«BACILLUS Spp IN SUGAR CANE FERMENTATION MEDIA»

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Bacterial activities are known to be related to rum technology. Most high-grade rums (600-1000 gr/hl AP) are made by the mixed fermentation associated with **Shizosaccharomyces pombe** and various other bacteria species. A certain number of works (Hall, 1935 : Allan, 1939 ; Mayeux 1960 ; Bevan and Bond, 1971) concerning the participation of bacterial activity in rum's manufacture have been reported, However, an exhaustive study on the different species occurring in fermentation media, is not known.

The study on rum-associated bacteria is specially interesting in the French Antilles, where the production is mostly based on sugar cane juice, the aromatic characteristics of such juice are suitable for bacterial growth. We have been concerned with the analysis of the bacterial activities present in sugar cane fermentation media (Ganou, 1984), and the present work provide evidences for the occurrence of species of the Bacillus genus as a constitutive part of such activity.

MATERIALS AND METHODS

Sampling was made at different stages during rum elaboration ; raw materials consisted of sugar cane stalks, workrooms for milling and fermentation.
Following a thermic shock, thermolabile bacterias were discarded and the bacterial suspension diluted.
The inoculum was smeared upon a nutritive agar (3g/l meat extract, 5g/l peptone, 15g/l agar).
Culture media were adjusted to pH 6,6 and sterilized for 20 min at 120°C. In some cases, 5mg/l of manganese sulphate were added in order to favor sporulation.

Taxonomic studies on Bacillus strains were carried out according to Bergey (8th edition) and Starr et al. (1981).

Different media were used for studying the biochemical activity of Bacillus strains.

a) Semi-synthetic medium for acidification studies, consisting of ; 1g/l $(NH_4)\ 2HPO_4$, 0,2 g/l KCl, 0,2 g/l $MgSO_4$, $7H_20$, 0,2g/l yeast extract, 15g/l agar and 20 ml of an aqueous solution (0,004%) of bromocresol purple red. This medium was completed with 18 different sources of carbon at a concentration of 0,5% and sterilized for 20 min at 120°C, pH was 7,0.

b) Fermentation medium.
   — M63 medium ; 13,6 g/l KH_2PO_4, 2g/l $(NH_4)2\ SO_4$. 0,2 g/l $MgSO_4$, $7H_20$, 0,5 mg/l $FeSO_4$, $7H_2O$.
   Carbon sources were either glucose, saccharose or glycerol at a concentration of 10 g/l. pH was 6,5 and sterilizing conditions were the same used above. Germ's development was verified on a control medium where 10 g/l of yeast extract were used as source of carbon.
   — M63 + ethanol medium, used for studying the utilization of ethanol as a source of carbon.
   — Sugar cane juice medium. Fresh sugar cane juice (pH 5,3) at two different concentrations of total sugar (10g/l and 85g/l) were sterilized at 115°C for 20 min. Certain assays required a 40 hour, prefermentation by mean of a **Saccharomyces cerevisiae** strain (seeding ratio, 0,5 g/l).
   Chromatographic methods for the analysis of higher alcohols and fatty acids have been previously described (Ganou-Parfait 1984).

EXPERIMENTAL RESULTS

The occurrence of Bacillus has not been reported by Mayeux (1960) in sugar cane galeries hollowed by the Borer, and we have only found it exceptionally.
Various strains are present during grinding and their frequency increases when filling the tuns and the begining of fermentation. Howewer, the fermentation-associated anaerobiosis enables their exhaustion.
Bacillus are also found in fermented products when late distillation has taken place, producing a veil appearance on the surface of wines. In fact these «veils» contain many spores. We have demonstrated the consumption of ethanol by all strains, a fact that can explain the occurence of such strains on wines that have been stored for long periods before distillation. It is worth noting, that at this stage, Bacillus can provoke a large loss of alcohol. Bacillus have also been isolated from slopes.

The Bacillus strains that we have studied consist of well visible egg-shaped spores, resistant to 60°C for 30 min and to 80°C for 10 with an optimal growing temperature near 37°C, optimal pH around 6-6,5 and good resistance to ethanol at around 10°GL.
Respiration is aerobic with catalase production.
The presence of sodium thioglycolate in the glucose-containing medium still allows acidification to take place, with (F2 strain) or without gas (B_2).
However, bacterial growth is totally prevented at strict anaerobiosis.

Proteolytic activity is evident (gelatin and casein are both attacked) but no attack is observed on phenylalanine or tyrosine. No production of indole and H_2S is recorded. Urea is neither produced.
Table 1 summarizes the main features of the three strains representing the most frequent biological types.

TABLE 1
Main features of the three strains

Strains	B_2	F_2	M_{21}
Origin (0)	Acid wine of «agricole» distillery	Wine of «agricole» distillery	Canes hollowed by Borer
Colonies (C)	White, plates, irregular edges	Brown, mucous plates with irregular edges	White, mucous plates
Microscope examination (ME)	0,9 x 1,5 µm restless and non-destorted central spores	0,8 x 3 µm non-destorted sub-terminal spores with little mobility	1 x 4,2 destorted and notionless sub-terminal spores
Lysozyme resistance (LR)	LR below 11,250 units	+	Below 6750 units
Peroxydase	+	+	—
Oxydase	—	+	+
Nitrate reduction	—	+	—
Sodium hippurate test	+	+	—
Acetoine	—	+	—
Methyl red test	—	—	—
Growing response on lactate	+	+	—
Starch hydrolysis	+	+	—
Aesculine hydrolysis	—	+	—
Sodium azide resistance	—	+	—
Growing response in 7% Nacl	+	—	+
Growing response at 50°C	—	+	—
NaF resistance	—	+	+

F_2 strain presents acidification when cultured on glucose medium as well as on various carbonated substrates. This strain would appear to have some relation to **Bacillus subtilis.**

B_2 strain is able to metabolize various carbon-containing substrates, with acidification. It would appear that this strain is related to **Bacillus megaterium.**

M_{21} strain fails to produce acid or gas when cultured on glucides. This strain would be related to **Bacillus sphaericus.**

These three biological types are most frequent in fermentation tuns. By contrast, **Bacillus cereus** has only been found in sugar canes when harvesting and at grinding, just before anaerobiosis has taken place. All three strains fail to develop at stric anaerobiosis. When cultured in tubes containing liquid medium, no acidification is observed after aeration.
However, in sloped agar tubes, where aeration is assured, acidification does occur (Table 2).

TABLE 2
Acidification test on carbon substrates in sloped agar

Tested Sugars u/s strains	B_2		F_2		M_{21}	
Glucose	+	+	+	+	—	—
Saccharose	+	+	+	+	—	—
Fructose	+	+	+	+	—	—
Maltose	+	+	+	+	—	—
Lactose	—	—	+	+	—	—
Arabinose	—	—	+	+	—	—
Galactose	+	—	+	+	—	—
Rhamnose	—	—	+	—	—	—
Mannose	+	+	—	—	—	—
Raffinose	+	+	+	—	—	—
Solicine	+	+	+	—	—	—
Xylose	—	—	+	+	—	—
Adonitol	—	—	+	+	—	—
Dulcitol	—	—	—	—	—	—
Glycérol	+	+	+	+	—	—
Inositol	—	—	+	—	—	—
Mannitol	—	—	+	+	—	—
Starch	+	+	+	+	—	—

+ + Total acidification
— — No acidification
+ — Only surface acidification

All three strains are able to produce small-chain fatty acids (acetic, propionic, isobutyric, butyric, isovaleric, valeric, caproîc, and ethyl 2-methyl 3-butyric acids) and high alcohols (propanol, isobutanol, 1-butanol, 2-butanol, isopentanol).

In addition, it seems that they can also metabolize at least some of these products, as it occurs with ethanol.

DISCUSSION AND CONCLUSION

Evidences are presented for the occurence of Bacillus species among the bacterial flora of fermentation media in rum distillery. The absence of **Bacillus thuringiensis** is due to the the lack of utilization of such strain for biological lutte in the French Antilles. Other Bacillus known to be pathogenic for parasite insects of the sugar cane (Krieg, 1981) are neither present.
Bacillus cereus is only found in the vegetal material grinder before fermentation. The most relevant species are : **B. subtilis, B. sphaericus** and **B megaterium.** Not many Bacillus are present during the anaerobic phase of fermentation. The resistance to ethanol is a remarkable feature among these species.
B_2 is more sensitive to sodium fluorure that the others, since it comes from a factory where this antiseptic is never used.

According to our results, we can evaluate the effect of Bacillus on rum technology.

The fermentation procedures used in French Antilles for the manufacture of rum, are based on the use of both bacteria and yeast. Although Bacillus strains consume fairly large amounts of ethanol, we could not assure they are the sole to account for the low efficiency observed in «agricole» distillery.

They are also related to the formation and evolution of higher alcohols and fatty acids of fermented products.

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ABSTRACT

The occurence of Bacillus Spp. in Sugar Cane Juices for «agricole» Rum production is reported.
Four species are described : **B. cereus, B. megaterium, B. subtilis** *and* **B. sphaericus,** *as well as their influence upon rum technology.*
The consumption of ethanol is pointed out as a more relevant factor, than the production of volatile fatty acids. Bacillus spp. are not the sole to account for bacterial activity in rum industry.

SAMENVATTING

De aanwezigheid van Bacillus Spp. in suikerriet-sap voor «landbouw»- rum productie wordt besproken.
Vier species worden beschreven : **B. cereus, B. megaterium, B. subtilis en B. sphaericus,** *alsook hun invloed op rum technologie.*
Het alcoholverbruik is wordt aangeduid als een relevantere factor dan de productie van vluchtige vetzuren. Bacilli spp. zijn niet de enige verantwoordelijken voor bacteriele activiteit in de rum industrie.

RESUME

Les Bacillus Spp sont présents dans les milieux fermentaires à base de jus de canne à sucre rentrant dans la production des rhums agricoles.
Les propriétés de 4 souches: **B. cereus, B. megaterium, B. subtilis, B. sphaericus** *sont étudiées sur la base de leur importance en technologie rhumière.*
Dans ces conditions l'utilisation de l'éthanol peut avoir des conséquences plus graves que la production d'acides gras volatils. Les Bacillus à eux seuls ne rendent pas compte de l'intensité de l'activité bactérienne en rhumerie.