Bull. Food Res. (Bratislava) Special issue, 71—80, 1986

Mutagen analysis by the SOS chromotest

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Summary. The work contains some characteristics of SOS chromotest such as: relationship between concentration of cells and mutagen sensitivity, influence of incubation time upon intensity of bacterial response, kinetic optimization of determination of indicating enzymes. Under the optimized assay conditions the mutagenicities of some known agents were determined and the results were compared with those obtained by Ames test. The mutagenic potency of ten new furylethylenes (amides and esters of 5-nitro-2-furylacrylic acid) was proved. The convenience of the SOS chromotest for the mutagenic testing of nitrovin in chicken meat and 5-nitro-2-furylacrylic acid, in wine was examined. The mutagenic activity of these compounds in the food-stuff samples does not differ markedly from the activity of pure compounds.

In order to test chemical agents from the view point of their ability to induce changes in the genetic material of cells and organisms, various models are used : bacteria, fungi, somatic mammalian cells, germ cells of higher plants and animals and intact animal and plant organisms. The fact that the chemical features of DNA are almost identical for all organisms authorizes us to use them for genetic analysis of any organism or its cells.

Bacteria are widely used as the indicator organisms in the test systems for occurrence of genetic toxins [13]. They offer practical advances and may provide insights into basic mechanisms of genotoxicity and their consequences. The preference of employing bacteria as testing organisms to search for mutagenicity and carcinogenity consists in their time and economical unpretentiousness as well as in good correlation to mammalian [13].

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Bacteria response to DNA damage by switching on the SOS system, which promotes repair of DNA and of other cellular functions [6]. The most competent study on this mechanism has been performed in E. coli. The basic structure of the SOS system is set of subordinate genes (SOS genes) that are repressed under the normal conditions by a single repressor (lex A protein) and are derepressed by a second regulator (rec A protein) in response to DNA damage. This fact has been implied in the construction of the so called „SOS chromotest" for the detection of mutagenic agents [4, 10, 11]. In this test system the expression of SOS genes by monitoring the level of ß-galactosidase in the sfi A : : lac Z fusion strain E. coli PQ 37 can be easily detected. To increase the response to certain DNA-damaging agents, the strain has been made deficient in excision repair (uvr A) and to allow better difusion of chemical compounds into cells, the lipopolysacharide (rfa) has been made also deficient.

The direct assay consists in incubation of the tested strain with agents in various concentrations. After some time which is necessary for protein synthesis ß-galactosidase activity is measured using o-nitrophenyl-ß-galactoside as chromosubstrate. The compounds tested may exert toxic effects in certain concentrations leading to the underestimation of induced ß-galactosidase activity. To avoid this possibility, the total protein synthesis during incubation period has been estimated. The strain has been made constitutive for alkaline phosphatase. This enzyme, non-inducible by DNA damaging agents, is assayed simultaneously with ß-galactosidase. The ratio of both activities is taken as a measure of the specific activity of ß-galactosidase.

In the present paper we describe some biological characteristics of SOS chromotest and the possibilities of its application in the analysis of mutagens.

Materials and methods

Chemicals. The structures of the studied mutagens are shown in Tables 1 and 3. Nitrofurylacrylic acid (Na salt) was obtained from Slovakofarma, Hlohovec, nitroquinoline-N-oxid kindly supplied Dr. Bahna, Institut of Experimental Onkology SAV, Bratislava, nitrovin was from Chemapol Praha and furylfuramid was a gift of Dr. T. Matsushima, Institut of Medical Sciences, University of Tokyo (Japan). Alkylesters of 5-nitro-2-furylacrylic acid were synthesized according to [5] and amides of 5-nitro-2-furylacrylic acid according to [12]. Enzyme substrates o-nitrophenyl-ß-D-galactopyranoside (ONPG) and *p*-nitrophenyl phosphate disodium (PNPP), were purchased from Lachema, Brno.

Microorganism. The strain E. coli K-12, recombinant PQ 37, granted by Dr. M. Hofnung (Institute Pasteur, Paris) has been used. The strain has genotype thr, leu, his-4, pyrD, thi, galE, galK (or galT), lac △U169, srl-300: : Tn10 (Tc^r), rpoB (Rf^r), rpsL (Sm^r), uvrA, rfa, trp : : MUC^+, sfiA : : Mud (ap, lac). It is constitutive for alkaline phosphatase, resistant for phage C21 and sensitive for sodium deoxycholate.

Media, buffers and reaction mixtures. Cultivation of bacteria proceeded in Lamp medium (10g tryptone, 5g yeast extract and 10g NaCl per 1) supplemented with ampicillin in 20 μg per ml. ß-Galactosidase was assayed in „Z buffer" containing 16.1g $Na_2HPO_4.7H_2O$, 5.5g $NaH_2PO_4.H_2O$, 0.75g KCl, 0.25g $MgSO_4.7H_2O$ and 2.7 ml ß-mercaptoethanol (pH 7) per 1. Alkaline phosphatase was assayed in „T buffer" containing 121g Tris-(hydroxymethyl)-aminomethane (TRIS, pH 8.8 adjusted with HCl). The next reaction solution used have been : 0.1% sodium dodecylsulphate, 2 mol HCl per 1, 1 mol Na_2CO_3 per 1, 2 mol TRIS per 1.

Test procedure. The fresh overnight culture of the strain from 0.05 ml of frozen culture added to 5 ml of the Lamp medium was 50 fold diluted with the same medium and was grown at 37°C with stirring in „L"-test tube of inner diameter 13 mm, till the value of $A_{600}^{1cm} = 0.2$ (2.2×10^8 cells per ml) was reached. The culture was then diluted 10 fold in the Lamp medium and the fractions of 0.6 ml were pipetted into Wassermann tubes containing the tested compounds in various concentrations (in volumes 10—70 μl). After two hours of incubation at 37°C the fractions of 0.1 ml were transferred into new tubes and the reactions were terminated by adding of the appropriate buffer. The first series were used for ß-galactosidase activity and the second one for the assay of alkaline phosphatase activity.

Enzyme assays. ß-Galactosidase was tested as follows. To 0.1 ml of the culture incubated for two hours with the tested chemical 0.9 of Z buffer was added. Cell membranes were disintegrated by adding 0.03 ml of 1% sodium dodecylsulphate and 0.05 ml of chloroform. The tubes were rigorously agitated for 10 sec and the tubes were then incubated at 28°C for 10 min. For ß-galactosidase assay 0.2 ml of stock solution (4mg per ml) of o-nitrophenyl-ß-galactoside (ONPG) was added. After appropriate time of incubation the reaction was stopped by addition of 0.65 ml Na_2CO_3 (1 mol per 1). The intensity of yellowish colour was measured against blank at 420 nm. Blank contained 20 μl of 100 fold diluted dimethylsulphoxide (DMSO) a solvent for tested agents as well as cells, which were not incubated at 37°C and to which the tested agents were not added.

The assay of alkaline phosphatase was done in the same way as the ß-galactosidase assay, except that Z buffer was replaced by T buffer and as the substrate *p*-nitrophenylphosphate (PNPP) was employed. The reaction was terminated by adding of 0.33 ml of 2 mol per 1 HCl. After 5 min pH of the reaction mixture was alkalized by adding of 0.33 ml of 2mol per 1 TRIS to restore the yellow colour, which intensity was measured at 420 nm.

The positive control for assays was nitrofurantoin.

The preparation of real food stuffs samples. To 10g of chicken meat 5 ml of distilled water was added and the mixture was homogenized in mortar with pestle. To this suspension 10 fold diluted 10 mmol/l nitrovin in DMSO was added and the mixture was further diluted by distilled water. 10 mmol/l 5-nitro-2-furylacrylic acid in DMSO was 10 fold diluted with wine and in further diluted with distilled water.

The evaluation of enzyme activities and mutagenicity. The enzyme activities were expressed in the units defined by [8] as follows.

$$\text{Number of units} = 1000\,\frac{A_{420}}{t.v.A_{600}}$$

A_{420} is the absorbance at 420 nm of the incubation mixture, A_{600} is the absorbance of the cell culture before the assay, t is the time of incubation in the presence of substrates (ONPG or PNPP) in the volume v of culture in ml.

The ratio R of the activities of *β-galactosidase and alkaline phosphatase* reflects the induction of the sfiA gene even when the inhibition of protein synthesis occurrs [10, 11]. This ratio can be calculated as follows

$$R = \frac{A_{420\beta} \cdot t_p}{A_{420p} \cdot t_\beta}$$

where t_β and t_p are the reaction times for the ß-galactosidase and alkaline phosphatase assay, respectively.

To compare the results obtained in different experiments, it is convenient to normalize the ratio R(c) dividing by the value R(o) in zero concentration of the compound tested. The induction factor I(c) is the ratio $\frac{R(c)}{R(o)}$. The SOS inducing potency (SOSIP) is calculated from the linear region of dose-response curve. The SOSIP is a single parameter which represents the induction factor per mass unit or per nmol of compound tested [10, 11].

Results and discussion

The introductory experiments in our work were focused to the closer characteristics of the SOS chromotest. We have dealed with the relationships between the concentration of cells in the test and their sensitivity to mutagens, with the influence of the time of the mutagen effect to the intensity of mutagenic reply and with kinetic optimization of the enzymes activities determination.

At first the growth curve of the tested strain *E. coli* K-12 has been constructed (time of doubling cca 75 min) and a relationship between the absorbance of suspension and the quantity of cells has been find out (Fig. 1). The relationship between the concentration of cells and their sensitivity towards the mutagens has been determined applying 3-(5-nitro-2-furyl)-acrylic acid (NFAA) (Fig. 2).

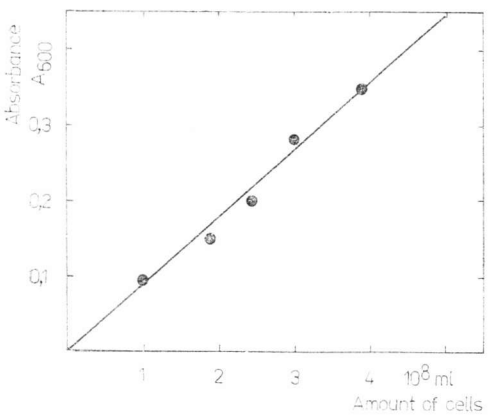


Fig. 1. Relationship between the optical density of suspension *E. coli* K-12 and the amount of cells. The cells were incubated at 37 °C in „L" test tubes under shaking. During growth A_{600} was measured densitometrically and the amount of cells was counted using Coulter Counter.

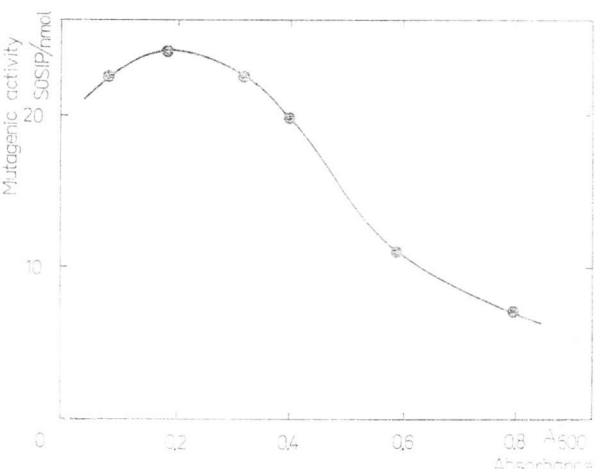


Fig. 2. Relation between concentration of cells of E. coli employed in the SOS chromotest and mutagenic response. The amid of 5-nitrofurylacrylic acid in concentration $1.6.10^{-7}$ — $1.6.10^{-6}$mol.l^{-1} was used as the mutagen. The cells were in concentration of 10^8—10^9 per ml. (Optical density at 600 nm in range 0.1—0.8).

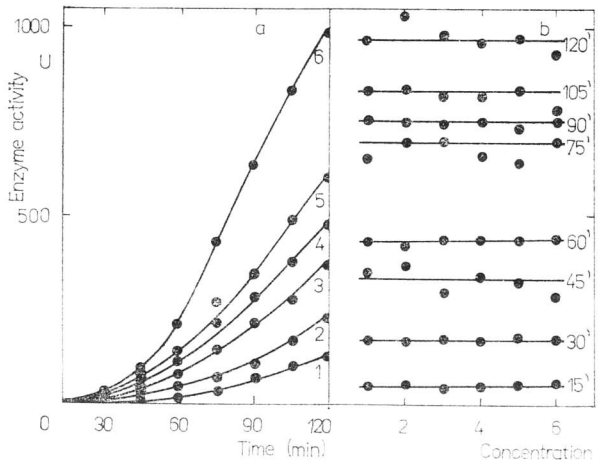


Fig. 3. Relationship between time of incubation of the mutagen with cells employed in the SOS chromotest and the level of indicating enzymes β-galactosidase (a) and alkaline phosphatase (b). The amid of 5-nitrofurylacrylic acid was used as the mutagen and was applied in final concentrations 0(1), $3.2.10^{-7}$(2), $6.4.10^{-7}$(3), $9.6.10^{-7}$(4), $1.6.10^{-6}$(5) and $3.2.10^{-6}$(6) mol.l^{-1}

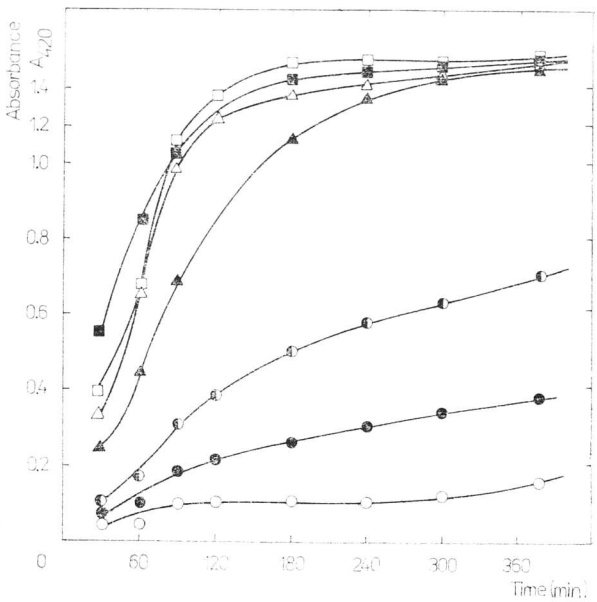


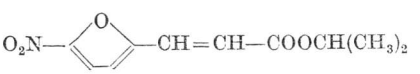
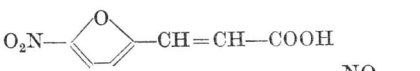
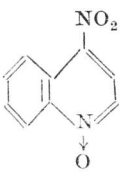
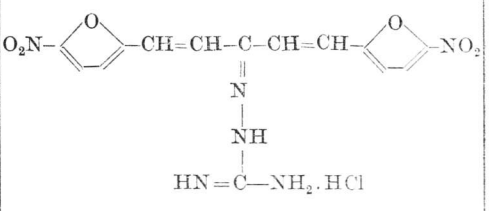
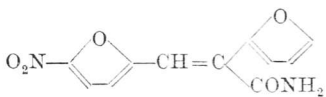
Fig. 4. Influence of incubation time of tested enzymes upon their activities. The nitrofurantoin ($1.6.10^{-7}$ — $1.6.10^{-6}$ mol.l^{-1}) was used as mutagen. The concentrations of nitrofurantoin: 0(○), $1.6.10^{-7}$(●), $3.2.10^{-7}$(◐), $6.4.10^{-7}$(▲), $9.6.10^{-7}$(△), $1.6.10^{-6}$(□) mol. l^{-1} for measuring of β-galactosidase activity. The alkaline phosphatase (■) was measured in $1.6.10^{-6}$mol.l^{-1} concentration of nitrofuratoin. The enzyme activities are expressed in units of colour density of solution (A_{420}).

With increasing amount of cells the SOSIP parameter decreases. The optimal cocentration of cells is $2.0—2.5.10^8$ per ml ($A_{600} = 0.2 — 0.25$). In further the influence of the affecting mutagen on the activity of indicating enzymes ß-galactosidase and alkaline phosphatase has been studied. The amid of NFAA has

been used in the role of mutagen. During two hours of incubation in the presence of mutagen the intensity of ß-galactosidase synthesis has been gradually increased. During the first hour of incubation the mutagenic response was not sufficient enough to be significant for analysis in further experiments (Fig. 3). Therefore as the time limit of incubation, two hours were fixed. The level of activity of alkaline phosphatase increased as the consequence of the further growth of cells in the optimal conditions.

The plot of relation between activity of enzymes and duration of incubation with substrate under the definite conditions has linear region, which enables to compare the activity of enzyme under the different concentrations of mutagen. Therefore we tried to find out the optimal time for testing of enzyme released from the cells of E. coli. The cells were incubated with nitrofurantoin, which was used as positive control. Rhe results obtained from this experiment are presented in Fig. 4, on the basis of which the optimal time of the incubation of enzymes with substrate was fixed to one hour.

Tab. 1. Structure of tested compounds

Common name	Structure
1 Furapropymidium	
2 Nitrofurylacrylic acid	
3 Nitroquinoline-N-oxid	
4 Nitrovin	
5 Furylfuramid (AF-2)	

For the verification of the SOS chromotest five chemicals with known mutagenic effects [7] were chosen (Table 1). Nitrovin is used as anabolic agent for

domestic animals, furylfuramide (AF 2) has been used in Japan as food preservative, 5-nitro-2-furylacrylic acid as the wine stabilizer, furapropymidium is used as the agent with antischistosomal effect and 4-nitroquinoline-N-oxid is a known compound developing skin cancer.

Table 2 contains the results obtained by testing the abovementioned compounds in the SOS chromotest and in the Ames test. Further we have compared the mutagenicity of two homogenous series of furylethylenes (amides and

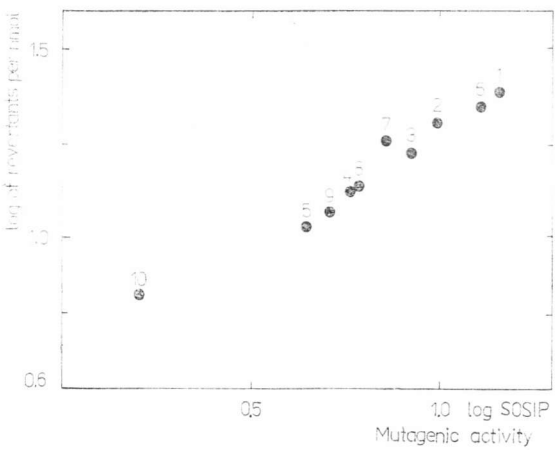


Fig. 5. Correlation of mutagenicity characterized by the SOS chromotest and the mutagenic potency determined by the Ames test of the compounds mentioned in Table 3.

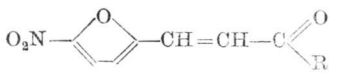
Tab. 2. Mutagenic activity of some chemical compounds characterized by SOS chromotest and Ames test. Results from the Ames test were obtained from citated references and are valid for the strain Salmonella typhimurium TA 98

Compound	SOSIP nmol	Ames test revertants nmol	Ref.
1 Furapropymidium	1.15	12	12
2 Nitrofurylacrylic acid	4.92	19	12
3 Nitroquinoline-N-oxid	16.20	2 100	10,11
4 Nitrovin	28.50	7 145	2
5 Furylfuramid (AF-2)	753.00	237.000	3

esters of 5-nitro-2-furylacrylic acid, Table 3) by using both SOS chromotest and the Ames test [1] (Fig. 5). With prolongation of the side alkyl chain of 5-nitrofurylacrylic acid esters the mutagenicity of the derivatives decreases. The correlation of mutagenicity tested by SOS chromotest and the Ames test was very good and this fact authorizes use of this test for detection of mutagens.

Furhter we have paid attention to some basic analytical characteristics of the SOS chromotest and to the possibilities of its application to the analysis

Tab. 3. Structure of tested esters and amides of 5-nitro-2-furylacrylic acid



No	R	No	R
1	$-OCH_3$	6	$-NHCH_3$
2	$-OC_2H_5$	7	$-NHC_2H_5$
3	$-OC_3H_7$	8	$-NHC_3H_5$
4	$-OC_4H_9$	9	$-NHC_4H_9$
5	$-OC_5H_{11}$	10	$-NHC_5H_{11}$

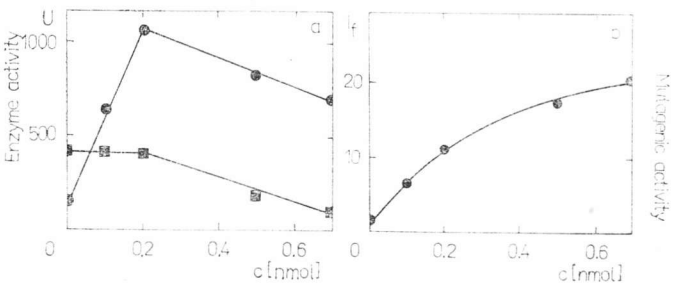


Fig. 6. Relationship between intensity of mutagenic response and concentration of the mutagen in the SOS chromotest. The amid of 5-nitrofurylacrylic acid in concentration $1.6.10^{-7}$—$1.6.10^{-6}$ mol.l^{-1} was used as mutagen. a — the activity of β-galactosidase (●) and alkaline phosphatase (■) in relation to concentration of mutagen. b — the relation of inducing factor I_f to concentration of mutagen.

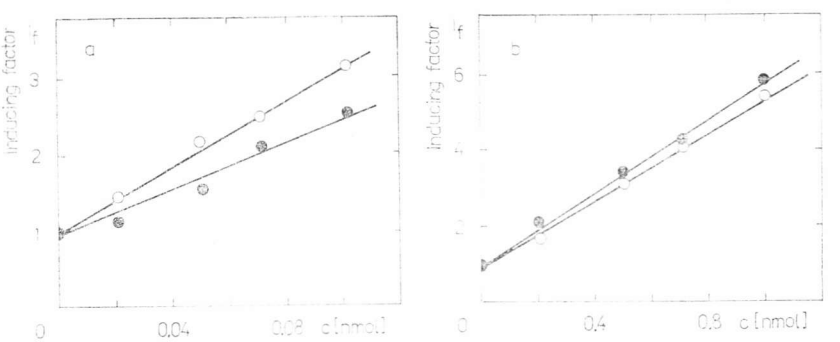


Fig. 7. Determination of mutagens in food-stuffs samples. a — nitrovin in chicken meat homogenate (●). The concentrations of mutagen $3.2.10^{-8}$—$1.6.10^{-7}$mol.l^{-1}. b — nitrofurylacrylic acid (NFAA) in wine (●), concentrations $3.2.10^{-7}$—$1.6.10^{-6}$mol.l^{-1}. Mutagenicity of alone compounds (○).

of mutagens in real food — stuffs samples. The dose response curve $I_f = t(n)$ has linear region, the slope of which is a measure of the mutagenic activity of the compound. This part of the curve could be described by the equation $I_f = a + bn$ (n is the amount of substance). For various chemicals different regions of linearity were found (furylethylenes : $n = 3.2.10^{-7} — 1.6.10^{-6}$mol. $.l^{-1}$ except of AF 2, where $n = 1.6.10^{-9} — 1.6.10^{-8}$mol.$l^{-1}$ and nitrovin with $n = 1.6.10^{-8} — 1.6.10^{-7}$mol.$l^{-1}$, nitrosoethylurea : $n = 3.2.10^{-4} — 3.2.10^{-1}$ mol.l^{-1}, aromatic compounds : $n = 1.6.10^{-8} — 1.6.10^{-7}$mol.$l^{-1}$). Higher concentrations were found to inhibit protein synthesis (Fig. 6).

In further we also studied mutagenicities of heterogenous and homogenous mixtures of the mutagens which may occur in the human environment. Occuring of mutagens in foodstuffs is especially dangerous to human health. That's why we tried to use the SOS chromotest for the detection of mutagenic compounds in the food-stuffs samples. We chose two examples : 5-nitro-2-furylacrylic acid in wine and nitrovin in chicken meat. Both chemicals were and are being used currently in the foodstuff preservation and production (NFAA in wine as antimicrobial agent and nitrovin for speeding up growth of domestic animals). The results obtained from this experiment (Fig. 7a,b) offer the possibility to determinate the occurrence of these chemicals in foodstuffs samples. However, it is necessary in each new case to verify the convenience of the SOS chromotest for analysis of appropriate mutagen in appropriate real sample.

The SOS chromotest did not enable to detect the mutagens only qualitatively, but also made possible to quantitate offects of mutagens which is its main advantage among the short-term tests which are used for determination of mutagenicity.

Acknowledgements. We are deeply grateful to Dr. Maurice Hofnung, Institut Pasteur, for providing the bacterial strain E. coli PQ 37.

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Мутагенный анализ хромотестом СОС

Резюме

В работе приводятся некоторые биологические характеристики хромотеста СОС (отношение между концентрацией клеток и их чувствительностью к мутагенам, влияние инкубационного периода на интенсивность бактериального ответа, кинетическая оптимализация определения индикаторных ферментов). В оптимализированных условиях была определена мутагенность известных соединений и результаты были сравнены с данными полученными с помощью Амес — теста. Дана характеристика мутагенного действия десяти новых фурилэтиленов (амидов и эфиров 5-нитро-2-фурилакриловой кислоты). Была проверена также уместность СОС хромотеста для анализа мутагенов в реальных пробах (нитровин в курином мясе, 5-нитро-2-фурилакриловая кислота в вине). Мутагенная активность этих соединений в приведенных пробах не отличается существенным образом от мутагенности самых веществ.

Mutagénna analýza SOS chromotestom

Súhrn

V práci sa uvádzajú niektoré biologické charakteristiky SOS chromotestu (vzťah medzi koncentráciou buniek a ich citlivosťou na mutagény, vplyv inkubačnej doby na intenzitu bakteriálnej odpovede, kinetická optimalizácia stanovenia indikačných enzýmov). Za optimálnych testovacích podmienok sa stanovili mutagenity známych zlúčenín a výsledky sa porovnali s údajmi získanými pomocou testu Ames. Určila sa mutagénna účinnosť 10 nových furyletylénov (amidov a esterov kyseliny 5-nitro-2-furylakrylovej). Vyskúšala sa aj vhodnosť SOS chromotestu na analýzu mutagénov v reálnych vzorkách (nitrovin v kuracom mäse, kyselina 5-nitro-2-furylakrylová vo víne). Mutagénna aktivita týchto zlúčenín v uvedených vzorkách sa významne nelíši od mutagenity samých látok.