Assessment of lactic acid bacteria sensitivity to terpenoids with the Biolog methodology

Assessment of lactic acid bacteria sensitivity to terpenoids by means of Biolog methodology (Short title): Study of LAB-terpenoid interaction by Biolog

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Abstract

Terpenoids are plant metabolites which can be found in traces in the milk of animals fed with fresh forages. To these compounds many biological properties, including antimicrobial activity, have been recognized. However, no information about the sensitivity of lactic acid bacteria (naturally occurring in milk and dairy products) to terpenoids are currently available. Biolog methodology, which is traditionally used for the metabolic characterization of microorganisms, has also been found suitable for the evaluation of the activity exerted by plant components against bacterial consortia, allowing to establish the duration of antimicrobial activity (if present) and its resulting effect on microorganisms viability. Thus, in the present work, this approach was employed to study the effect of six oxygenated terpenoids (geraniol, linalool, alpha-terpineol, terpinen-4-ol, carvone and menthone), which can be found in dairy products, towards 27 lactic acid bacterial strains (thermophilic or mesophilic, homo- or hetero-fermenting cocci), previously isolated from raw goat milk. Results showed that microorganisms were variously affected by the selected molecules. In some cases terpenoids seemed to have a stimulating action, while in others a transient antimicrobial activity was highlighted, without evident relationship with the metabolic/physiologic groups to which the tested bacterial strains belonged.

Keywords

Terpenoids, antimicrobial activity, lactic acid bacteria, Biolog methodology.

1 Introduction

Terpenoids are secondary plant metabolites that may be present, free or conjugated, in milk obtained by animals fed with fresh forages (Coulon et al., 2004). Therefore, they have been studied in depth and proposed as biomarkers for dairy products produced from animals fed on mountain pasture (Coulon et al, 2004; Cornu et al., 2005; Fernandez Garcia et al., 2008; Renna et al., 2012). They were also indicated as capable to indirectly affect cheeses' sensory properties due to their antimicrobial effects on autochthonous or added microorganisms, mainly lactic acid bacteria (LAB), during cheese manufacture and ripening (Mariaca et al., 1997; Buchin et al., 1999; Bugaud et al., 2001; Martin et al., 2005). However, terpenoids cannot be considered as absolute xenobiotic compounds for LAB, as these bacteria proved to be able both to synthesize and degrade such molecules (Imhof et al., 1995; Belviso et al., 2011a). These evidences suggest that a bi-directional relationship exists between terpenoids and LAB.

Raw milk, especially that collected from animals fed with fresh forages, is a typical environment in which LAB and terpenoids can occur at the same time. The way by which these two elements interact could have important outcomes on the resulting dairy products, but such investigations are not currently available. The sensitivity of LAB, selected from milk obtained by fed-pasture animals, to terpenoids, commonly found in the same type of milk, is the first step to understand the mechanisms at the basis of this relation. Due to high number of terpenoids and strains which have to be analysed for this purpose, a method able to detect rapidly this response could be desirable.

The traditional methods for the assessment of microbial sensitivity to chemical compounds are the so-called MIC (Minimum Inhibiting Concentration) and antibiogram tests (Barry, 1976; Alos and Rodriguez-Bano, 2010). For both, inconvenience due to terpenes' low solubility and high volatility has been indicated. Moreover, such methods are suitable for the sensitivity assessment of a single microbial species in standard conditions, while they are less indicated for mixed populations subjected to the influence of various factors (Burt, 2004). An alternative approach is represented by the Biolog methodology, a modified form of which has been developed and successfully applied to monitor the metabolic activity of total microbiota isolated from salad in presence of volatile terpenic compounds with antimicrobial activity (Bertolone et al., 2011). Moreover, by monitoring the metabolism over the whole growth curve, it is able to provide useful pieces of information about temporary and residual effect of compounds with possible antimicrobial activity on microorganisms (Bertolone et al., 2011). In the present work, such application of Biolog methodology has been used to study the effect of six terpenoids (geraniol, linalool, alpha-terpineol, terpinen-4-ol, carvone and menthone) on the metabolic activity of 27 lactic acid bacterial strains (thermophilic or mesophilic, homo- or hetero-fermenting cocci) previously isolated from raw goat milk, in view to provide a contribution to the knowledge of the possible bi-directional relationship between terpenoids and LABs.

2 Materials and methods

2.1 Bacterial strains and culture conditions

Twenty seven lactic acid bacterial strains isolated from goat milk in previous researches have been used for the tests. These strains came from the culture collection of the Department of Agricultural, Forest and Food Sciences, University of Turin, and were all cocci (which are prevalent in goat's as in cow's milk) belonging to the following physiologic/metabolic categories: a) thermophilic homofermenters (*Streptococcus thermophilus*, strains I1, I2, I3, I4, I18); b) mesophilic homofermenters (*Lactococcus* sp. *lactis*, strains I5, I6, I7, I8, I13, I14, I15, I16, I19, I30, I31); c) homofermenting enterocci (*Enterococcus* sp., strains I9, I10, I11, I12, I17, I26); d) heterofermenters (*Leuconostoc* sp., strains I21, I22, I23, I24, I25). They were checked for purity and maintained on M17 agar (Oxoid, Milan, Italy). At the moment of their utilization, they were cultivated (at 30°C and 40°C for mesophilic and thermophilic strains, respectively) on BHI broth (Oxoid, Milan, Italy) and successively transferred into M17 broth (Oxoid, Milan, Italy).

2.2 Terpenoids

The following terpenoids were selected: geraniol (purity higher than 96%), (-)-linalool (purity higher than 95%), alpha-terpineol (purity higher than 99%), (+)-terpinen-4-ol (purity higher than 99%), (+)-carvone (purity higher than 98.5%) and (-)-menthone (purity higher than 99%) were purchased from Fluka (Sigma-Aldrich, Milan, Italy). Terpenoids were selected among those previously found in dairy products (Cornu et al., 2005; Belviso et al., 2011b). Moreover, on the basis of our unpublished data and as reported by other Authors (Abilleira et al., 2010) these concentrations can be really found in milk.

2.3 Biolog analysis

Biolog MT2 microplates (Biolog Inc., CA, USA) with wells (120 µL capacity) containing tetrazolium violet as redox indicator were used. Terpenoid solutions were obtained by dissolving each terpenoid in absolute ethanol at the concentration of 1000 $mg \cdot L^{-1}$ and separately added to each well at two different final concentrations, 0.1 and 1 $mg \cdot L^{-1}$. These values were chosen on the basis of previous studies (Abilleira et al., 2010; Bertolone et al., 2011; Belviso et al., 2011a). Cultures of each strain were taken from M17 pre-cultures at the end of the exponential phase of growth and separately inoculated at two different final concentrations, 10^3 and 10^4 $cfu \cdot mL^{-1}$. M9 broth (Oxoid, Milan, Italy) was used to fill the well capacity.

The following blanks were also prepared: 1) microplates containing M9 broth and terpenoids; 2) microplates containing M9 broth and strain cultures. The plates were incubated in the dark at suitable temperatures (30 °C and 40 °C for mesophilic and thermophilic strains, respectively) and the colour changes induced in the wells by microbial activity recorded as absorbance (Ab) values by spectrophotometric readings at 590 nm on Biolog E-Max reader (Biolog Inc., CA, USA). The evolution of the microbial activity was followed for one week and in particular during the first two days, the readings were made every 2 hours, to catch the lag and exponential phases, and successively, when the stationary phase was reached, every 24 hours. Each assay was carried out in triplicate.

2.4 Data treatment

Ab values were converted into the index "Average Well Colour Development" (AWCD) (Garland, 1997). AWCD values plotted against time resulted in a curve similar to a microbial growth curve, in which the usual growth phases (lag, exponential and stationary) are recognizable (Fig. 1). These experimental curves were then modelled by using Tablecurve 2D software (Systat Inc., city, USA) according to Gompertz's modified equation (Zwietering et al., 1990). Gompertz's modified equation is the following:

$$LogN=LogN_0+A*exp(-exp((\mu_{max}*e/A)*(\lambda-t)+1)$$

where: λ is the duration of the lag-phase; μ_{max} the growth velocity in the exponential phase; A the maximum value of microbial growth at the end of the exponential phase. Such parameters were recorded and used to quantify the effect of terpenoids on LABs.

2.5 Statistical analysis

Values of λ, μ_{max} and A parameters of each curve were submitted to factorial analysis of variance, using strain, inoculum density, terpenoid, terpenoid concentration and their interactions as independent factors, and Duncan test for mean comparison (Statistica for Windows ver. 7.1, StatSoft Inc., USA).

3 Results and discussion

Results of factorial analysis of variance performed on λ, μ_{max} and A values were reported in Table 1. Parameter λ indicates the duration of the lag-phase in a growth curve (Fig. 1). For a given microorganism, at a given inoculum density, a significantly higher λ value in presence than in absence of a specific terpenoid can be considered as indicative of an antimicrobial effect exerted by that compound. On the other hand, a significantly lower value suggests that such terpenoid has a favourable effect on the metabolic activity of that particular strain. Nevertheless, such a positive or negative effect is only temporary as an exponential growth phase normally follows the lag-phase. . Concerning λ values, significant differences were detected for each factor and some of their interactions (strain/inoculum density and strain/terpenoid) (Table 1). In particular, from Duncan test (data not shown) no significant differences resulted for most of the microorganisms tested, with the exception of strains I 22, I 24, I 25, and I 31. In fact, when inoculated with terpenoids, such strains presented a lag-phase duration significantly higher than that obtained in absence of terpenoids. Results concerning these strains are presented in Table 2 and hereby synthesized. I 22 (heterofermenter) resulted sensitive to all terpenoids, at the two concentrations and inoculum densities. I 24 (heterofermenter) was found sensitive to all terpenoids, with the exception of α-terpineol at the inoculum density of 10^3 cfu·mL^{-1}. I 25 (heterofermenter) was found sensitive only to geraniol at 1 mg·L^{-1} and inoculum density of 10^3 cfu·mL^{-1}. I 31 (mesophilic homofermenter) was found sensitive only to carvone at 1 mg·L^{-1} and inoculum density of 10^3 cfu·mL^{-1}. The longer duration of the lag-phase observed in presence of these terpenoids, for strains I22, I24, I25, and I31, suggests that, at the concentrations and inoculum densities above reported, they exert a strong although temporary anti-microbial activity.

Parameters μ_{max} and A indicate the vitality of a strain after the lag-phase. In particular, if μ_{max} and A values recorded in presence of terpenoids are higher than those registered in their absence, a stimulating effect of terpenoids on the microorganism can be suggested especially when associated to a shorter lag-phase,while if μ_{max} and A values were lower, a persisting antimicrobial action can be indicated especially in association with a longer lag-phase. On the other hand, values, not significantly different from those obtained in presence of terpenoids, indicate that the microorganism maintains its specific functionality. As an example a representative curve (Fig. 2) is shown to see the two opposite effects on the growth phases; in the specific case linalool has a promoting effect on the growth of the strain under study (I 10) while menthone did not favour its growth especially in the exponential phase. Values of μ_{max} obtained in this work are significantly affected by all factors , except the terpenoid one, and some of their combinations (Table 1). No differences were seen for the combination in which the four factors are linked, while high significance resulted for the combination "strain/inoculum density/terpenoid". In addition, Duncan test (data not shown) highlighted that strains I 14, I 16, I 19 (*Lactococcus lactis* subsp. *lactis*) and I 18 (*Streptococcus thermophilus*) cultivated in absence of terpenoids were characterized by μ_{max} valuesparticularly high and similar to those obtained when they were cultivated in their presence with the only exception of alpha-terpineol for which a lower value was observed (Table 2).Thus, the tested terpenoids, except alpha-terpineol, did not show antimicrobial effect on the exponential growth phase of the mentioned strains. Such finding suggests this to be a strain-linked behaviour, not affected by the presence of some terpenoids.

Concerning A values significant differences within each single factor (except for inoculum density), and also for some of their combination (strain x inoculum density, strain x terpenoid, inoculum density x terpenoid, strain x terpenoid concentration, strain x inoculum density x terpenoid x terpenoid concentration) have been picked out. In this case, the subsequent Duncan test (data not shown) showed that strains I 11 and I 12 (homofermenting enterococci) in presence of linalool (0.1 $mg{\cdot}L^{-1}$) and carvone (1 $mg{\cdot}L^{-1}$), respectively, had values significantly lower than those obtained without the addition of terpenoids (Table 2) and also lower than those obtained for the other strains under study. This seems to indicate that a residual antimicrobial effect is present.

4 Conclusions

The adopted Biolog methodology confirmed its ability to provide useful information about the terpenoids effect on lactic acid cocci, in particular about the transience/persistence of such effect.

Some of the strains belonging to the heterofermenters resulted affected by nearly all the terpenoids tested, undergoing a delay in their metabolic activity. Also one mesophilic homofermenting strain (I 31) resulted positively affected, but only by carvone at 1 $mg \cdot L^{-1}$ and inoculum density of 10^3 $cfu \cdot mL^{-1}$.

Although the diffusion of such sensitivity to terpenoids within lactic acid bacteria in general cannot be established here, our results indicate that at least some of their species (mainly heterofermenting cocci) are liable to undergo a depressing effect in consequence of the presence of some terpenoids. Such effect resulted to be in general temporary, in the sense that after a certain time practically all strains regained their "normal" growth rate, namely the one shown in terpenoids' absence. Nevertheless, also the persistence of a residual antimicrobial effect was suggested for two *Enterococcus* sp. strains. No specific relationship was ascertained between terpenoids and physiologic/metabolic groups. This suggests the hypothesis that sensitivity to terpenoids is a "mixed" feature among lactic acid cocci, that are variously (positively or negatively, temporarily or persistently) affected by various terpenoids.

Even if only temporarily, the possible antimicrobial action of terpenoids (naturally occurring in milk at the concentrations tested in this work) is likely to have not irrelevant effects on the activity of a mixed lactic acid microflora (such as the "natural" one present in some artisanal dairy products), slowing the growth of some of its components and thus favouring others, with an influence of the final characteristics of the product.

Some of the microbial strains tested (I14, I16 and I19, *Lactococcus lactis* subsp. *lactis*, and I18, *Streptococcus thermophilus*) showed a metabolic activity particularly high even in presence of terpenoids. While this finding was interpreted as a strain-linked characteristic, the possibility that some lactic acid bacteria can metabolize terpenoids (with a favourable effect on their activity) cannot be excluded. Such confirmation requires further tests, which could be performed with the same Biolog methodology applied in the present research, cultivating the microorganisms under study with terpenoids as the only carbon source.

References

Abilleira E, de Renobales M, Nájera AI, Virto M, de Gordoa JCR, Pérez-Elortondo FJ, Albisu M, Barron LJR (2010) An accurate quantitative method for the analysis of terpenes in milk fat by headspace solid-phase microextraction coupled to gas chromatography–mass spectrometry. Food Chem 120: 1162-1169.

Alos JI and Rodriguez-Bano J (2010) Which antibiotics should we report in an antibiogram, and how? Enferm Infecc Microbiol Clin 28(10): 737-741.

Barry AL (1976) The Antimicrobics Susceptibility Test: Principles and Practices. Lea and Febiger, Philadelphia, USA.

Belviso S, Giordano M, Dolci P, Zeppa G (2011a) Degradation and biosynthesis of terpenoids by lactic acid bacteria isolated from cheese: First evidence. Dairy Sci Technol 91(2): 227-236.

Belviso S, Giordano M, Coppa M, Lombardi G, Zeppa G (2011b) Individuazione di biomarker terpenoidici per la differenziazione di prodotti caseari di montagna [Terpenoids as biomarkers of mountain dairy products]. Sci Tec Latt Casearia 62(6), 433-440.

Bertolone E, Minati JL, Zanoni B, Ambrosoli R (2011) Biolog methodology for the antimicrobial activity evaluation of essential oils' volatile compounds against foodborne microflora of fresh-cut salad. Ital J Food Sci 23: 289-301.

Buchin S, Martin B, Dupont D, Bornard A, Achilleos C (1999) Influence of the composition of Alpine highland pasture on the chemical, rheological and sensory properties of cheese. J Dairy Res 66 :579-588

Bugaud C, Buchin S, Hauwuy A, Coulon JB (2001) Relationships between flavour and chemical composition of Abondance cheese derived from different types of pastures. Lait, 81: 757-773.

Burt S (2004) Essential oils: properties and potential applications in foods - a review. Int J Food Microbiol 94: 223-253.

Cornu A, Kondjoyan N, Martin B, Verdier-Metz I, Pradel P, Berdarguè JL, Coulon JB (2005) Terpene profiles in Cantal and Saint-Nectaire-type cheese made from raw or pasteurised milk. J Agric Food Chem 85:2040–2046.

Coulon JB, Delacroix-Buchet A, Martin B, Pirisi A (2004) Relationship between ruminant management and sensory characteristics of cheeses: a review. Lait 84:221–241.

Fernández García E, Imhof M, Schlichtherle-Cerny H, Bosset JO, Nuñez M (2008) Terpenoids and benzenoids in La Serena cheese made at different seasons of the year with a Cynara cardunculus extract as coagulant. Int Dairy J 18:147–157.

Garland JL (1997) Analysis and interpretation of community-level physiological profiles in microbial ecology. FEMS Microbiol Ecol 24: 289-300.

Imhof R, Glatti H, Bosset JO (1995) Volatile organic compounds produced by thermophilic and mesophilic single strain dairy starter culture. Lebensm Wiss Technol 28:78–96.

Mariaca RG, Berger TFH, Gauch R, Imhof MI, Jeangros B, Bosset JO (1997) Occurrence of volatile mono- and sesquiterpenoids in highland and lowland plant species as possible precursors for flavor compounds in milk and dairy products. J Agric Food Chem 45: 4423-4434.

Martin B, Verdier-Metz I, Buchin S, Hurtaud C, Coulon JB (2005) How do the nature of forages and pasture diversity influence the sensory quality of dairy livestock products? Animal Sci B1: 205-212.

Renna M, Cornale P, Lussiana C, Giordano M, Belviso S, Zeppa G, Battaglini LM (2012) Efficacy of fatty acids and terpenoids and weakness of electronic nose response as tracers of Asiago d'Allevo PDO cheese produced in different seasons. Dairy Sci Technol 92(3): 203-218.

Zwietering MH, Jongenburger I, Rombouts FM, Van'T Riet K (1990) Modeling of the bacterial growth curve. Appl Environ Microbiol 56: 1875-1881.

Figure captions

Fig. 1 A typical growth curve with the three parameters, λ (lag-phase duration), μ_{max} (velocity in the exponential phase) and A (maximum value of microbial growth at the end of the exponential phase). (original source: Zwietering MH, Jongenburger I, Rombouts FM, Van'T Riet K (1990) Modeling of the bacterial growth curve. Appl Environ Microbiol 56: 1875-1881)

Fig. 2 A representative growth curve fitted to Gompertz modified model of I 10 strain inoculated at 10^3 cfu·mL^{-1} without terpenoid and with linalool and menthone at 1 mg·L^{-1}.

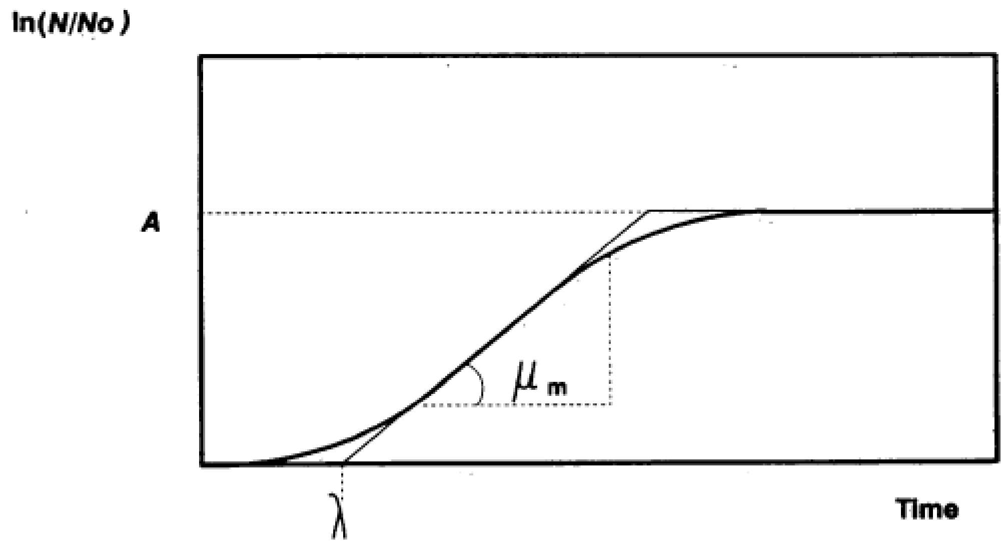
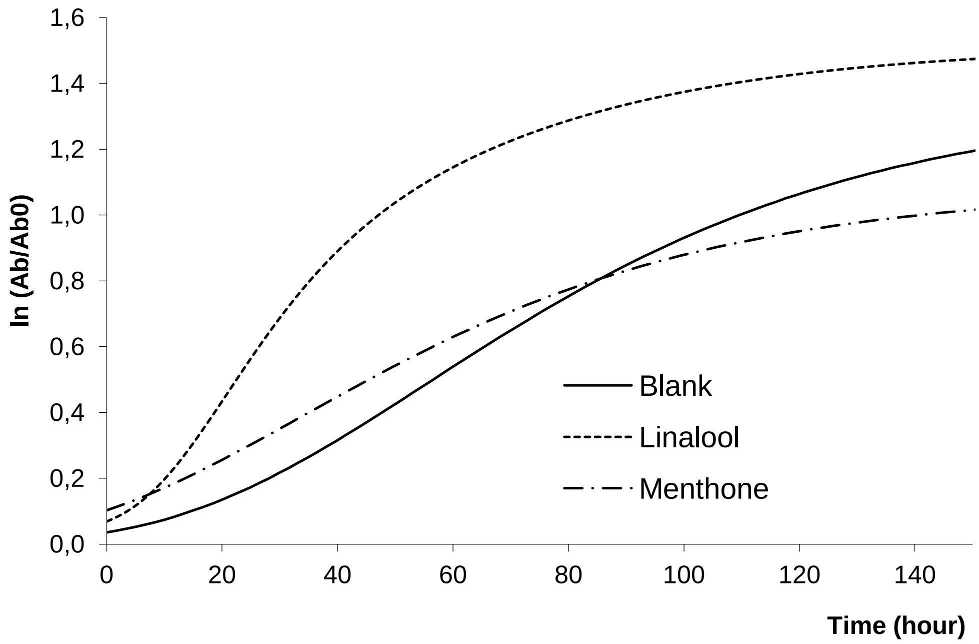


Fig. 1



1,6
1,4
1,2
1,0
0,8
0,6
0,4
0,2
0,0
ln (Ab/Ab0)
0
20
40
60
80
100
120
140
Time (hour)
Blank
Linalool
Menthone

Fig. 2

Table 1. Results of factorial analysis of variance performed on values of λ (lag-phase duration), μ_{max} (velocity in the exponential phase), A (maximum value of microbial growth at the end of the exponential phase) parameters, taking into account the factors strain (1), inoculum density (2), terpenoid (3), terpenoid concentration (4) and their interactions.

Factors and interactions		*P*	
	λ	μ_{max}	A
(1) Strain	****	****	****
(2) Inoculum density	****	****	ns
(3) Terpenoid	****	ns	*
(4) Terpenoid concentration	*	****	*
1 x 2	****	****	****
1 x 3	****	****	****
2 x 3	*	*	*
1 x 4	*	****	*
2 x 4	ns	ns	ns
3 x 4	ns	ns	ns
1 x 2 x 3	ns	****	ns
1 x 2 x 4	ns	ns	ns
1 x 3 x 4	ns	ns	ns
2 x 3 x 4	ns	ns	ns
1 x 2 x 3 x 4	****	ns	****

*P≤0.05; **P≤0.01; ***P≤0.005; ****P≤0.001

ns not significant

Table 2. Mean values of λ (lag-phase duration) for strains I 22, I 24, I 25, I 31, of μ_{max} (velocity in the exponential phase) for strains I 14, I 16, I 18, I 19 and of A (maximum value of microbial growth at the end of the exponential phase) for strains I 11 and I 12 , , in absence of terpenoids (blank) and in presence of geraniol, linalool, alpha-terpineol, terpinen-4-ol, carvone and menthone.

			Blank	Geraniol		Linalool		alpha-Terpineol		Terpinen-4-ol		Carvone		Menthone	
	Strain			c 1	c 2	c 1	c 2	c 1	c 2	c 1	c 2	c 1	c 2	c 1	c 2
λ															
	I 22	i.d. 1	12.67	199.16	117.50	153.09	147.54	112.50	110.47	211.37	212.11	243.20	185.34	128.93	84.23
		i.d. 2	15.30	59.29	117.03	153.09	147.54	112.50	107.56	64.90	109.31	56.62	54.27	47.72	22.34
	I 24	i.d. 1	45.22	143.31	166.98	150.91	222.88	**48.42**	**44.78**	146.95	134.52	307.84	284.63	130.69	280.07
		i.d. 2	1.61	30.52	26.18	94.16	50.54	53.10	32.89	37.91	42.78	10.14	15.78	11.47	13.78
	I 25	i.d. 1	1.23	2.04	**74.88**	1.11	2.57	2.81	1.35	1.09	0.88	1.09	0.93	1.09	1.02
		i.d. 2	1.26	2.01	1.06	1.43	1.47	1.04	1.04	2.48	1.17	1.10	1.19	1.29	1.38
	I 31	i.d. 1	3.38	2.69	3.07	3.36	3.43	3.40	3.31	3.37	3.39	3.36	**90.92**	3.29	3.28
		i.d. 2	3.41	3.31	3.27	3.39	3.11	3.35	3.39	3.38	3.38	3.08	3.38	3.40	3.32
μ_{max}															
	I 14	i.d. 1	0.84	0.84	0.80	0.79	0.79	0.73	0.80	0.80	0.83	0.79	0.84	0.81	0.80
		i.d. 2	0.45	0.55	0.42	0.52	0.43	0.48	0.46	0.38	0.45	0.51	0.44	0.48	0.47
	I 16	i.d. 1	0.62	1.08	0.47	0.74	0.50	0.66	0.45	0.70	0.81	0.60	0.45	0.59	0.52
		i.d. 2	0.63	0.86	0.71	0.64	0.63	0.60	0.61	0.82	0.84	0.64	0.65	0.62	0.60
	I 18	i.d. 1	0.20	0.19	0.18	0.23	0.21	0.15	0.20	0.18	0.24	0.20	0.31	0.39	0.27
		i.d. 2	0.05	0.09	0.07	0.08	0.06	0.03	0.04	0.07	0.05	0.05	0.08	0.06	0.04
	I 19	i.d. 1	0.92	0.77	0.85	0.98	0.84	**0.08**	**0.04**	0.91	0.96	0.75	0.91	0.97	0.94
		i.d. 2	0.06	0.04	0.07	0.06	0.06	0.06	0.04	0.06	0.06	0.08	0.10	0.05	0.09
A															
	I 11	i.d. 1	19.13	14.52	20.20	**3.12**	14.98	20.15	20.10	20.12	20.23	20.51	19.97	20.15	20.17
		i.d. 2	21.77	20.61	20.29	**2.52**	21.63	21.01	21.09	19.16	21.25	19.16	21.26	21.17	21.40
	I 12	i.d. 1	7.58	8.46	7.28	8.01	7.40	8.34	7.52	8.11	8.24	8.25	**3.41**	8.17	7.70
		i.d. 2	8.70	8.51	7.14	8.97	8.14	8.45	9.10	9.60	7.98	8.36	**2.94**	9.12	9.23

i.d. 1 inoculum density at 10^3 cfu·mL^{-1}; i.d. 2 inoculum density at 10^4 cfu·mL^{-1}; c1 terpenoid concentration at 0.1 mg·L^{-1}; c2 terpenoid concentration at 1 mg·L^{-1}