Optimised Screening of Antibiotic Producing Strains*

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In memoriam Prof. Emeritus Vera Johanides

To recognise microbial strains capable to produce substances with antimicrobial activity the culture conditions enabling, both, the microorganism growth and the expression of antibiotic production, should be applied. Since the optimal culture conditions vary depending on properties of particular strains one can recommend the application of various culture conditions, designed on the basis of different factorial plans, in order to increase the probability of expression of antibiotic production and/or to enhance antibiotic biosynthesis efficiency. In the case of microbial colonies, values of inhibition zone diameters and colony diameters could be considered that they reflect strain properties. In performed experiments different microbial strains were applied. *Streptomyces rimosus* R6–500 as parental strain and its derivative strains MV9R-1 and MV9R-2, were used as antibiotic (oxytetracycline) producers. Additionally, *S. rimosus* R6-ZGL1 as producer of new substances with antibiotic activity and *S. rimosus* MV25W as the strain showing not detectable antibiotic activity, were used also. *Bacillus cereus* ATCC 11778 was applied as test microorganism sensitive to antibiotic action. Different cultivation media were used, their composition being defined according to different factorial plans.

As expected, antimicrobial activity expressed differently in different media. Properties of media reflected differently on particular strains. Results showed that the use of different media can markedly increase the probability of recognising strains with antimicrobail activity. When comparing activities of microbial colonies one can observe that strains *S. rimosus* R6–500 and *S. rimosus* MV9R-2 behaved similarly. Antimicrobial activity was not expressed in 50–56 % of cases. The strain *S. rimosus* MV9R-1 expressed its activity even in 81 % of cases and showed to be more efficient than both *S. rimosus* R6–500 and *S. rimosus* MV9R-2. In contrast to the colony cultivation on solid media, in submerse cultures the strain MV9R-1 showed to be inferior with respect to other two strains mentioned, which showed their similarity also when cultivated as submerse cultures. Correlation between values of potency index and corresponding antimicrobial activities in corresponding submerse cultures was not found to be significant. The main purpose of performed experiments was to test new strains and to detect their possible biological activity. Accordingly, the strain R6-ZGL1 was tested, and its antibiotic activity compared with strains R6–500 and MV25W, the later being used as negative control. In 75 % of cases this strain showed biological activity, in some cases even higher than R6–500.

It was important to test divergence of different isolates of the same strain to verify whether there is any variation in their properties. Three types of experiments were performed: a) only one isolate was cultivated on 16 different media, b) 4 isolates were put on 16 different agarized media (each of them on 16/ 4 = 4 different media) and c) 16 isolates were put on 16 different media, i.e. every isolate was cultivated applying only one of 16 different media. Potency indexes were statistically analysed and excellent correlation was established. So it can be concluded that there is no divergence between randomly chosen isolates of strain MV9R-1, i.e. investigated microbial population showed to be of highly homogenous properties.

Key words: Screening of strains • antibiotics • method evaluation

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Emeritus prof. Vera Johanides observed the complete final text of the poster. Her opinion referring to the poster was that this was well done and giving very interesting data and that the applied approach to the screening of antibiotic producing microorganisms could be accepted.

Introduction

In general, product formation kinetics depends on microbial strain properties and culture conditions. Therefore, to recognize microbial strains capable to produce substances with antimicrobial activity the culture conditions enabling both the microorganism growth and the expression of antibiotic production should be applied. Since the optimal culture conditions vary depending on properties of particular strains one can recommend the application of various culture conditions, designed on the basis of different factorial plans, in order to increase the probability of expression of antibiotic production and/or to enhance antibiotic biosynthesis efficiency. Efficiency of screening procedures largely depends on the recognition of strains showing the capability to synthesize antibiotic substances, especially in cases when strains with antibiotic activity appear with extremely low frequency in whole investigated population.

In the case of microbial colonies the antibiotic synthesis kinetics can be roughly described applying the following mathematical equations:[1]

$$dD_C/dt = k_{1D} - k_{2D} \cdot D_C \quad (1)$$

and

$$dD_Z/dt = q_{D2} \cdot D_C^2/D_Z \quad (2)$$

or

$$dD_Z/dt = q_{D3} \cdot D_C^3/D_Z \quad (3)$$

Where D_C = colony diameter, D_Z = inhibition zone diameter, t = cultivation time, k_{1D} and k_{2D} = colony growth kinetic coefficients, q_{D2} and q_{D3} = specific rates of inhibition zone increase, depending whether colony surface area or colony volume is relevant for antibiotic synthesis. Values of kinetics quantities (k_{1D}, k_{2D}, q_{D2} and q_{D3}) vary depending on strain properties and colony cultivation conditions. Equations (1) – (3) summarize adequately the previous statements.

Therefore, values of inhibition zone diameters and colony diameters could be considered to reflect strain properties. One could recommend their values to be used in recognizing strains with antibiotic synthesis capability. Many authors[1–3] recommended the potency index (D_Z/D_C) to be strain selection criterion.

Materials and methods

Microorganisms:

The parental strain *Streptomyces rimosus* R6-500,[4] and its mutants MV9R-1 and MV9R-2,[5] were used as oxytetracycline producers. The strain *S. rimosus* R6-ZGL1 produces some new substances of undefined antibiotic activity,[6] while *S. rimosus* MV25W is the strain that shows not detectable antibiotic activity and was used as negative control.[7] *Bacillus cereus* ATCC11778 was applied as test microorganism.

Nutrient media:

Different cultivation media were used (Tables 1 and 2), their composition was performed according to different factorial plans.[8,9]

Table 1 – *Factorial plan of 16 experiments (15 factors on two levels).*

Tablica 1 – *Faktorski plan za 16 pokusa (15 faktora na dvije razine).*

Factors Faktori	Level 1 Razina 1	Level 2 Razina 2
glucose glukoza	0 g L^{-1}	10 g L^{-1}
maltose maltoza	0 g L^{-1}	10 g L^{-1}
dextrin dekstrin	0 g L^{-1}	10 g L^{-1}
sorbitol	0 g L^{-1}	10 g L^{-1}
glycerol glicerol	0 g L^{-1}	10 g L^{-1}
CSL filtrate filtrat CSL-a	0.5 g L^{-1}*	1 g L^{-1}*
yeast extract kvaščev ekstrakt	0.5 g L^{-1}*	1 g L^{-1}*
casein kazein	0.5 g L^{-1}*	1 g L^{-1}*
$NaNO_3$	0.5 g L^{-1}*	1 g L^{-1}*
$(NH_4)_2SO_4$	0.5 g L^{-1}*	1 g L^{-1}*
KH_2PO_4	0 g L^{-1}*	30 mg L^{-1}*
mineral salts mineralne soli	0 g L^{-1}	conc. 1
Ca-salts	$CaCO_3$ 0 g L^{-1}	$CaCO_3$ 5 g L^{-1}
Ca-soli	$CaCl_2$ 0.05 g L^{-1}	$CaCl_2$ 0.1 g L^{-1}
pH	6 – 7	7 – 8
temperature temperatura	28 °C	37 °C

Table 2 – *Factorial plan of 27 experiments (12 factors on three levels).*

Tablica 2 – *Faktorski plan za 27 pokusa (12 faktora na tri razine).*

Factors Faktori	Level 1 Razina 1	Level 2 Razina 2	Level 3 Razina 2
glucose glukoza	1 g L^{-1}	5 g L^{-1}	20 g L^{-1}
maltose, dextrin maltoza, dekstrin	0 g L^{-1}	20 g L^{-1} maltose	20 g L^{-1} dextrin
poliols polioli	0 g L^{-1}	10 g L^{-1} sorbitol	10 g L^{-1} glycerol
CSL filtrate filtrat CSL-a	0.2 g L^{-1*}	0.5 g L^{-1*}	0.8 g L^{-1*}
yeast extract kvaščev eksrakt	0.2 g L^{-1*}	0.5 g L^{-1*}	0.8 g L^{-1*}
casein kazein	0.2 g L^{-1*}	0.5 g L^{-1*}	0.8 g L^{-1*}
$NaNO_3$	0.2 g L^{-1*}	0.5 g L^{-1*}	0.8 g L^{-1*}
$(NH_4)_2SO_4$	0.2 g L^{-1*}	0.5 g L^{-1*}	0.8 g L^{-1*}
KH_2PO_4	0 g L^{-1}	15 mg L^{-1*}	30 mg L^{-1*}
Ca – salts Ca-soli	$CaCO_3$ 0 g L^{-1} $CaCl_2$ 0 g L^{-1}	$CaCO_3$ 3 g L^{-1} $CaCl_2$ 0.05 g L^{-1}	$CaCO_3$ 10 g L^{-1} $CaCl_2$ 0.1 g L^{-1}
mineral salts mineralne soli	0 g L^{-1}	conc. 1	conc. 2
pH	6.0 – 6.5	6.5 – 7.0	7.0 – 8.0

* – calculation based on protein nitrogen
* – calculation based on inorganic phosphorus
$CaCO_3$ is applied for liquid cultures, $CaCl_2$ for solid cultures

* – proračunato prema proteinskom dušiku
* – proračunato prema anorganskom fosforu
$CaCO_3$ se primjenjuje u tekućim, a $CaCl_2$ u krutim podlogama

Results and discussion

For an appropriate evaluation of proposed approaches the starting experiments were based on strains with already known relevant characteristics. *Streptomcyes rimosus* parental and mutant strains were used differing by their growth characteristics and their kinetics of oxytetracycline production. Common cultivation procedure for mentioned strains takes six days, but preliminary experiments have shown that biological activity could be detected even after two days of cultivation.

As expected, antimicrobial activity expressed differently in different media. Properties of media reflected differently on particular strains. If applied medium is inadequate for given strain it can happen that the antibiotic activity cannot be expressed. Since the used strains differed in their properties they showed differences in their behaviour during their cultivation in different media. Data suggest that the use of different media can markedly increase the probability to recognise strains with antimicrobial activity. When comparing activities of microbial colonies one can observe that strains *S. rimosus* R6–500 and *S. rimosus* MV9R-2 behaved similarly (Fig. 1 and Fig. 3). Antimicrobial activity was not expressed in 50–56 % of cases. The strain *S. rimosus* MV9R-1 expressed its activity even in 81% of cases and showed to be more efficient than both *S. rimosus* R6–500 and *S. rimosus* MV9R-2. In contrast to the colony cultivation on solid media, in submerse cultures the strain MV9R-1 showed to be inferior with respect to other two strains mentioned which showed their similarity also when cultivated as submerse cultures (Fig. 2 and Fig. 3). Superior (bolded) values could become important in selecting isolates of improved antibiotic activity (Table 3).

Correlation between values of potency index and corresponding antimicrobial activities in corresponding submerse cultures was not found to be significant (Table 4). Statistical results presented in Table 4 also confirm similarity between strains R6–500 and MV9R-2.

Different expression of antibiotic activity in submerse and solid cultures could be due to the differences in antibiotic synthesis induction and process kinetics (Fig 4).

Since in comparison with submerse cultures the method of cultivating microbial colonies undoubtedly is much more convenient for screening of high number of strains, there is no need to apply submerse cultures in the first screening step. They could be recommended for the second step, for the screening of optimal culture conditions to achieve microbial cultures with such amount of bioactive substance, which enables its identification and its biological activity.

Strains R6–500 and MV9R-1 were also tested applying the system of 27 different media (Fig. 5). Strain MV9R-2 was excluded from this experiment because in previous experiments it showed rather high correlation with strain R6–500 (Fig. 1).

The main purpose of performed experiments is to test new strains and to detect their possible biological activity. Accordingly, the strain R6-ZGL1 was tested, and its antibiotic activity compared with strains R6–500 and MV25W, the later being used as negative control (Fig. 6). In 75% of cases this strain

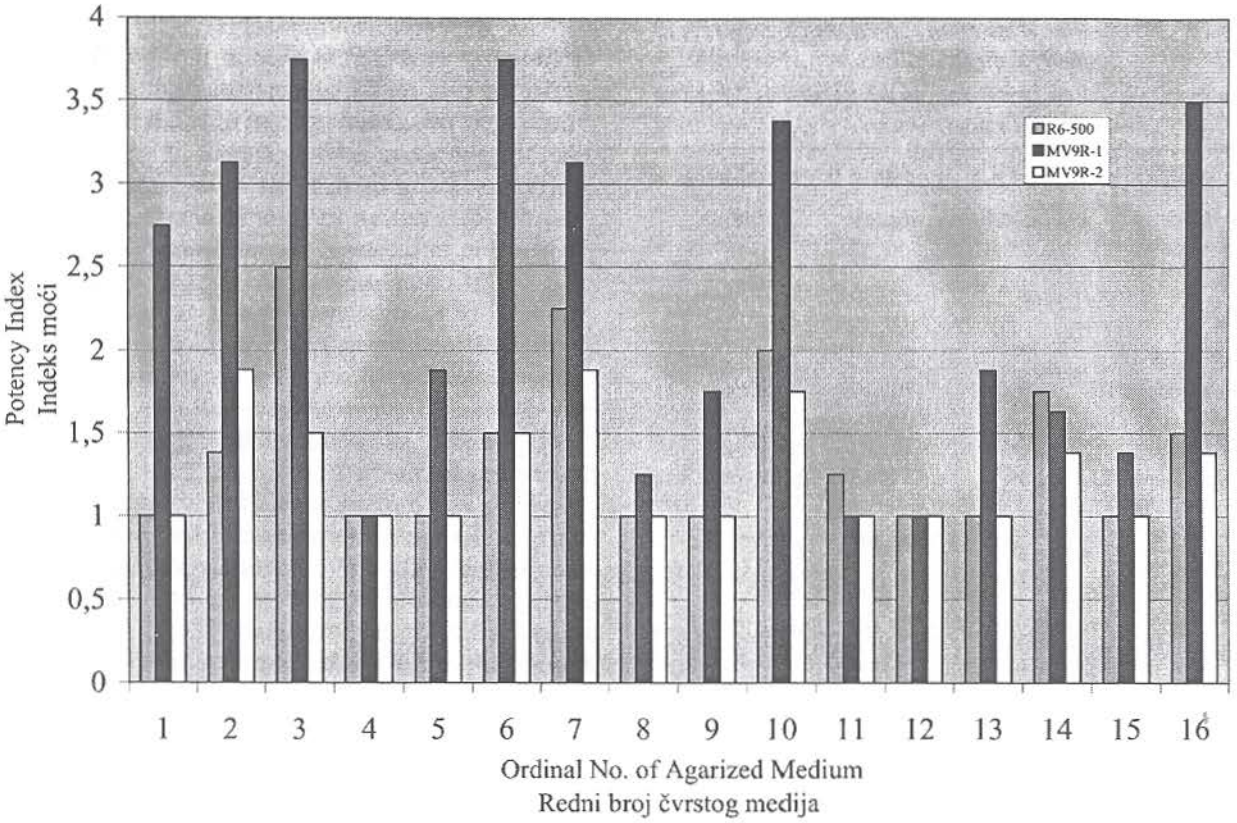

Fig. 1 – *Potency indexes for tested strains cultivated on 16 different agarized media*
Slika 1 – *Vrijednosti indeksa moći testiranih sojeva uzgojenih na 16 različitih čvrstih podloga*

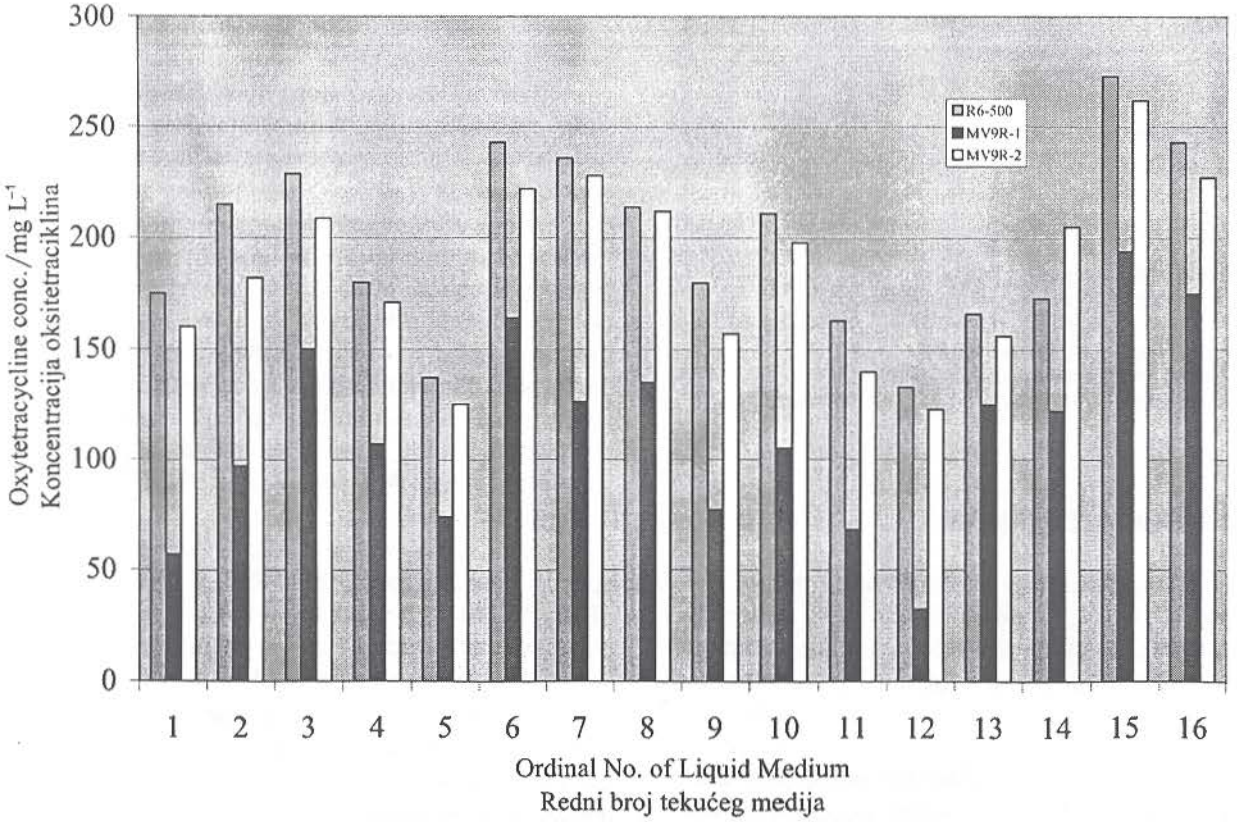

Fig. 2 – *OTC production of tested strains cultivated on 16 different media*
Slika 2 – *Proizvodnja OTC-a s pomoću istraživanih sojeva uzgojenih na 16 različitih podloga*

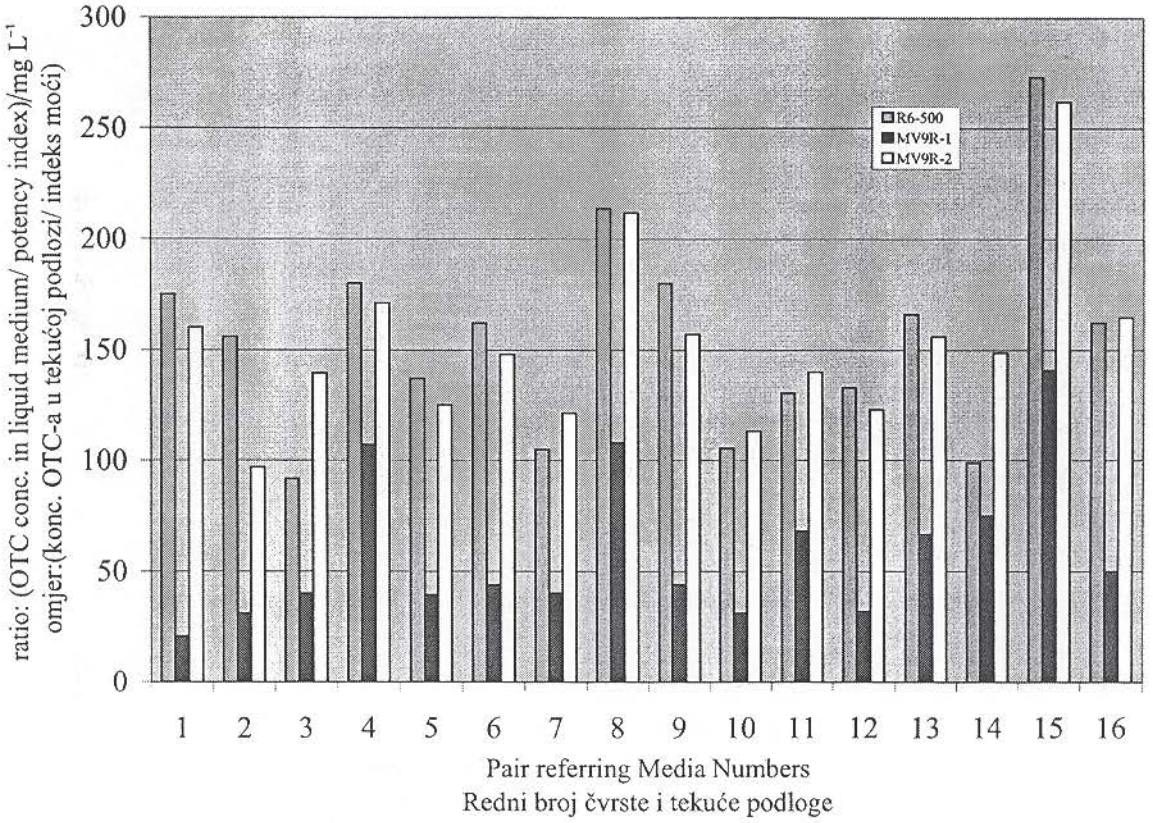

Fig. 3 – *Correlation between activities in solid and liquid media as a function of strain properties and cultivation conditions*

Slika 3 – *Korelacija između aktivnosti na čvrstim i u tekućim podlogama kao funkcija svojstava sojeva i uvjeta uzgoja*

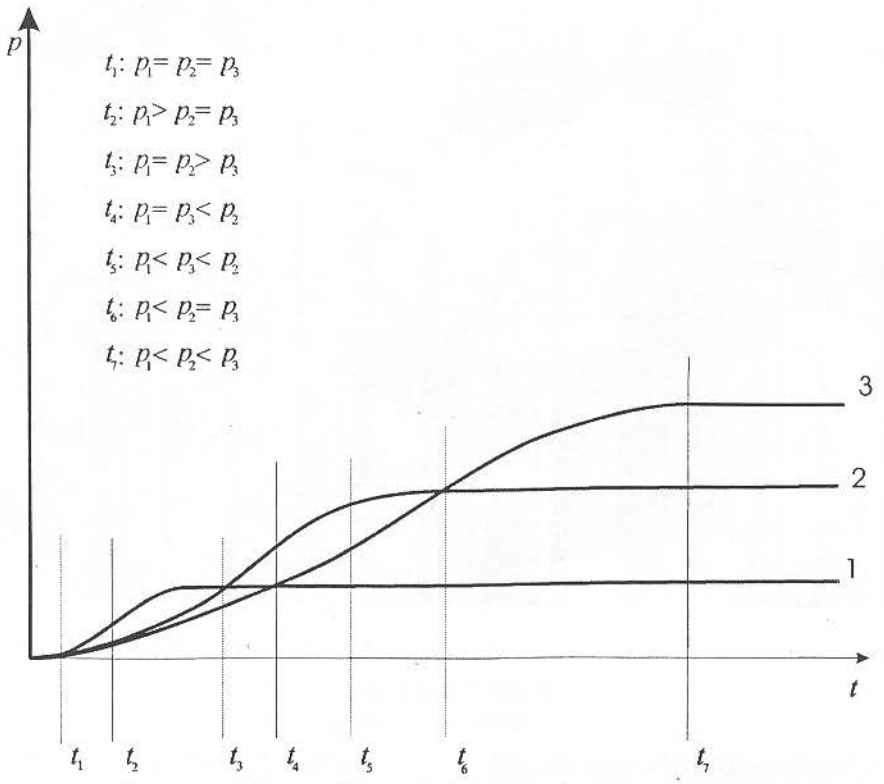

Fig. 4 – *Some of possible kinetics of product formation*

Slika 4 – *Neke od mogućih kinetika tvorbe produkta.*

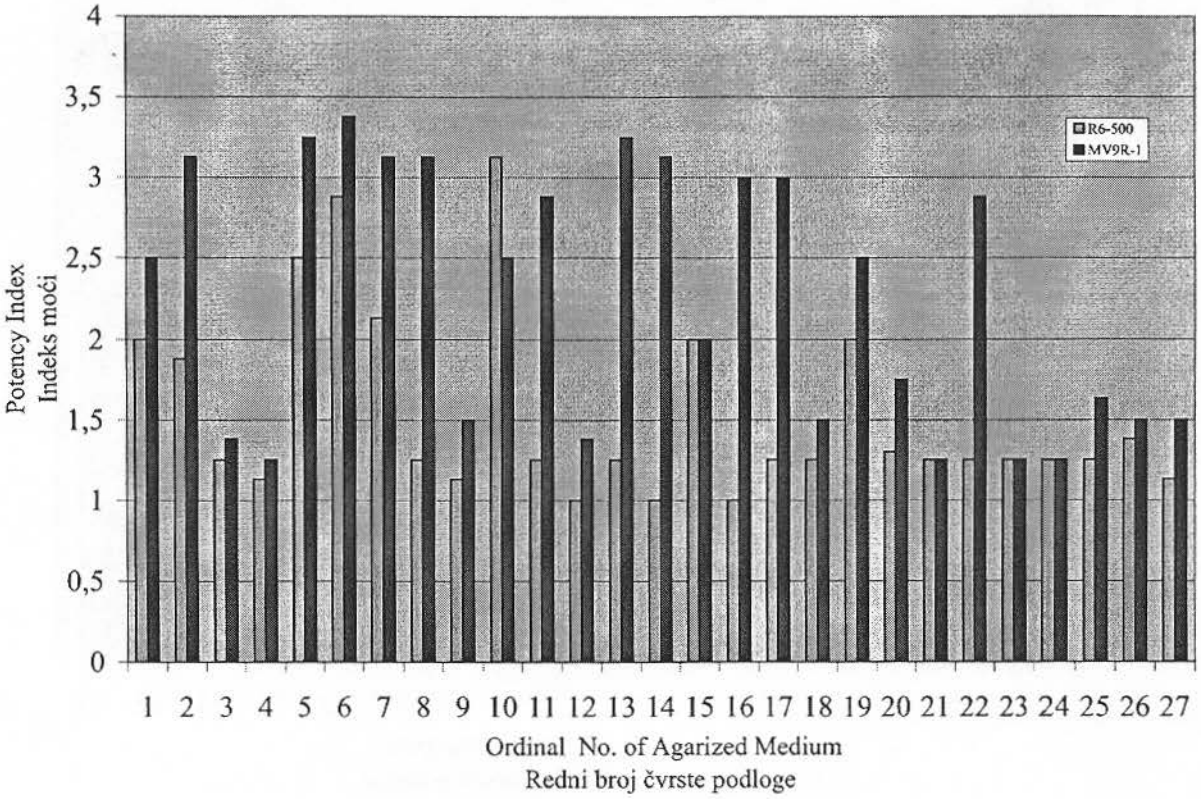

Fig. 5 – *Potency indexes of tested strains cultivated applying the system of 27 different agarized media*
Slika 5 – *Vrijednosti indeksa moći testiranih sojeva uzgojenih na 27 različitih čvrstih podloga*

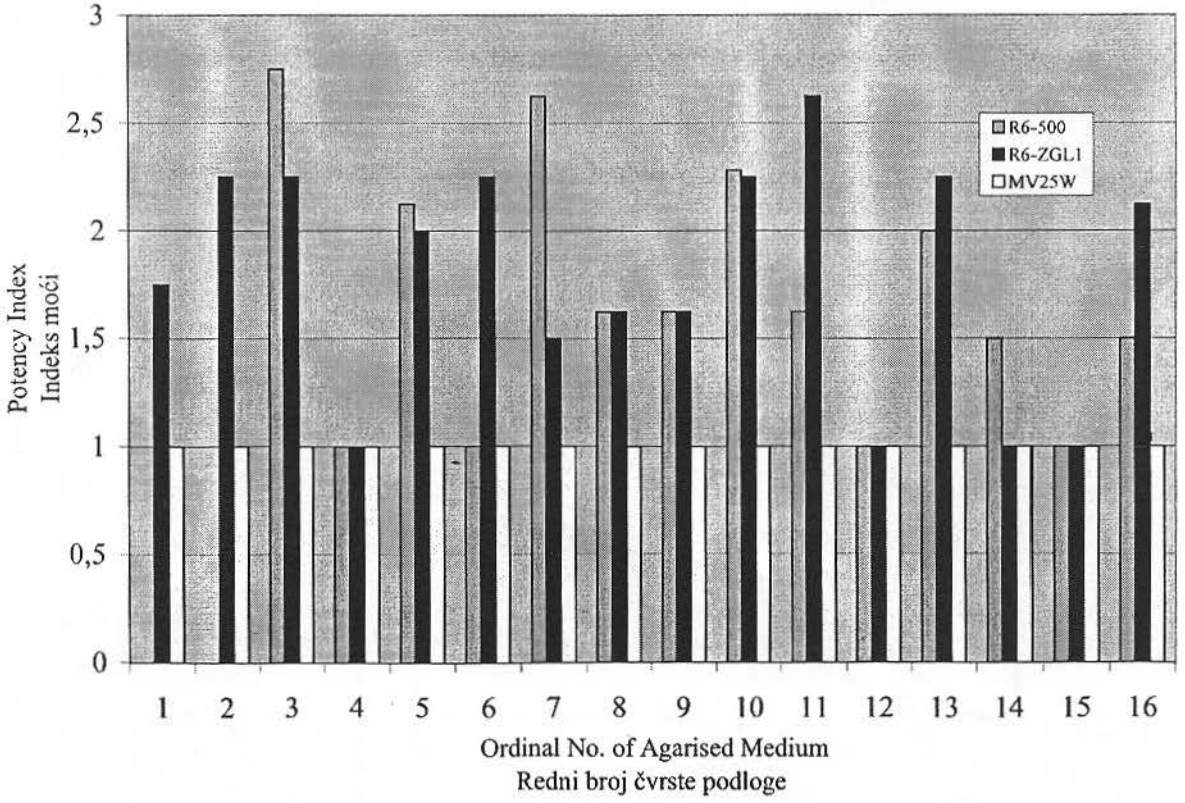

Fig. 6 – *Potency indexes of strains R6–500, R6-ZGL1 and MV25 W cultivated applying 16 different agarized media*
Slika 6 – *Vrijednosti indeksa moći sojeva R6–500, R6-ZGL1 i MV25 W uzgojenih na 16 različitih čvrstih podloga*

Table 3 – *Comparison of strains on the basis of their potency indexes. Superior values are bolded*
Tablica 3 – *Usporedba sojeva prema vrijednostima indeksa moći. Najbolje vrijednosti su istaknute*

Medium Podloga	D_Z/D_C of MV9R-2	Normalized potency indexes Normalizirani indeks moći		
		MV9R-2	MV9R-1	R6-500
1	1.00	1.00	**2.75**	1.00
2	1.88	1.00	**1.66**	0.73
3	1.50	1.00	**2.50**	1.67
4	1.00	1.00	1.00	1.00
5	1.00	1.00	**1.88**	1.00
6	1.50	1.00	**2.50**	1.00
7	1.88	1.00	**1.66**	1.20
8	1.00	1.00	**1.25**	1.00
9	1.00	1.00	**1.75**	1.00
10	1.75	1.00	**1.93**	1.14
11	1.00	1.00	1.00	**1.25**
12	1.00	1.00	1.00	1.00
13	1.00	1.00	**1.88**	1.00
14	1.38	1.00	1.18	**1.29**
15	1.00	1.00	**1.38**	1.00
16	1.38	1.00	**2.54**	1.50

Table 4 – *Statistical evaluation (linear regression analysis) of screening parameter relationships*
Tablica 4 – *Statistička ocjena (analiza linearne regresije) zakonitosti odabira*

Variable Varijabla		Linear regression coefficients Koeficijent linearne regresije		
X	*Y*	slope nagib (a)	intercept odsječak (b)	correlation korelacija (r2)
potency index indeks moći (R6-500)	OTC conc. in liquid media Konc. OTC-a u tekućoj podlozi (R6-500)	34.77	150.10	0.18
potency index indeks moći (MV9R-1)	OTC conc. in liquid media Konc. OTC-a u tekućoj podlozi (MV9R-1)	8.47	89.00	0.04
potency index indeks moći (MV9R-2)	OTC conc. in liquid media Konc. OTC-a u tekućoj podlozi (MV9R-2)	53.66	118.08	0.20
potency index indeks moći (R6-500)	potency index indeks moći (MV9R-1)	1.43	0.28	0.47
potency index indeks moći (R6-500)	potency index indeks moći (MV9R-2)	0.54	0.52	0.62

showed biological activity, in some cases even higher than R6–500. Those results (based on the use of media No. 6 and 11) might serve as a good start for selecting isolates of strain with improved antibiotic activity.

It is rather important to test divergence of different isolates of the same strain to verify whether there is any variation in their properties. Three types of experiments were performed: a) only one isolate was cultivated on 16 different media, b) 4 isolates were put on 16 different agarized media (each of them on 16/4 = 4 different media) and c) 16 isolates were put on 16 different media, i.e. every isolate was cultivated applying only one of 16 different media (Fig. 7). Potency indexes were statistically analysed and excellent correlation was established (Table 5). So it can be concluded that there is no divergence between randomly chosen isolates

Table 5 – *Statistical evaluation of screening parameter relationships for different isolates of strain MV9R-1*
Tablica 5 – *Statistička ocjena zakonitosti odabira za različite izolate soja MV9R-1*

Variable Varijabla		Linear regression coefficient Koeficijent linearne regresije		
X	*Y*	slope nagib (a)	intercept odsječak (b)	correlation korelacija (r2)
potency index indeks moći 1/16	potency index indeks moći 4/16	0.99	0.02	0.99
potency index indeks moći 1/16	potency index indeks moći 16/16	0.99	0.01	0.98

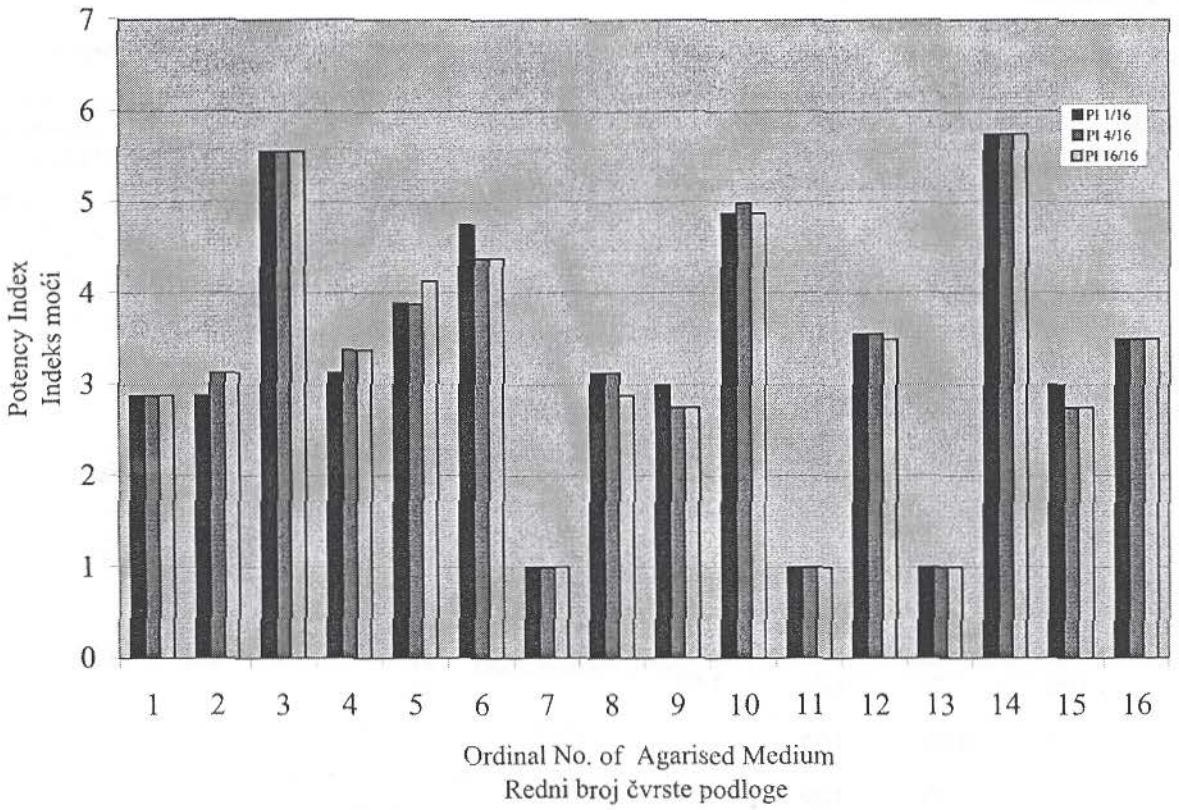

Fig. 7 – *Potency indexes of different isolates of strain MV9R-1.*
Slika 7 – *Vrijednosti indeksa moći različitih izolata soja MV9R-1.*

of strain MV9R-1, *i.e. investigated microbial population showed to be of highly homogenous properties.*

Conclusions

– efficiency of recognition of biologically active strains can be significantly increased when different culture conditions are applied,

– application of systems of different agarized media can be recommended as the first step in screening strains with antibiotic activity,

– degree of similarity between microbial isolates (particular strains) can be evaluated statistically comparing results of corresponding screening parameters.

ACKNOWLEDGMENT

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SAŽETAK

Optimirani odabir mikroorganizama s antibiotičkom aktivnošću

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Budući da proizvodnja antibiotika ovisi o svojstvima mikrobnog soja i uvjetima uzgoja, proučavani su učinci različitih uvjeta uzgoja na izražavanje tvorbe antibiotika, radi optimiranja metode odabira mikrobnih sojeva koji su sposobni proizvoditi antibiotike. Kako bi se ocijenila pouzdanost primjenjenih metoda odabira, roditeljski soj *Streptomyces rimosus* R6–500 i njegovi derivatni sojevi poznatih svojstava uzgojeni su pri različitim uvjetima uzgoja u skladu s primjenom različitih frakcijskih faktorskih planova. Antibiotičke aktivnosti određivane su mjerenjem zona inhibicije rasta test mikroorganizma *Bacillus cereus* ATCC 11778. Rezultati pokusa analizirani su statistički. Usporedbom rezultata dobivenih uzgojem na čvrstim agariziranim pločama s onima postignutim odgovarajućim dubinskim uzgojem u tikvicama na tresilici ustanovljeno je da postoji slaba korelacija između različitih metodologija. Svaki soj pokazao je svoju osobitost glede optimalne proizvodnje antibiotika pri testiranim uvjetima. Prema tome, potreban je niz testova da bi se povećala mogućnost izražavanja sposobnosti tvorbe antibiotika u sojeva nepoznatih svojstava. Za provedbu određenih različitih uvjeta uzgoja preporučljivo je primijeniti frakcijski faktorski plan za 8–16 kombinacija, ako se želi provesti odabir antibiotički aktivnih mikroorganizama u «prosijavanju» mješovitih populacija velikog broja različitih mikroorganizama.

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