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PAPER

OCCURRENCE OF WINE YEASTS ON GRAPES SUBJECTED TO DIFFERENT PESTICIDE TREATMENTS

PRESENZA DI LIEVITI VINARI SU UVE MARCHIGIANE SOTTOPOSTE A DIVERSI TRATTAMENTI ANTIPARASSITARI

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ABSTRACT

As a contribution to the study and preservation of indigenous wine yeast populations, we initiated a programme of isolation and characterization of yeast strains from grapes of the Italian region of Marche. During the 1996 vintage, grape samples were collected from three groups of vineyards differing in the pesticide treatments given. Of the 279 yeasts isolated, thirty-nine were assigned to the species *Saccharomyces cerevisiae*. The majority of the isolates and all thirty-nine S. *cerevisiae* came from vineyards which had not been treated with systemic pesticides, indi-

RIASSUNTO

Per contribuire allo studio e alla conservazione delle popolazioni indigene di lieviti vinari, che possono avere un importante ruolo nella determinazione delle caratteristiche organolettiche di molti vini di qualità, abbiamo iniziato un programma di isolamento e caratterizzazione di ceppi di lievito da uve delle Marche. Durante la vendemmia 1996 le uve sono state prelevate da tre gruppi di vigneti, sottoposti a diversi trattamenti antiparassitari. Dei 279 lieviti isolati trentanove sono stati assegnati alla specie *Saccharomyces cerevisiae*. È interessante notare che la

- Key words: biodiversity, fermentative capabilities, *Saccharomyces*, wine yeast isolation -

cating that these pesticides may exert a negative effect on the biodiversity of the grape microflora, and in particular on the occurrence of *S. cerevisiae* on grapes. The thirty-nine *S. cerevisiae* isolated were subjected to microfermentation trials, in which ten of them showed promising fermentative capabilities.

maggior parte dei lieviti, e tutti i *S. cerevisiae*, sono stati isolati da uve provenienti da vigneti che non hanno subito trattamenti con antiparassitari sistemici. Questi risultati suggeriscono che l'utilizzo di tali antiparassitari può avere un effetto negativo sulla biodiversità della microflora delle uve, ed in particolare sulla presenza di *S. cerevisiae* sulle stesse. I trentanove *S. cerevisiae* sono stati saggiati in prove di microfermentazione in beuta, nelle quali dieci isolati hanno dimostrato promettenti capacità fermentative.

INTRODUCTION

The organoleptic characteristics of wine are directly influenced not only by the grapes and the technologies utilized in the wine-making process, but also by the yeasts involved in the fermentation. In fact, within the *Saccharomyces cerevisiae* species, responsible for the major part of the alcoholic fermentation, different strains vary greatly in their production of compounds responsible for flavour and aroma development. This partly depends on the type of substrate and the process conditions, such as the temperature at which the fermentation is carried out (RANKINE, 1968; RIBÉREAU-GAYON, 1971; RADLER and SHÜTZ, 1982; FATICHENTI et al., 1984; HERRAIZ et al., 1990). Recent studies have also indicated that the activities of some non-*Saccharomyces* yeasts normally present in musts, particularly *Hanseniaspora uvarum* (*Kloeckera apiculata*) and *Candida stellata*, may positively influence the composition of wines (HERRAIZ et al., 1990; FLEET and HEARD, 1993; LEMA et al., 1996; CIANI and FERRARO, 1998), not only in natural, but also in inoculated fermentations, where pre-existing microflora have been shown to survive for longer periods of time than previously thought (FLEET et al., 1984; PARDO et al., 1989). Thus, it is not surprising that the activity of indigenous fermenting yeasts has been implicated in the expression of the typical organoleptic characteristics of local wines (LEMA et al., 1996).

An increasing proportion of wine production in Italy is based on the utilization of a small number of *S. cerevisiae* strains as starter cultures that have been mainly isolated in France from commonly used grapes such as Cabernet, Pinot, Chardonnay and Sauvignon Blanc. While these starters have undoubtedly valuable oenological characteristics and are able to ensure fast and reliable fermentation, they may be unable to fully develop the typical organoleptic characteristics of wines originating from different grapes grown in other areas. Moreover, the wide use of these commercial strains may reduce, and eventually eliminate, the autochthonous yeast populations that have been selected through years of traditional wine making.

While musts are known to be good sources of *S. cerevisiae* able to carry out alcoholic fermentation, it has been questioned if there are good fermenting *S. cerevisiae* strains on grapes. On the basis of the great difficulties in isolating *S. cerevisiae* from grape berries, MARTINI (1993), and MARTINI et al. (1996) concluded that this yeast is not a normal resident of grapes, but instead colonizes the winery environment, from which it passes into the must when the grapes are crushed. The opposite conclusion was drawn by TOROK et al. (1996) who used more elaborate isolation methods and PCR, classical genetic and electrophoretic karyotyping analyses to show that vineyards are the main source of *S. cerevisiae* yeasts in Northern California wineries.

As a contribution to the study and preservation of these indigenous populations, we started a three-year programme to isolate and characterize wine yeasts from some traditional wine-producing areas in the Italian region of Marche. The principal grapevine cultivars cultivated in these areas are Verdicchio, Montepulciano and Sangiovese which are protected by specific legislation and Pecorino and Passerina which are autochthonous varieties that are currently being evaluated by local wine producers.

The aims of the project are: i) to establish a collection of indigenous wine yeasts; ii) to select starter cultures among the indigenous yeasts isolated; iii) to investigate the occurrence of *S. cerevisiae* yeasts on grapes; iv) to evaluate the effect of pesticide treatments used in the vineyards.

In this paper we present the results from the first year of the isolation campaign from grapes, during which 279 yeasts were collected. Thirty-nine of these were assigned to the species *S. cerevisiae* and further characterized by means of microfermentation tests in order to identify potentially good fermenters.

MATERIALS AND METHODS

Media and growth conditions

The solid media initially used for isolation of the yeasts from grapes were YM (yeast extract 3 g/L, malt extract 3 g/L, peptone 5 g/L, glucose 10 g/L, agar 18 g/L) and WL Nutrient Agar (Oxoid). Chloramphenicol (to a final concentration of 100 mg/L) was added to both media after autoclaving to inhibit bacterial growth, and similarly, when required, dichloran (to a final concentration of 2 mg/L) was added to inhibit mould development (KING et al., 1979). WL is a differential medium which enhances the differences existing between different yeast genera (CAVAZZA et al., 1992). Having determined that the total microbial counts on these two media were not significantly different, WL was used throughout the isolation campaign. Chloramphenicol proved to be effective, with some bacterial growth being observed only rarely. On the other hand, moulds were inhibited only partially by dichloran, and in heavily contaminated samples (e.g. time 0 from grapes, see below) they rapidly overtook all other colonies.

The solid media used for yeast identification were: AA (Agar Acetate, sporulation medium containing potassium-acetate 9.8 g/L, yeast extract 2.5 g/L, glucose 1 g/L, agar 18 g/L, and adjusted to pH 8.4 with NaOH); CYM (YM with 20 g/L $CaCO_3$); YMD (YM with cycloheximide (Sigma Co.) added to a final concentration of 100 mg/L to the autoclaved medium from a filter sterilized aqueous stock solution 0.8 g/L stored at -20°C); AL (Lysine Medium, Oxoid); AN, AC, AE (glucose 20 g/L, Yeast Nitrogen Base w/o a.a. (Difco) 7 g/L, agar 18 g/L, and, respectively, $NaNO_3$ 400 mg/L, cadaverine 700 mg/L, or ethylamine 0.6 mL/L); Ma (D-mannitol 20 g/L, Yeast Nitrogen Base w/o a.a. (Difco) 7 g/L, agar 18 g/L). Growth at 37°C was assessed in liquid GYNB (glucose 20

g/L, Yeast Nitrogen Base w/o a.a. (Difco) 7 g/L, agar 18 g/L).

Unless otherwise stated, cultures were incubated at 28°C, with shaking (200 rev/min) for liquid cultures.

Isolation

Grapes from five different cultivars (Sangiovese, Montepulciano, Verdicchio bianco, Passerina, Pecorino) were collected from two groups of vineyards, group 1 and group 2, situated in the same area but differing in the pesticide treatments received. In addition, Verdicchio and Sangiovese grapes were collected from a vineyard that had been abandoned for six years (group 3). The pesticide treatments used in the three groups of vineyards are reported in Table 1. From each vineyard three bunches of grapes for each grapevine cultivar were collected in sterile bags during vintage, and transported in refrigerated containers (4°C) to the laboratory where the grapes were manually crushed in the same bag, and transferred to sterile 250 mL flasks within 24 h after collection. The flasks were shaken at room temperature for 60 min, after which time the first aliquot (time 0) was diluted and plated onto WL agar in duplicate (direct isolation). The flasks were then incubated statically at 25°C, and aliquots were diluted and plated after 2, 4 and 8 days (enrichment stages 1, 2 and 3).

Four colonies/plate showing the *Saccharomyces* morphology and two colonies/plate for each other morphology were collected from WL, and purified by repeated (at least twice) streaking to single colonies on YM plates. Long-term storage of the purified isolates was at –80°C as cellular suspensions in YM with

Table 1 - Pesticide treatments used in the vineyards before the 1996 vintage.

	Grapevine cultivar	Type and number of treatments	
Group 1	Montepulciano, Sangiovese, Pecorino, Passerina	Copper oxychloride	5
		Sulphur	4
		Myclobutanil	2
		Fenitrothion	1
	Verdicchio	Sulphur	10
		Copper sulphate	2
		Copper oxychloride	5
		Metalaxil	1
		Penconazole	1
		Vinclozolyn	2
		Fenitrothion	2
Group 2	Montepulciano, Sangiovese, Pecorino, Passerina	Copper Sulphate	7
		Sulphur	4
		Bacillus thuringiensis toxin	2
	Verdicchio	Sulphur	11
		Copper oxychloride	2
		Copper sulphate	4
		Bacillus thuringiensis toxin	2
Group 3	Sangiovese, Verdicchio	No treatments	

20% glycerol. Short-term storage for further characterization was on YM plates at 4°C; the isolates were reinoculated onto fresh plates every three months.

Phenotypic characterization

A tentative identification was performed on all the isolates on the basis of cell shape and division (observed in cultures freshly grown on YM, at 40x magnification), colony morphology on WL plates, and ability to grow on YMD and AL (CAVAZZA et al., 1992; BOULTON et al., 1996). Yeasts provisionally assigned to the genus *Saccharomyces* on the basis of these analyses were further identified according to KURTZMAN and FELL (1998), utilizing the dichotomic key proposed by BOULTON et al. (1996) for wine-related yeasts. To discriminate between the species within this class, physiological tests were performed utilizing the *S. cerevisiae* CBS422 and CBS1171, *S. paradoxus* CBS5829, *S. bayanus* CBS380 and *S. pastorianus* CBS1260 strains as controls.

The assimilation tests were conducted by replica plating on the appropriate solid media, and, as controls, on the same media without the carbon or nitrogen sources to be tested.

Microfermentations

Microfermentations were performed in 100 mL flasks stoppered with sulphuric acid containing valves to allow only CO_2 to escape from the system (CIANI and ROSINI, 1987). Each flask was filled with 70 mL of steam-treated (90°C for 30 min) Pinot must, pH 3.10, sugar content of 18.5% (w/v), supplemented with sucrose to a final concentration of 27% (w/v). The must was inoculated with 5% (v/v) preinoculum grown in the same medium at 25°C without shaking for 48 h. The flasks were then incubated at 25°C without shaking, and weighed every day until no variations for two consecutive days were measured (end of fermentation). A *S. cerevisiae* strain (ScCG96) used as a fermentation starter by local wineries was included as control.

RESULTS AND DISCUSSION

Isolation of indigenous yeasts

In the course of the isolation campaign conducted during the 1996 vintage, 279 indigenous yeasts were isolated from Verdicchio, Sangiovese, Montepulciano, Pecorino and Passerina grapes from the Italian region of Marche. These isolates were initially ascribed to 8 classes, on the basis of shape and division pattern of vegetative cells, appearance of colonies on WL, resistance to cycloheximide, and ability to assimilate lysine (Table 2).

According to the simplified scheme proposed by CAVAZZA et al. (1992) and the wine-related yeast descriptions of BOULTON et al. (1996), the isolates belonging to classes A-F were provisionally identified as follows: class A, *Hanseniaspora/Kloeckera* (29.75% of the total yeasts isolated); class B, *Saccharomyces* (15.1%); class C, *Dekkera-Brettanomyces* (16.1%); class D, *Metschnikowia pulcherrima* (13.6%); class E, *Saccharomycodes ludwigii* (3,9%); class F, *Schizosaccharomyces* (1.4%). Classes G and H are likely to contain other wine-related genera, such as *Debaryomyces*, *Cryptococcus*, *Candida*, *Pichia*, *Kluyveromyces*, *Zygosaccharomyces*.

The great majority of the isolates included non-*Saccharomyces* yeasts. Among these, classes A and D were relatively well represented, as expected from the available ecological data which indicate apiculate yeasts and the low ethanol tolerant species *M. pulcherrima* as the dominant flora of grapes (ROSINI et al., 1982; FLEET *et al.*, 1984; PARDO *et al.*, 1989; MARTINI, 1993). Class B, which included colonies ascribed to the genus

Table 2 - Classification of the 279 isolates on the basis of morphological and physiological characteristics.

Character	Classes							
	A (83)	B (42)	C (45)	D (38)	E (11)	F (4)	G (18)	H (38)
Cell shape	a	e	o	e/s	a	ele	e/s	e/s
Cell division	b	m	m	m	b	f	m	m
Colony morphology on WL	green, flat	white/ light green, elevated	white	white, producing a reddish/brown pigment	green	green, small	green	white/ light green,
Cycloheximide resistance	+	-	+	-	-	-	+	-
Lysine assimilation	+	-	+	+	+	+	+	+

Values in parentheses refer to the number of strains belonging to each class.
+ and - indicate the presence or absence of the character considered;
e/s, elliptical or spherical; a, apiculate; e, elliptical; o, small cells, sometimes ogival; ele, elongated;
b, bipolar; m, multipolar; f, fission.
See text for the presumptive attribution of the classes to genera.

Saccharomyces, had forty-two isolates and was unexpectedly numerous. This could be due to the isolation procedure used, where four colonies presenting the *Saccharomyces* morphology were chosen against two for any other morphology (see Materials and Methods).

Table 3 shows that the majority of the isolates came from vineyards belonging to group 2 (47.3% of the total yeasts isolated), while only seventy-seven (27.6%) and seventy of them (25.1% of the total) were isolated from groups 3 and 1 vineyards. Moreover, *Saccharomyces* yeasts were totally absent from vineyards belonging to group 1. These results suggest that the number and combination of pesticide treatments used in group 1 vineyards may have had a negative effect on the presence of yeasts on the grapes. Moreover, since the isolation strategy adopted is able to show the presence of *Saccharomyces* yeasts, even if they are present at very low numbers on grapes, this effect seems to be particularly pronounced on yeasts ascribed to this genus. Pesticide treatments could either affect the frequency/presence of yeasts on grapes directly, via a toxic effect on yeast cells, or indirectly, by inhibiting insects, which have been shown to be the principal vectors for the transportation of yeasts (PHAFF *et al.*, 1956; MILLER *et al.*, 1962; STEVIC, 1962).

Identification of *S. cerevisiae* among the isolates

The forty-two isolates preliminarily ascribed to the genus *Saccharomyces* were further characterized. On the basis of spore formation and shape, ability to assimilate nitrate, cadaverine and ethylamine, and to solubilize $CaCO_3$, all of them were confirmed as belonging to the genus *Saccharomyces*, validating, at least in this case, the simplified identification scheme previously used.

These forty-two yeasts isolated from grapes were then subjected to physiological tests to identify the species within the genus. All forty-two isolates were

Table 3 - Number of the isolates ascribed to each class on grapes from vineyards receiving different pesticide treatments.

Vineyards	Number of isolates	Isolates ascribed to different classes							
		A	B	C	D	E	F	G	H
Group 1	70	19	0	14	6	4	1	8	18
Group 2	132	49	28	19	13	2	3	8	10
Group 3	77	15	14	12	19	5	0	2	10

able to grow at 37°C, and three were able to assimilate mannitol; thus these three isolates (5E9, 5L9 and 5G9) were identified as *S. paradoxus*, while the other thirty-nine were assigned to the *S. cerevisiae* species (KURTZMAN and FELL, 1998).

The ability to discriminate *S. cerevisiae* and *S. paradoxus* on the basis of physiological tests has been questioned (RODRIGUES DE SOUSA *et al.*, 1995; TORNAI-LEHOCZKI *et al.*, 1996). However, molecular analyses conducted utilizing mitochondrial DNA restriction analyses grouped 5E9, 5L9, 5G9 and nineteen of our *S. cerevisiae* into two separate clusters, confirming their identification as different species (GUERRA, 1998)

All the isolates ascribed to the species *S. cerevisiae* came from Passerina and Montepulciano (group 2 vineyards), and from Sangiovese grapes (group 3 vineyards). As expected, the isolation of these yeasts was possible only at enrichment stages 2 and 3. Interestingly, no *S. cerevisiae* were isolated from Verdicchio or Pecorino regardless of the pesticide treatment given. The isolation of *S. cerevisiae*, reattempted on the last two grapevine cultivars during the vintage years 1997 and 1998, was successful for Verdicchio but not for Pecorino (data not shown). A possible explanation is that Pecorino grapes are an early-maturing variety with a very resistant skin; since yeasts preferentially colonize areas of the grape surface where some juice might escape (BELIN, 1972), these particular features of Pecorino grapes could explain why it was not possible to isolate *S. cerevisiae* from them. On the other hand, and supporting this hypothesis, Passerina, Sangiovese and Verdicchio cultivars all have a fragile skin, susceptible to cracking and juice secretion, therefore constituting a desirable environment for wine yeasts (COCCI-GRIFONI, 1996).

Microfermentations

The fermenting capabilities of the thirty-nine *S. cerevisiae* strains isolated from grapes were screened by means of microfermentation trials. The results are shown in Fig. 1 (A-H). The CO_2 produced at the end of the fermentations varied from 6.5 g to 8.5 g depending on the strain; the commercial starter used as control (ScCG96) gave a value of around 7.5 g. Values close to these were obtained from ten strains, namely 5E10, 5G10, 5I10, 7I1, 7I2 7I3, 7H2, L3, L7 and L19, originally isolated from two different bunches of Passerina grapes (group 2 vineyard) and from the abandoned vineyard. Thus, among the *S. cerevisiae* coming from grapes, two groups isolated from two different areas showed promising fermentative behaviour.

CONCLUSIONS

Two hundred and seventy-nine yeasts indigenous to some important wine-pro-

ducing areas of the Marche region were isolated from grapes as a basis for selecting autochthonous starter strains able to enhance the typical organoleptic characteristics of local wines. The vast majority of these isolates included non-*Saccharomyces* yeasts. However, utilizing a relatively simple enrichment technique and a differential medium for yeast identification, it was possible to obtain a relatively high percentage of *S. cerevisiae* out of the total number of isolates. Some of the thirty-nine *S. cerevisiae* isolated gave a CO_2 production comparable to that of the starter strain used as control. It follows that *S. cerevisiae* can indeed be

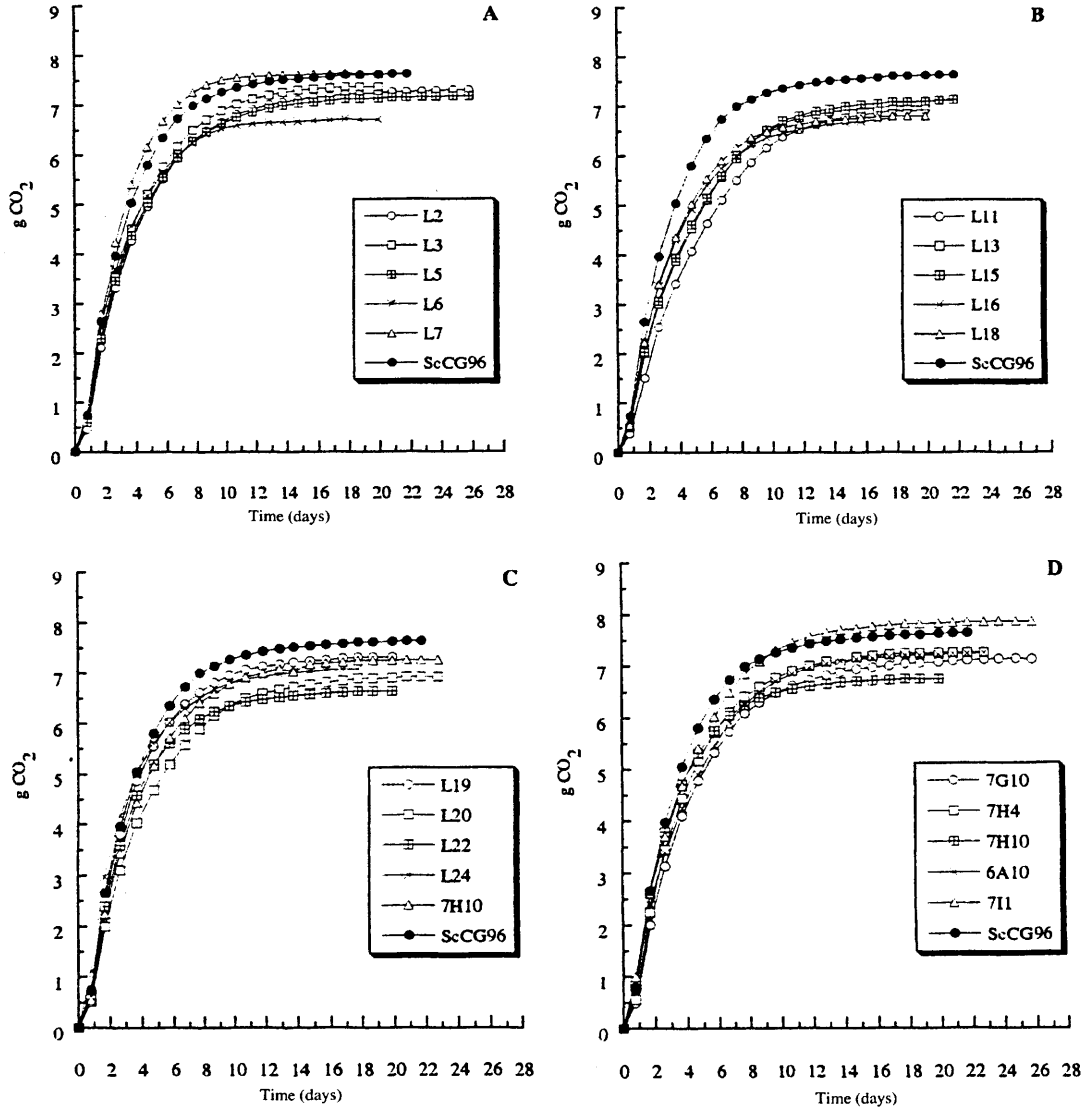

Fig. 1 - Fermentation curves of the 39 *S. cerevisiae* isolated from grapes. In each graph the fermentation curve of the control strain ScCG96 is shown for comparison.

isolated from grapes, and that these isolates can be subjected to clonal selection for identification of novel autochthonous starters.

The study of the composition of the yeast microflora of grapes collected from vineyards subjected to different pesticide treatments pointed out the negative effect that some systemic pesticides may exert on the biodiversity of wine yeasts on grapes (with a reduction in both the number of different genera and the numbers within each genus). Moreover, the repeated absence of *S. cerevisiae* on Pecorino grapes suggests that also the grape cultivar can affect the presence of this yeast.

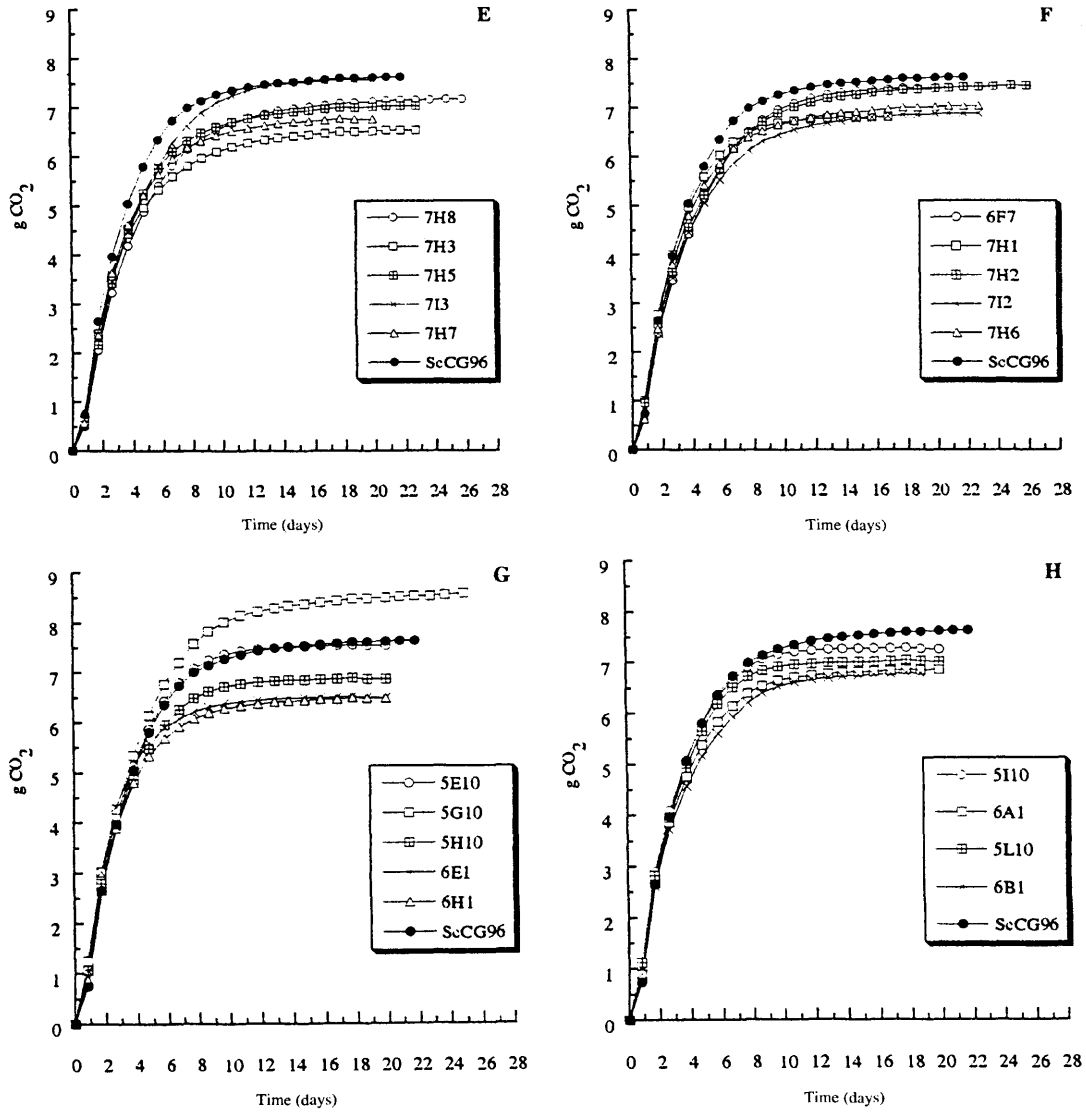

Fig. 1 - Continued.

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