system the interaction mechanism depends on the culture medium and can be characterized as comensalism, competition or neutralism.

Utilization of Different Cellulosic Residues

The mixed culture was able to use a great variety of cellulosic residues as sole carbon and energy source (Table 1). Even residues that are hardly degraded, like rice husks and coconut shells, were utilized. In the case of residues that were to a limited extent degraded, efficiency might be increased if an appropriate pretreatment was applied. The pretreatment could be a combination of different chemical treatments to extract the compounds that block the access of cellulases to cellulose. For example, in the specific case of the guayule bagasse it was necessary to extract the latex residues and swell the fibers, so that a single pretreatment with NaOH was not enough.
It is noteworthy that the mixed culture used 84.3% of pineapple bagasse that had not been pretreated. This indicates that some agricultural residues could be directly utilized.
Unfortunatelly there are few reports about the isolation of mixed cultures and the interaction mechanisms among the species than integrate them. Most of these studies are related to the isolation of cultures by continuous enrichment for the SCP production from methane or methanol In any case, symbiotic systems were frequently obtained (Harrison 1978). Molina (1980) isolated a mixed culture of *Cellulomonas sp.* and *Bacillus subtilis* for SCP production on bagasse pith but he did not study the interaction. Callihan and Dunlap(1971) studied the SCP production on sugar cane bagasse by *Cellulomonas sp.* and a mixed culture of this strain and *Alcaligenes faecalis* but the interaction mechanism was not established. Later, Han (1982)investigated the mixed culture of *Cellulomonas sp.* and *A. faecalis* and combinations of the *Cellulomonas* strain with yeasts that metabolize cellobiose. He found that the combination of *Cellulomonas* and *A. faecalis* produced almost six times as much as cell mass as *Cellulomonas* alone, while the effect with the yeasts was less pronounced. The mechanism of the symbiotic effect has not been fully elucidated. He proposed that the removal of cellobiose from the culture medium by *A. faecalis* increases the production of cellulases, and consequently increases the cell mass of both bacteria. However, the interaction might be more complex.

All the above mentioned reports and our results support the hypothesis that mixed cultures can be advantageous for some fermentation processes, especially for those where complex substrates are metabolized i.e. lignocellulosic residues. In addition, our results and

those obtained by Harrison (1978) agree with the statement that metabolically coupled mixed cultures can be isolated by enrichment techniques.

The isolated mixed culture is an interesting system to study microbial interactions and seems to be promising for the SCP production on cellulosic residues. Nevertheless, more studies are needed to assess its stability under different conditions, since stability is the key for using a mixed culture in industrial processes. Mateles (1980) mentioned in his discussion on the use of mixed culures in SCP processes, that if the nutritional and toxicological characteristics of the biomass produced by a mixed culture are kept relativily constant and the nutritional and toxicological test are satisfactory, it should not be inconvenient to use the biomass produced by well-characterized mixed cultures for animal feeding.

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