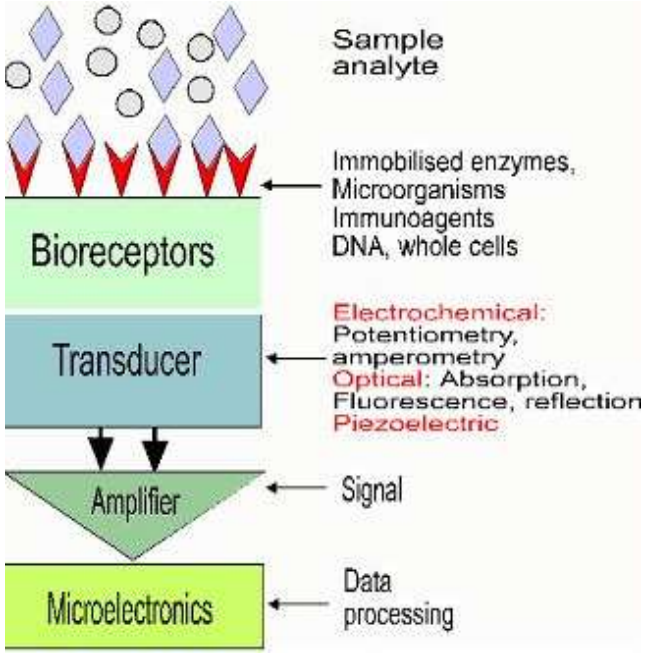
Molekularna biotehnologija: okoljske aplikacije

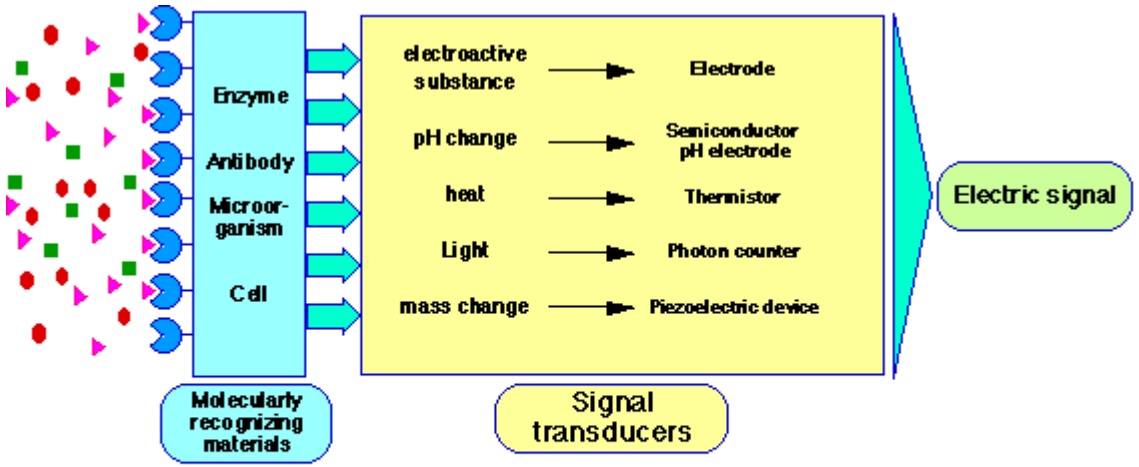
Biosenzorji

Biosenzor je analitska naprava, ki združuje biološki element za prepoznavanje tarčne snovi in fizikalni prenašalec (transducer), s katerim generiramo merljiv signal, ki je proporcionalen koncentraciji analita. Signal zaznavamo elektrokemijsko , optično, akustično, mehansko, kalorimetrično ali elektronsko, vrednost pa koreliramo s koncentracijo preiskovane snovi. Prvi tak senzor je bil za določanje koncentracije glukoze (Clarkov glukozni senzor; zasnova 1962). Aplikacije biosenzorjev segajo na področja javnega zdravja, monitoringa okolja, prehranske varnosti in državne varnosti.

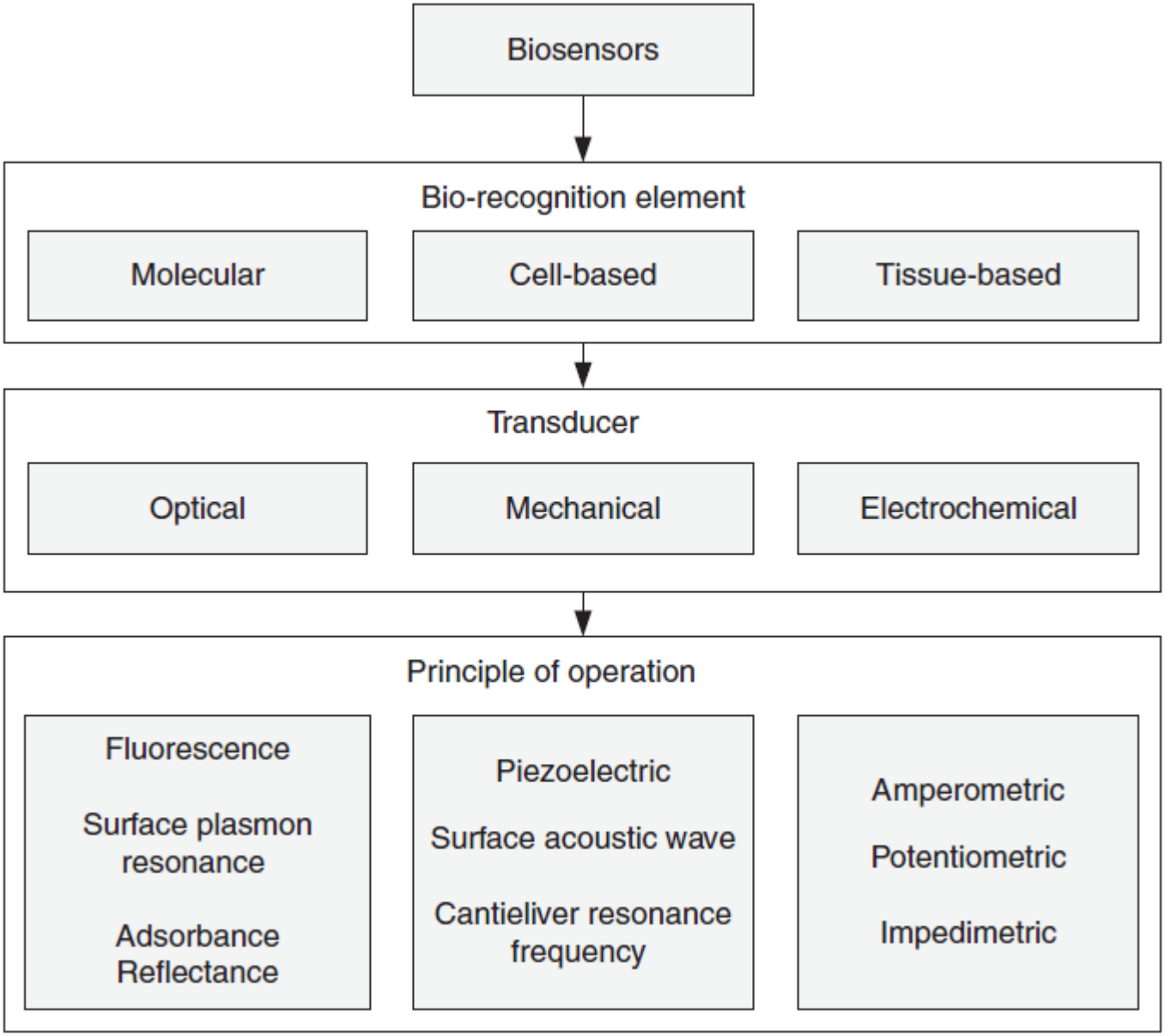


Brezcelični in celični biosenzorji

Biološki prepoznavni elementi so lahko kofaktorji, encimi, protitelesa, mikroorganizmi, organeli, celice višjih evkariontov, tkiva. Najpogosteje uporabljamo encime zaradi visoke specifičnosti in občutljivosti, vendar je izolacija čistih encimov zamuden in drag postopek, njihova uporaba in vitro pa lahko zmanjša aktivnost. Zato imajo prednost pred izoliranimi encimi mikrobne celice, saj take senzorske elemente ‚proizvajamo' preprosto z gojenjem. V primerjavi s celicami višjih evkariontov imajo prednost v tem, da omogočajo lažje genetsko spreminjanje, hkrati pa so bolj stabilne in dalj preživijo v pogojih poskusa. V celice lahko vgradimo več encimov / kofaktorjev in s tem pripravimo kombinirane detektorje za več snovi.



Principle of Biosensors



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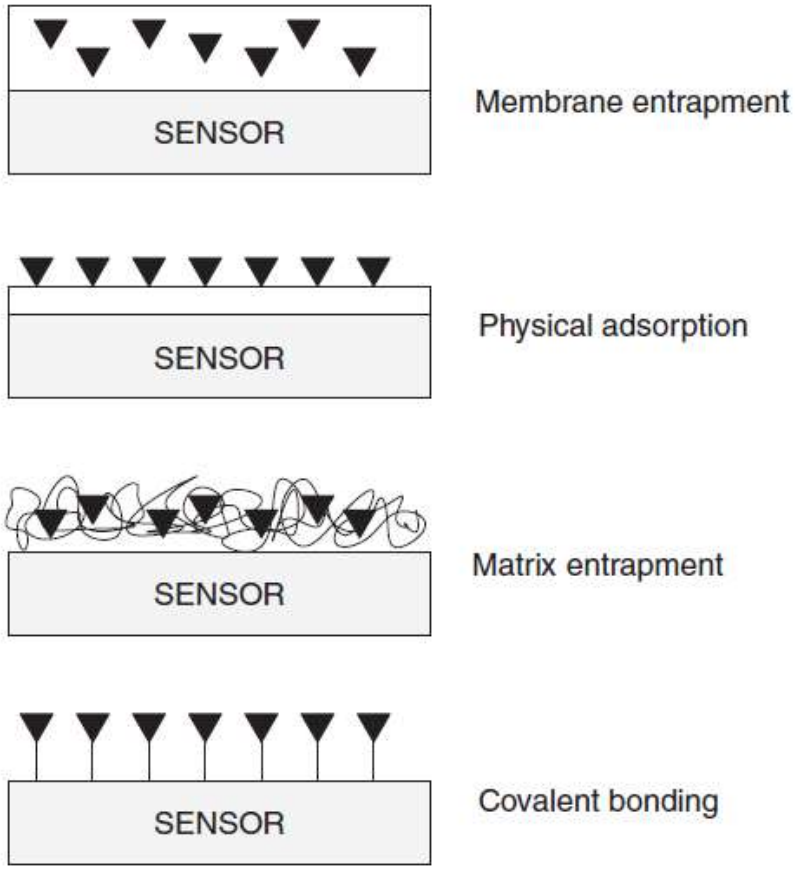
Biosenzorji: vezava na prenašalne elemente

Povezava s prenašalcem mora biti čimbolj tesna, kar dosežemo z imobilizacijo celic na prenašalec (adsorpcija, enkapsulacija, kovalentna vezava, zajem v matriks (entrapment), navzkrižno povezovanje (cross-linking)). Razvili so tudi kombinirane metode (npr. imobilizacija na prevodne polimere) in nove pristope (npr. mikrofluidika) z izboljšanimi lastnostmi senzorjev.



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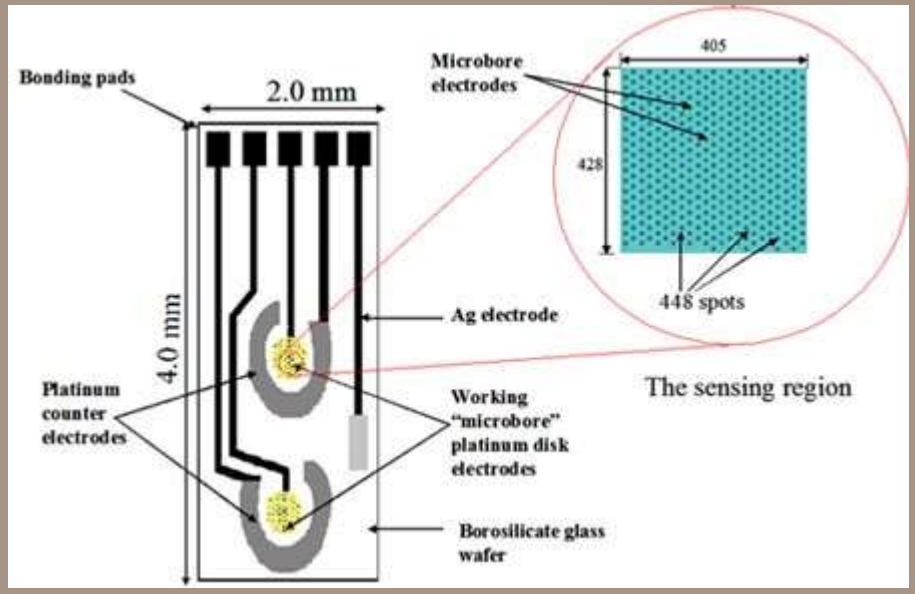


http://www.clemson.edu/c3b/inVivoOperation.html

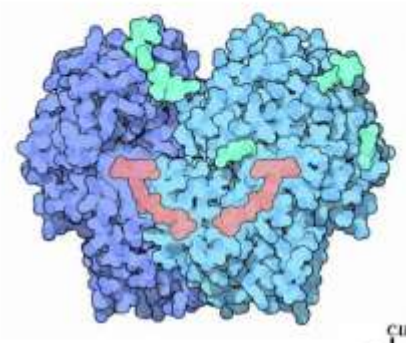
Schematic of the microfabricated, implantable, amperometric biochip device

Defining Events in the History of Biosensor Development

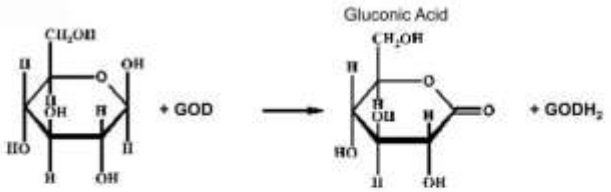
Date	Event
1916	First report on the immobilization of proteins: adsorption of invertase on activated charcoal
1922	First glass pH electrode
1956	Invention of the oxygen electrode
1962	First description of a biosensor: an amperometric enzyme electrode for glucose
1969	First potentiometric biosensor: urease immobilized on an ammonia electrode to detect urea
1970	Invention of the ion-selective field-effect transistor (ISFET)
1972–75	First commercial biosensor: Yellow Springs Instruments glucose biosensor
1975	First microbe-based biosensor First immunosensor: ovalbumin on a platinum wire Invention of the pO_2/pCO_2 optode
1976	First bedside artificial pancreas (Miles)
1980	First fiber optic pH sensor for in vivo blood gases
1982	First fiber optic-based biosensor for glucose
1983	First surface plasmon resonance (SPR) immunosensor
1984	First mediated amperometric biosensor: ferrocene used with glucose oxidase for the detection of glucose
1987	Launch of the MediSense ExacTech blood glucose biosensor
1990	Launch of the Pharmacia BIACore SPR-based biosensor system
1992	i-STAT launches hand-held blood analyzer
1996	Glucocard launched
1996	Abbott acquires MediSense for $867 million
1998	Launch of LifeScan FastTake blood glucose biosensor
1998	Merger of Roche and Boehringer Mannheim to form Roche Diagnostics
2001	LifeScan purchases Inverness Medical's glucose testing business for $1.3 billion
2003	i-STAT acquired by Abbott for $392 million
2004	Abbott acquires Therasense for $1.2 billion

Enzyme Biosensors. In: Methods in Biotechnology, Vol. 17: Microbial Enzymes and Biotransformations (2005)

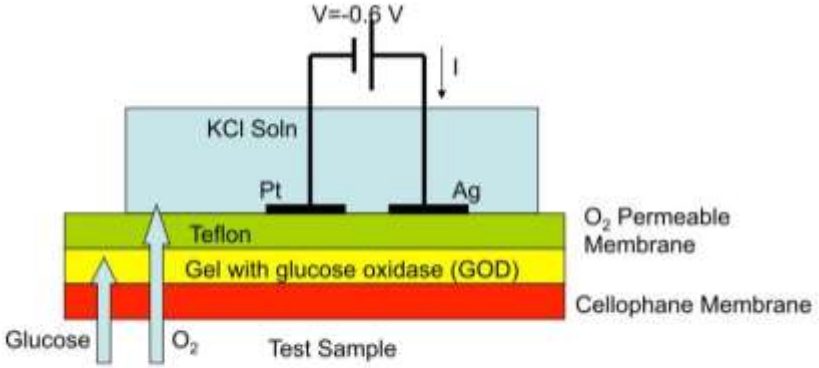
Glucose Oxidase



- Small stable enzyme
- Oxidizes glucose into glucolactone
- Converts O_2 to H_2O_2 in the process
- Found in honey
  - Natural preservative
  - H_2O_2 kills bacteria



$GODH_2 + O_2 \rightarrow GOD + H_2O_2$



Test sample contains Glucose and O_2

$[O_2]$ = CONSTANT

[Glucose] = VARIABLE

In presence of ENZYME (GOD)

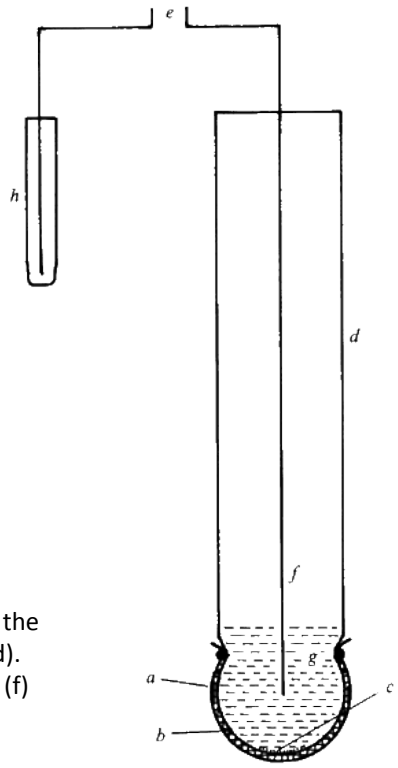
$$Glucose + O_2 \xrightarrow{GOD} GluconicAcid + H_2O_2$$

https://nanohub.org/resources/16947/watch?resid=17076

Zaznavanje z elektrokemijskimi tehnikami

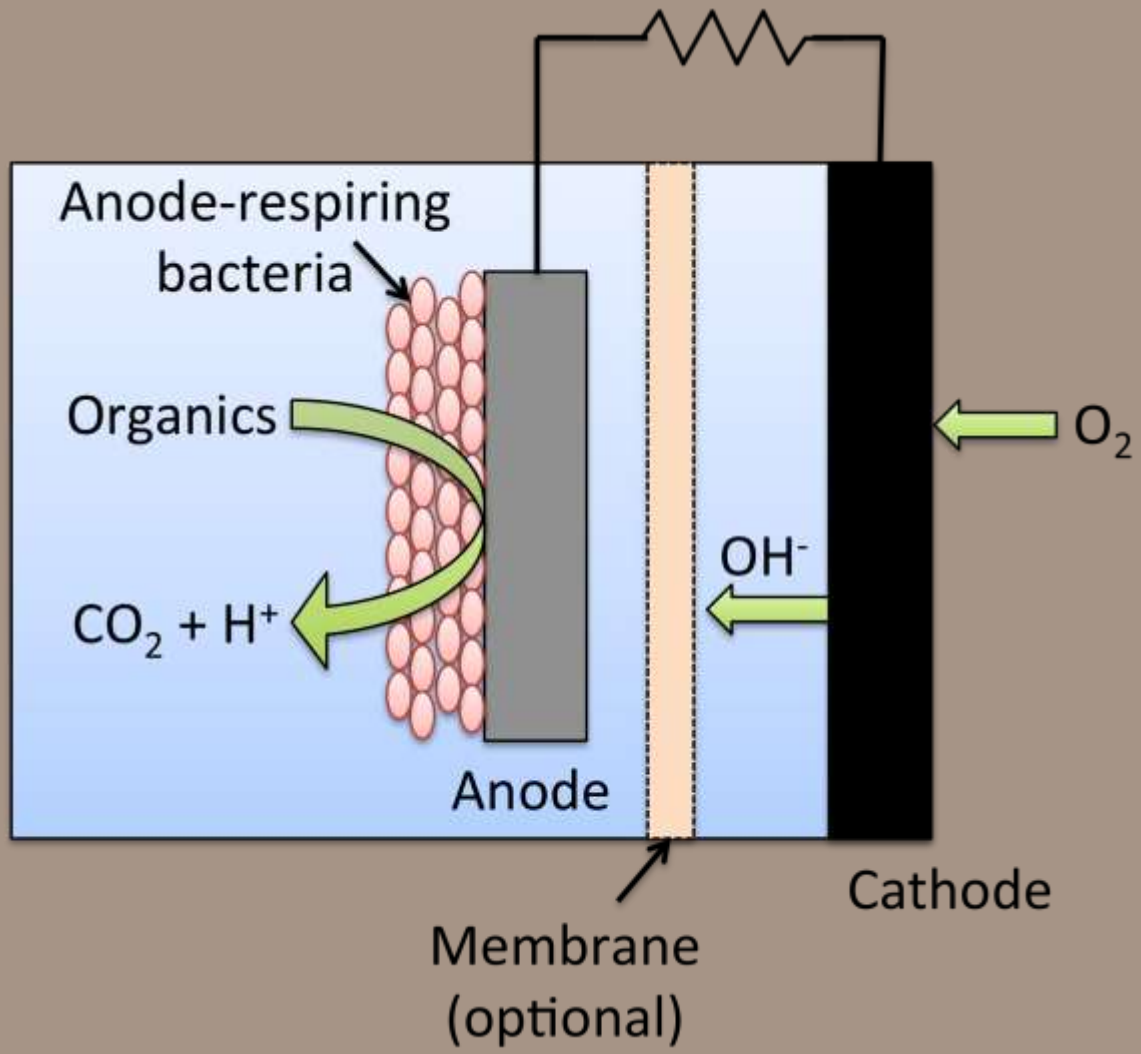
Zaznavamo lahko:

- Tok: pri danem potencialu in napetosti med referenčno in delovno elektrodo - merimo spremembe v prisotnosti tarčne snovi. Signal zaznavamo zaradi redukcije ali oksidacije elektroaktivnega metabolita ali intermediata na površini delovne elektrode. Občutljivost do ravni pA (ultraobčutljivi biosenzorji)
- Prevodnost: merimo spremembo prevodnosti v raztopini, kar je posledica proizvodnje ali porabe ionske zvrsti. Meritev je zelo hitra in ne zahteva referenčne elektrode, zato je primerna za miniaturizacijo. Slabost: nizka specifičnost (ne vemo, koncentracija katerega iona se je spremenila).
- Potencial: merimo spremembo potenciala med delovno in referenčno elektrodo. Prenašalec je največkrat selektivna elektroda (za plin ali ion), kar zagotavlja visoko občutljivost in selektivnost, vendar zahteva stabilno in natančno referenčno elektrodo.
- Napetost: spremljamo tok in potencial, zato je raven šuma nizka, zato je občutljivost visoka. Zaznavamo lahko različne snovi z različnimi vrhovi potencialov.
- Električno energijo, ki jo proizvede mikrobna gorivna celica. Metabolična aktivnost se po dodatku analita poveča (porabljanje) ali zmanjša (inhibitor metabolizma), s tem pa se spremeni izhodna energija.



A simple potentiometric biosensor. A semi-permeable membrane (a) surrounds the biocatalyst (b) entrapped next to the active glass membrane (c) of a pH probe (d). The electrical potential (e) is generated between the internal Ag/AgCl electrode (f) bathed in dilute HCl (g) and an external reference electrode (h).

http://www1.lsbu.ac.uk/water/enztech/potentiometric.html



http://www.thevoltreport.com/microbial-fuel-cell-to-clean-water-while-making-clean-energy/

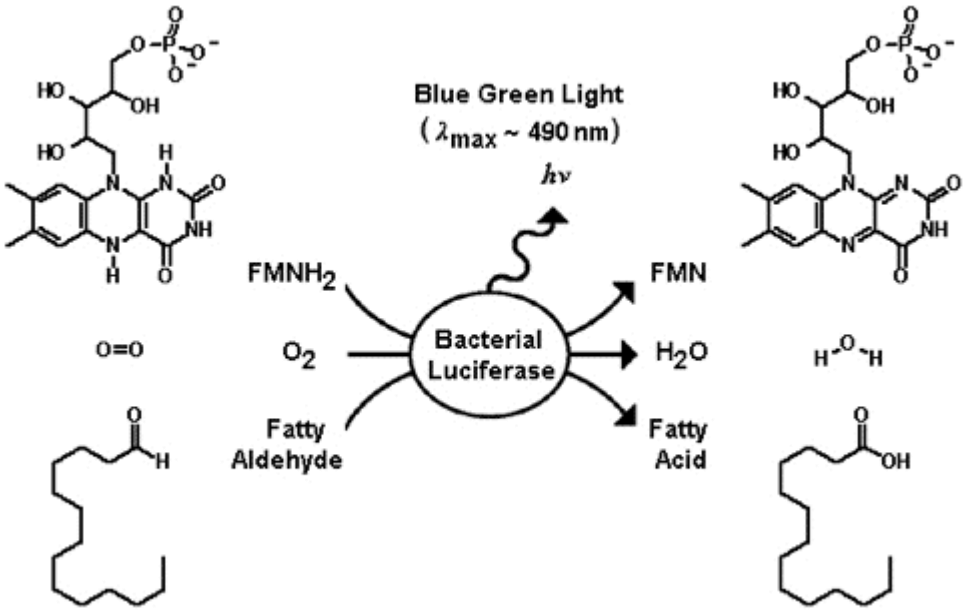
Zaznavanje z optičnimi tehnikami

Ob stiku mikroorganizma (največkrat GSO) z analitom pride do od doze odvisne spremembe signala, ki ga zaznavamo kot fluorescenco, svetlobo (luminiscenca), barvo (kolorimetrija). Največkrat delujejo na principu reporterskega gena, ki ga regulira inducibilni promotor. Optične tehnike so zelo primerne za visokozmogljive presejalne analize.

Fluorescenčni biosenzorji so uporabni in vivo (celica proizvaja fluorescirajočo molekulo) ali in vitro (fluorescirajoče molekule dodamo in spremljamo njihovo intenziteto po delovanju analita).

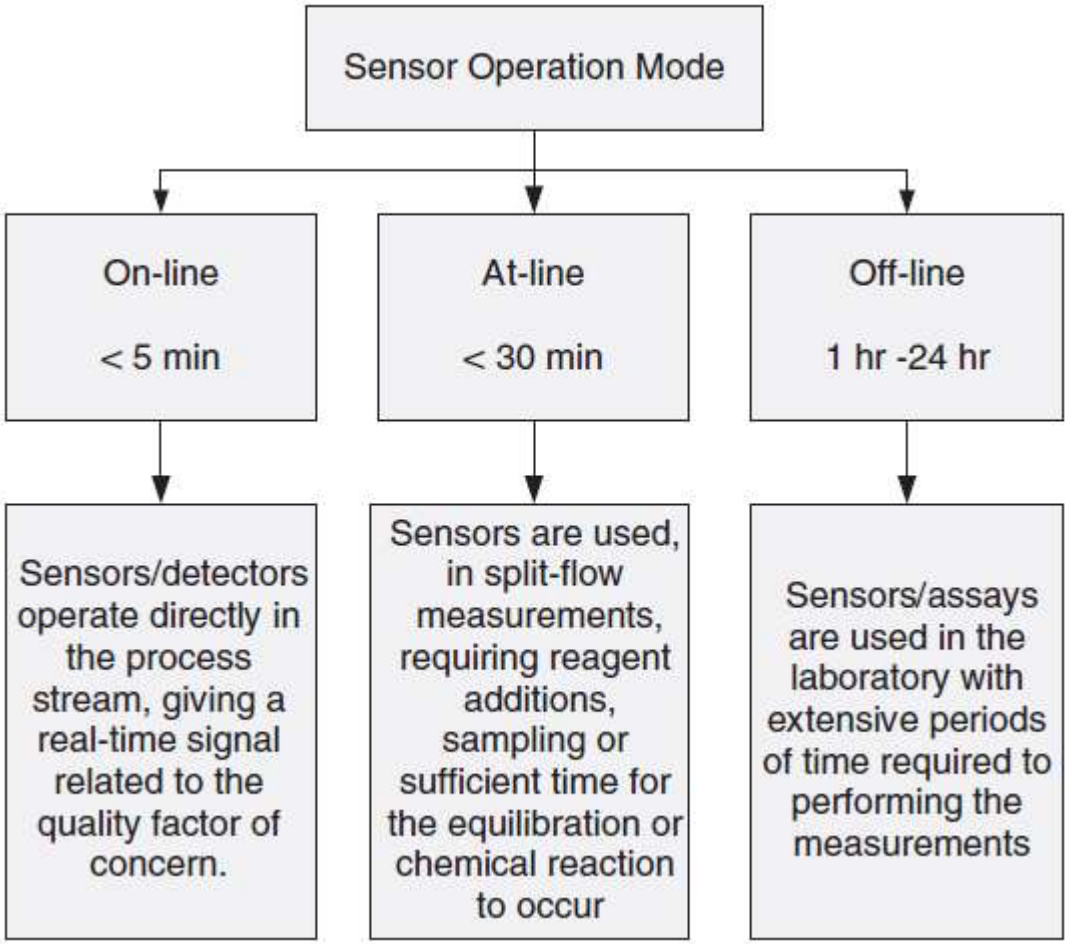
Kolorimetrične tehnike zahtevajo uporabo kromogenega substrata, ki se zaradi metabolične aktivnosti mikroba pretv ori v obarvano spojino.

Bioluminiscenčni biosenzorji temeljijo na delovanju luciferaz (npr. geni lux pri bakterijah).



Bioluminescence reactions in bacteria. Oxygen is used by luciferase to reduce long-chain aldehyde and reduced flavine mononucleotide to emit blue-green light.

https://microbewiki.kenyon.edu/index.php/Bioluminescent_Bacteria_in_Human_Settings



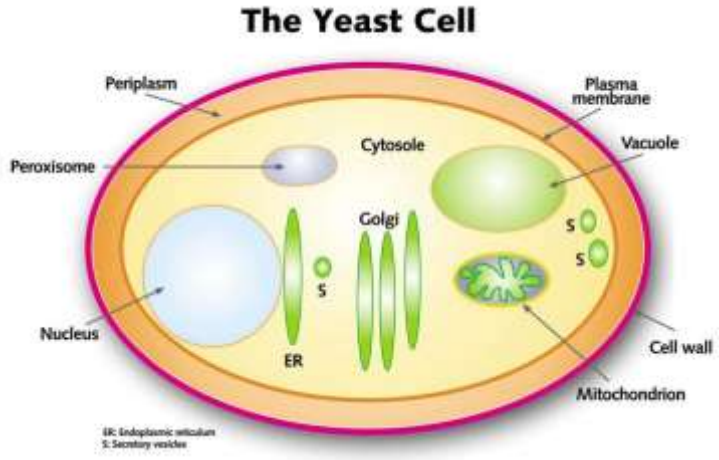
http://packagingtech.net/34-biosensor-technology-for-food-packaging-applications.html

Mikrobni biosenzorji

Za mikrobne biosenzorje uporabimo celice, ki so žive, ali pa so permeabilizirane in s tem neviabilne.
Pri živih celicah spremljamo spremembe v metabolizmu, pri čemer je analit substrat ali inhibitor.
Permeabilizirane celice so cenejši nadomestek izoliranih encimov. GS mikroorganizmi imajo uvedene gene za luminiscenco ali za fluorescirajoče proteine.

Celotne celice uporabljamo predvsem takrat, ko analit prehaja v notranjost in tam deluje na respiracijske procese (asimilacija – npr. vitamini, hranila, kisik,... ali inhibicija – okoljski polutanti). V primerjavi z encimskimi senzorji lahko pri mikrobnih ozko grlo predstavlja prenos snovi skozi membrano. Zato imajo permeabilizirane celice lahko prednost pred živimi. Permeabilizacijo dosežemo s fizikalnimi (zamrzovanje in tajanje), kemijskimi (organska topila ali detergenti) ali encimskimi (lizocim, papain) pristopi.

Celice lahko uporabimo brez permeabilizacije, če so encimi periplazemski (invertaza in katalaza pri kvasovkah, ureaza in fosfataze pri bakterijah). Z genskim inženirstvom lahko dosežemo, da se nek citoplazemski encim usmeri na drugo mesto v celici (periplazma ali na membrani).



Problem mikrobnih biosenzorjev je njihova nižja specifičnost v primerjavi z encimskimi, saj so v celici lahko prisotni številni encimi, ki lahko delujejo na iste ali podobne substrate.
Pri permeabiliziranih celicah je problem lahko odsotnost nizkomolekularnih efektorjev, ki so se zaradi naluknjanja membran sprostili iz celice.

Table 1
Microbial biosensors for environmental applications[a]

Analyte	Microorganism	Transducer/immobilisation	Detection limit	Reference
BOD	*Trichosporum cutaneum*	Miniature oxygen electrode (UV cross-linking resin (ENT-3400)	0.2–18 mg/l	Yang et al. (1996)
BOD	*T. cutaneum*	Miniature oxygen electrode array (photo cross-linkable resin)	<32 mg/l	Yang et al. (1997)
BOD	*T. cutaneum*	Oxygen electrode (entrapment)	10–70 mg/l	Marty et al. (1997)
BOD	*Ps. putida*	Oxygen electrode (adsorption on porous nitro cellulose membrane)	>0.5 mg/l	Chee et al. (1999)
BOD	Activated sludge (mixed microbial consortium)	Oxygen electrode/flow injection system (entrapped in dialysis membrane)	>3.5 mg/l	Liu et al. (2000)
BOD	Salt tolerant mycelial yeast *A. adeninivorans* LS3	Oxygen electrode (PVA)	2.61–524 mg/l	Tag et al. (2000)
Bioavailable organic carbon in oxic sediments	Yeast cells	Oxygen electrode (PVA)	Microscale	Neudoerfer and Meyer (1997)
Aniomic surfactants (linear alky benzene sulfonates (LAS)	LAS degrading bacteria isolated from activated sludge	Oxygen electrode, (reactor type sensor, ca-alginate)	<6 mg/l	Nomura et al. (1994)
Acrylamide; acrylic acid	*Brevibacterium* sp.	Oxygen electrode (free cells)	0.01–0.075 and 0.01–0.1 g/l	Ignatov et al. (1997)
Phenolic compounds	*Ps. putida*	Oxygen electrode (reactor with cells adsorbed on PEI glass)	100 μM	Nandakumar and Mattiasson (1999a)
Nitrite	*Nitrobacter vulgaris* DSM10236	Oxygen electrode (adsorption on Whatman paper)	>10 μM	Reshetilov et al. (2000)
Cyanide	*S. cerevisiae*	Oxygen electrode (PVA)	0.15–15 nM	Ikebukuro et al. (1996)
Chlorophenols	*Rhodococcus* sp.; *Trichosporon beigelii*	Oxygen electrode (PVA)	0.004–0.04 and 0.002–0.04 mM	Riedel et al. (1993, 1995)
3-Chloro-benzoate	*Ps. putida*	Oxygen electrode (PVA)	40–200 μM	Riedel et al. (1991)
Chlorinated and brominated hydrocarbons (1-chlorobutane and ethylenebromide)	*Rhodococcus* sp. DSM 6344	Ion selective electrodes (alginate)	0.22 and 0.04 mg/l	Peter et al. (1996)
Polycyclic aromatic hydrocarbons (Naphthalene)	*Sphingomonas yanoikuyae* B1 or *Ps. fluorescens* WW4	Oxygen electrode (polyurethane based hydrogel)	0.01–3.0 mg/l	Koenig et al. (1996, 1997a)
Organophosphate nerve agents (paraxon, methyl parathion, diazinon)	GEM[b] *E. coli* (organophosphorous hydrolase)	Potentiometric (adsorption on electrode surface)	0.055–1.8, 0.06–0.91 and 0.46–8.5 mM	Mulchandani et al. (1998a)
Organophosphate nerve agents (paraxon, parathion, coumaphos)	GEM[b] *E. coli* (organophosphorous hydrolase)	Fiber-optic (agarose)	0.0–0.6, 0.0–0.03 and 0.0–0.075 mM	Mulchandani et al. (1998b)
Pollutants such as diuron and mercuric chloride	*Synechococcus* sp. PCC 7942	Photoelectrochemical (photo cross linkable PVA bearing styrylpyridium group)	0.2 and 0.06 μM	Rouillon et al. (1999)
Herbicides[c] (diuron and atrazine)	Chloroplast/thylakoid membranes	Pt-electrode in microelectrochemical cell (photo cross linkable PVA bearing styrylpyridium group	2×10^{-5} and 2×10^{-4} mM	Rouillon et al. (1995)
Mono and polyphenols[c] (atrazine)	Potato (*S. tuberosum*) slices (polyphenol oxidase inhibition)	Oxygen electrode (tissue slice sandwitched between membranes)	20–130 μM	Mazzei et al. (1995)

[a] Polyvinyl alcohol.
[b] Genetically engineered microbes.
[c] Tissues or cellular organelle based.

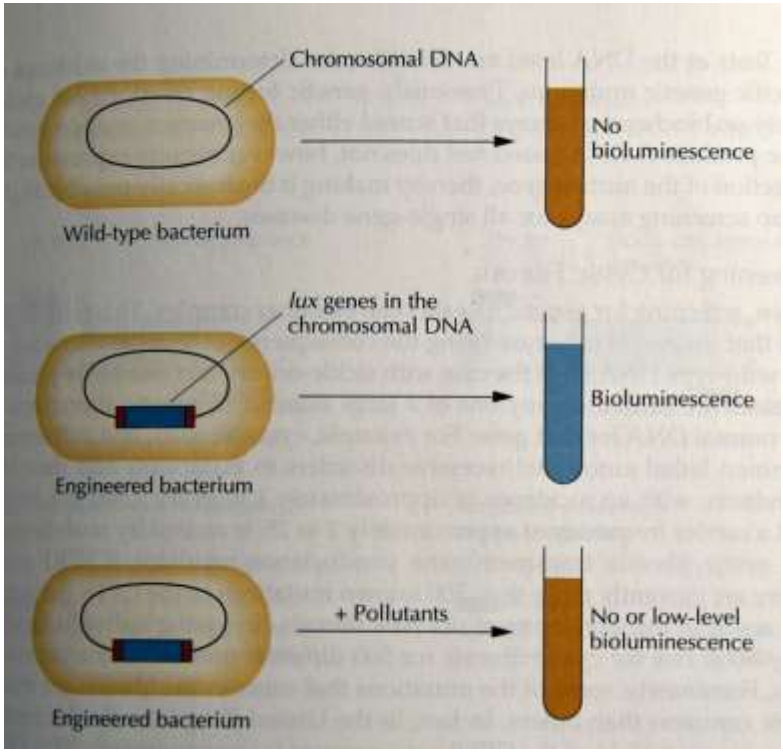
BOD = biological / biochemical oxygen demand
(biološka / biokemijska potreba po kisiku, BPK)
Količina kisika, potrebna za zadostitev biokemične oksidacije v določenem deležu vode pri dani temperaturi in časovnem obdobju. BPK je indeks deleža organskega onesnaženja v vodi. (Vir: LBC)

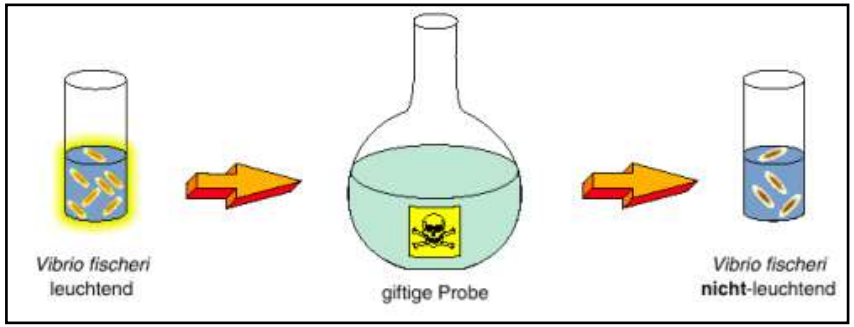
Applications of microbial biosensors in food, fermentation and allied fields

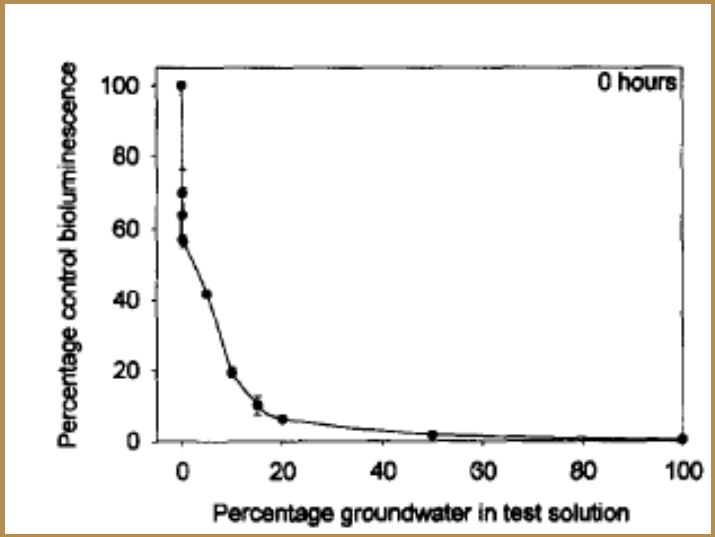
Analyte	Microorganism	Transducer/immobilisation	Detection limit	Reference
Alcohol	*Candida vini*	Oxygen electrode (porous acetyl cellulose filter)	2×10^{-2} -2×10^{-1} mM	Mascini et al. (1989)
Glucose	*A. niger* (glucose oxidase)	Oxygen electrode (entrapment in dialysis membrane)	>1.75 mM	Katrlik et al. (1996)
Glucose, sucrose, lactose	*G. oxydans* (D-glucose dehydrogenase),*S. cerevisiae* (invertase), *K. marxianus* (β-galactosidase)	Oxygen electrode (gelatine)	up to 0–0.8 mM	Svitel et al. (1998)
Sugars (glucose)	Psychrophilic *D. radiodurans*	Oxygen electrode (agarose)	0.03–0.55 mM	Nandakumar and Mattiasson (1999b)
Short chain fatty acids in milk (butyric acid)	*A. nicotianae* (acyl-CoA oxidase)	Oxygen electrode (Polyvinyl alcohol)	0.11–1.7 mM	Ukeda et al. (1992a,b)
Short chain fatty acids in milk (butyric acid)	*A. nicotianae* (acyl-CoA oxidase)	Oxygen electrode (Ca-alginate)	9.5–165.5 μM	Schmidt et al. (1996)
Phosphate	*Chlorella vulgaris*	Oxygen electrode (polycarbonate membrane)	8–70 mM	Matsunaga et al. (1984)
CO_2	CO_2 utilizing autotropic bacteria (*Pseudomonas*)	Oxygen electrode (bound on cellulose nitrate membrane)	0.2–5 mM	Suzuki and Karube (1987)
Vitamin B-6	*S. uvarum*	Oxygen electrode (adsorption on cellulose nitrate membrane)	0.5–2.5 ng/ml	Endo et al. (1995)
Vitamin B-12	*E. coli*	Oxygen electrode (trapped in porous acetyl cellulose membrane)	$5–25\times10^{-9}$ mM	Karube et al. (1987)
Peptides (aspartame)	*B. subtilis*	Oxygen electrode (filter paper strip and dialysis membrane)	0.07–0.6 mM	Renneberg et al. (1985)
Phenylalanine	*P. vulgaris* (Phenylalanine deaminase)	Amperometric oxygen electrode (Ca-alginate)	2.5×10^{-2}–2.5 mM	Liu et al. (1996)
Pyruvate	*Streptococcus faecium* (Pyruvate dehydrogenase complex)	CO_2 gas sensing electrode (direct immobilisation on sensor membrane)	0.22–32 mM	Di Paolantonio and Rechnitz (1983)
Tyrosine	*A. phenologenes* (Tyrosine-phenol lyase)	NH_3 gas sensing electrode (direct immobilisation on sensor membrane)	8.2×10^{-2}–1.0 mM	Di Paolantonio and Rechnitz (1982)
Enalapril maleate (angiotensin)	*B. subtilis*	Oxygen electrode	–	Fleschin et al. (1998)

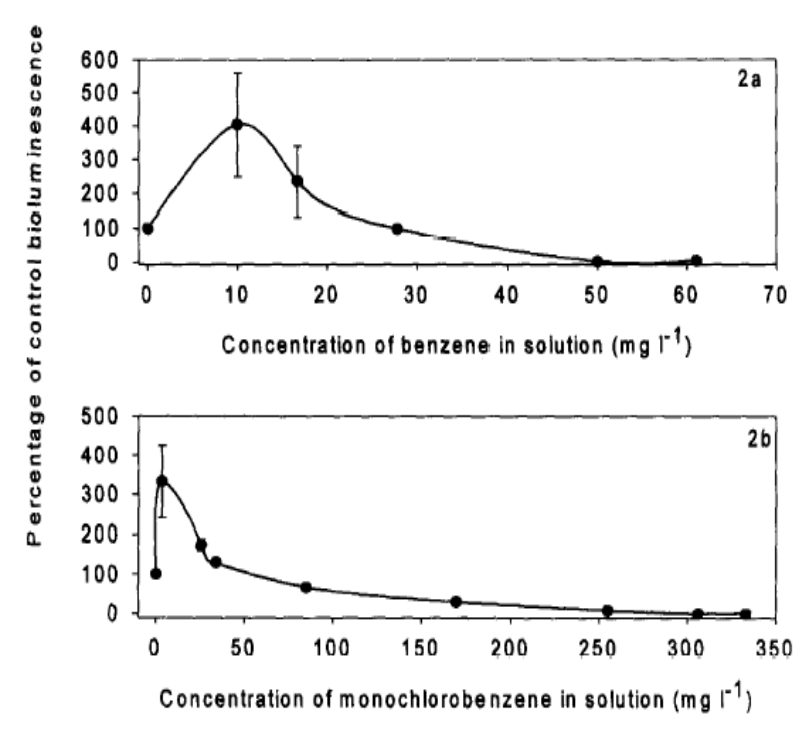
Senzorski organizmi

Potencialno nevarne snovi v okolju je mogoče zaznavati brez drage tehnološke opreme. Detekcijo lahko opravimo z ustrezno prilagojenimi (mikro)organizmi, npr. z bioluminiscečnimi bakterijami, kakršna je morska bakterija *Vibrio fischeri*. Za delo s sladko vodo so prenesli gene za bioluminiscenco (*lux*CDABE) prenesli v genom talne bakterije *Pseudomonas fluorescens*. Prenesli so jih brez promotorja, zato so luminiscenco opazili samo v primerih, ko je do vključitve prišlo tik za nekim dovolj močnim promotorjem. Izbrali so tiste, ki so kazale normalno rast in ki so močno luminiscirale. Testirali so jih na različne polutante (inkubacija 15 min v raztopinah organskih spojin ali kovin, nato meritev na luminometru).






0 hours
Percentage control bioluminescence
100
80
60
40
20
0
0
20
40
60
80
100
Percentage groundwater in test solution


Percentage of control bioluminescence
2a
600
500
400
300
200
100
0
0
10
20
30
40
50
60
70
Concentration of benzene in solution (mg l-1)
2b
500
400
300
200
100
0
0
50
100
150
200
250
300
350
Concentration of monochlorobenzene in solution (mg l-1)

Luminescence-based genetically engineered microbial biosensors

Application	Microorganism	Reference
Monitoring toxicity of compounds to eukaryotes	*S. cerevisiae* was genetically modified to express firefly luciferase	Hollis et al. (2000)
On-line monitoring of microbial growth	*E. coli* engineered for constitutive bioluminescence	Marincs (2000)
Toxicity of Zn, Cu and Cd, alone or in combination	*E.coli* HB101 and *Ps. fluorescens* 10586 genetically modified with *luxCDABE*	Preston et al. (2000)
Polycyclic aromatic hydrocarbons	*Ps. fluorescens* HK44 genetically modified with *luxCDABE*	Webb et al. (1997), Sayler et al. (1999), Ripp et al. (2000)
Ecotoxicity assessment of organotins and their initial breakdown products (tributyltin, dibutyltin, triphenyltin and diphenyltin)	Microtox and *luxCDABE* modified *Ps. fluorescens*	Bundy et al. (1997)
Ethanol as a model toxicant	*E. coli* TV1061, harboring the plasmid pGrpELux5	Gu et al. (1996), Rupani et al. (1996)
Monitoring of biocides	Bioluminescent strain of *E. coli* produced by recombinant DNA technology	Fabricant et al. (1995)
Metals, solvents, crop protection chemicals etc	*E. coli* heat shock promoters, *dnaK* and *grpE* were fused with *lux* genes of *V. fischeri*	Van Dyk et al. (1994)
Identifying constraints to bioremediation of BTEX-contaminated sites[a]	*luxCDABE* modified *Ps. fluorescens*	Sousa et al. (1998)
Assessment of the toxicity of metals in soils amended with sewage sludge	*luxCDABE* modified *Ps. fluorescens*	McGrath et al. (1999)

[a] Benzene, toluene, ethylbenzene,xylene.

Monitoring okolja

Spremljanje parametrov okolja vključuje tako fizikalne (npr. temperatura, vlaga, sevanje), kemijske (npr. konc. O_2, prisotnost kontaminantov) in biološke značilnosti (mikrobi, višji organizmi). Določamo trenutni status ali ugotavljamo trende kakovosti.

Okoljske vzorce lahko pregledujemo sistematično, da bi odkrili morebitna prikrita mesta kontaminacije, ali pa natančneje spremljamo samo določena mesta, kjer sumimo, da so za okolje nevarne snovi že prisotne. Uporaba mikrobov je možna in situ ali v laboratoriju, uporaba rastlin pa je predvsem primerna v naravi. Rastline, ki zaznajo prisotnost nevarnih snovi, lahko te tudi razgrajujejo (fitomonitoring in fitoremediacija). Primer nevarnih snovi: pesticidi, ki jih uporabljajo v kmetijstvu, hormonski motilci.

Monitoring se začne z izdelavo načrta, ki je odvisen od namena monitoringa. Sledi odvzem vzorcev (različne tehnike, tudi oddaljeno vzorčenje in meritve) in njihova analiza, sporočanje rezultatov itd.

Bioremediacija

Bioremediacija: biološki načini zmanjševanja strupenih odpadkov iz okolja.
Za okolje nevarne organske snovi odstranjujejo predvsem s sežiganjem, kar pa je drago, vprašljivo pa je tudi, ali pri sežigu ne nastajajo drugi okolju nevarni produkti. Ksenobiotiki: okolju tuje, sintetične in potencialno nevarne snovi. Primeri so: herbicidi, pesticidi, hladilne tekočine, topila,…

Okoli 1965 so odkrili, da naravni talni mikrobi lahko razgrajujejo nekatere tipe organskih molekul. Najbolj zmogljive so se izkazale bakterije iz rodu *Pseudomonas*, skupaj več kot 100 različnih organskih molekul, od tega nekateri sevi več podobnih spojin, iz katerih črpajo ogljik (edini vir C).

Metabolične poti, ki omogočajo razgradnjo organskih molekul, so zapisane bodisi na kromosomu, bodisi na plazmidu (enem ali več), kar je pogosteje. Na spekter tarčnih molekul lahko vplivamo s tem, da med seboj parimo seve s posameznimi lastnostmi oz. izoliramo in prenašamo ustrezne plazmide.

Table 13.1 *Pseudomonas* plasmids, their degradative pathways, and their sizes

Name of plasmid	Compound(s) degraded	Plasmid size (kb)
SAL	Salicylate	60
SAL	Salicylate	72
SAL	Salicylate	83
TOL	Xylene and toluene	113
pJP1	2,4-D	87
pJP2	2,4-D	54
pJB3	2,4-D	78
CAM	Camphor	225
XYL	Xylene	15
pAC31	3,5-Dichlorobenzoate	108
pAC25	3-Chlorobenzoate	102
pWWO	Xylene and toluene	176
NAH	Naphthalene	69
XYL-K	Xylene and toluene	135

Adapted from Cork and Krueger, *Adv. Appl. Microbiol.* 36:1–66, 1991.
Plasmids with the same name encode a similar degradative pathway even though they have different sizes and were described in different laboratories. 2,4-D, 2,4-dichlorophenoxyacetic acid.

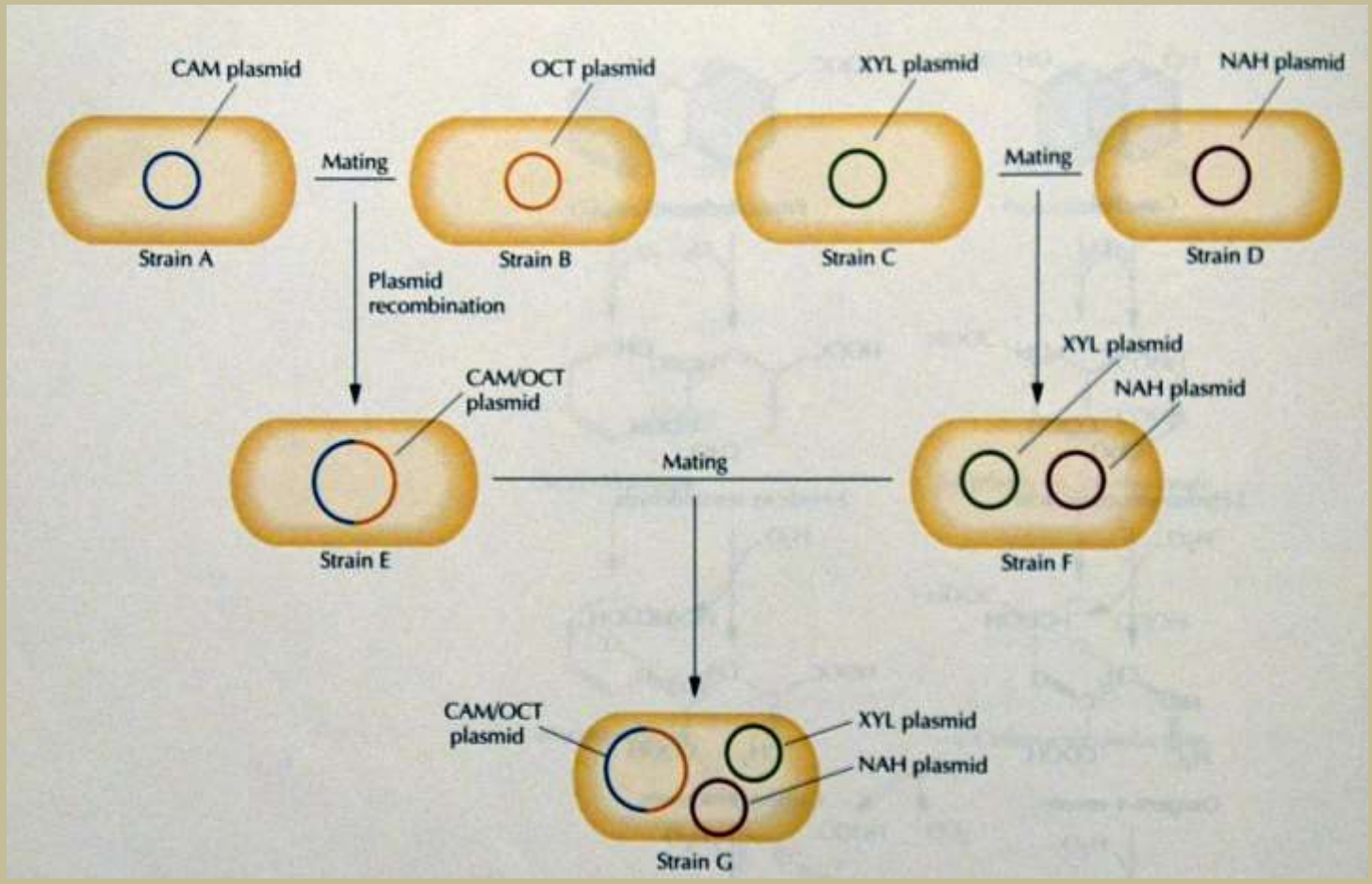

CAM plasmid
OCT plasmid
XYL plasmid
NAH plasmid
Mating
Mating
Strain A
Strain B
Strain C
Strain D
Plasmid recombination
CAM/OCT plasmid
XYL plasmid
NAH plasmid
Mating
Strain E
Strain F
CAM/OCT plasmid
XYL plasmid
NAH plasmid
Strain G

Table 13.2 Generation time of wild-type (nontransformed) and transformed psychrotrophic strains of *P. putida* on salicylate or toluate as the sole carbon source at various temperatures

Temp (°C)	Generation time (h) for:		
	Wild-type + salicylate	Transformant + salicylate	Transformant + toluate
37	No growth	No growth	No growth
30	2.2	2.5	2.0
25	2.1	3.2	1.3
20	2.6	3.8	1.9
15	3.2	4.2	2.9
10	6.3	5.6	3.3
5	13.9	12.9	12.2
0	18.6	18.1	24.4

Adapted from Kolenc et al., *Appl. Environ. Microbiol.* **54**:638–641, 1988.

The wild-type strain is unable to utilize toluate for growth at any temperature because it lacks the enzymes to metabolize this compound.

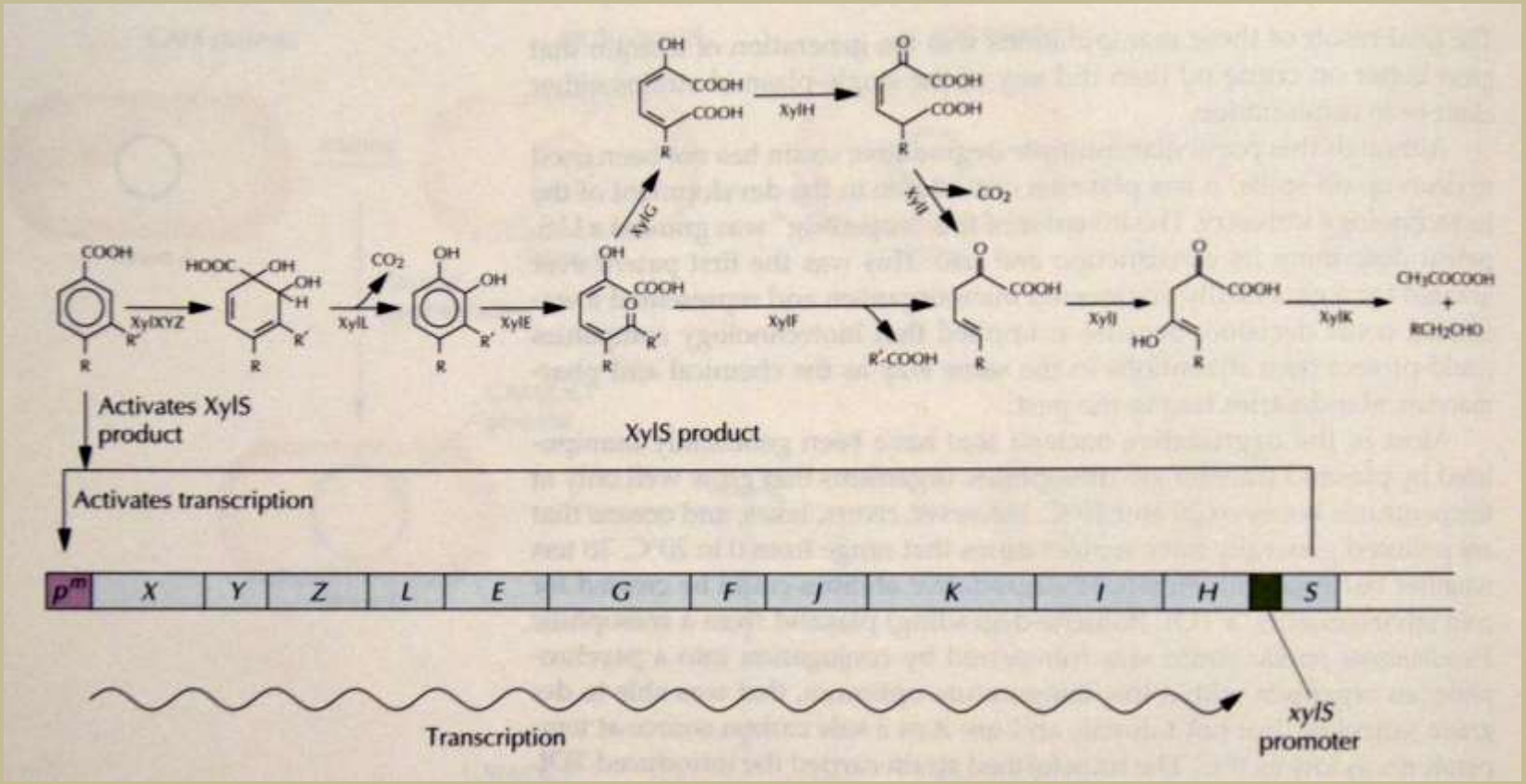
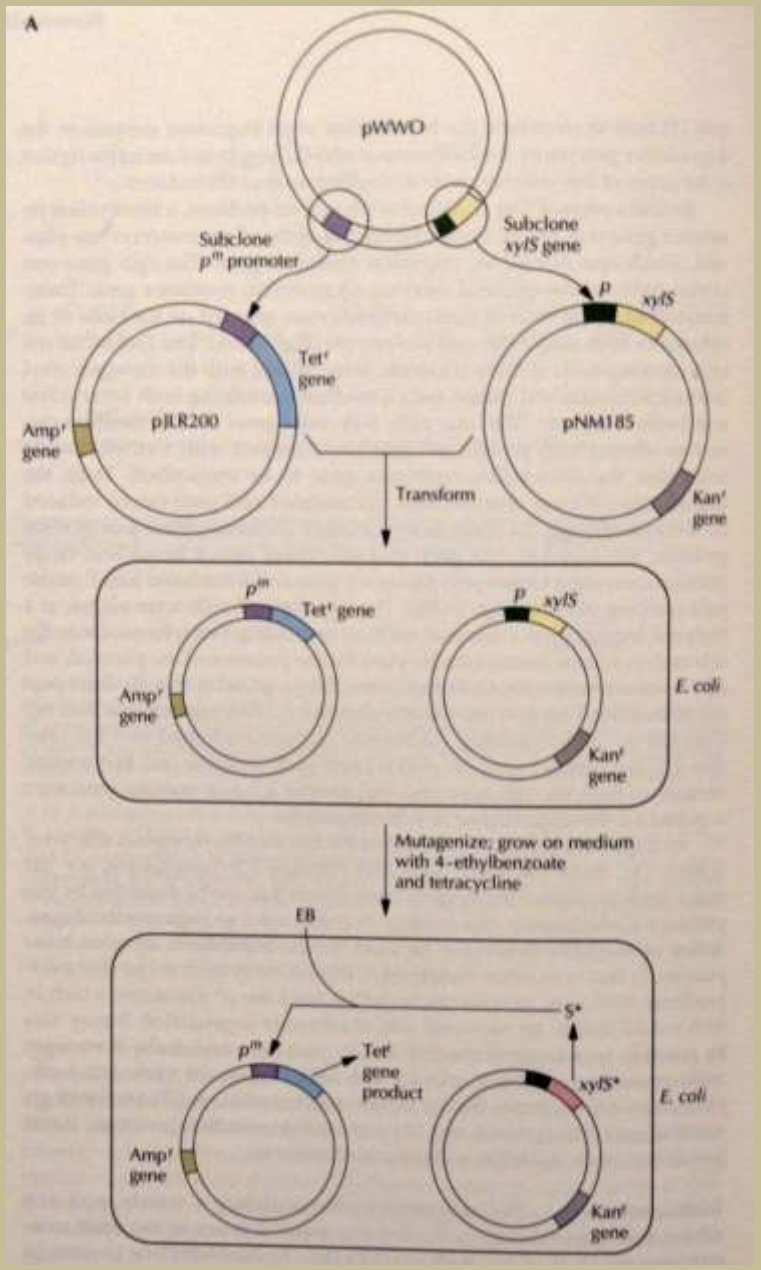
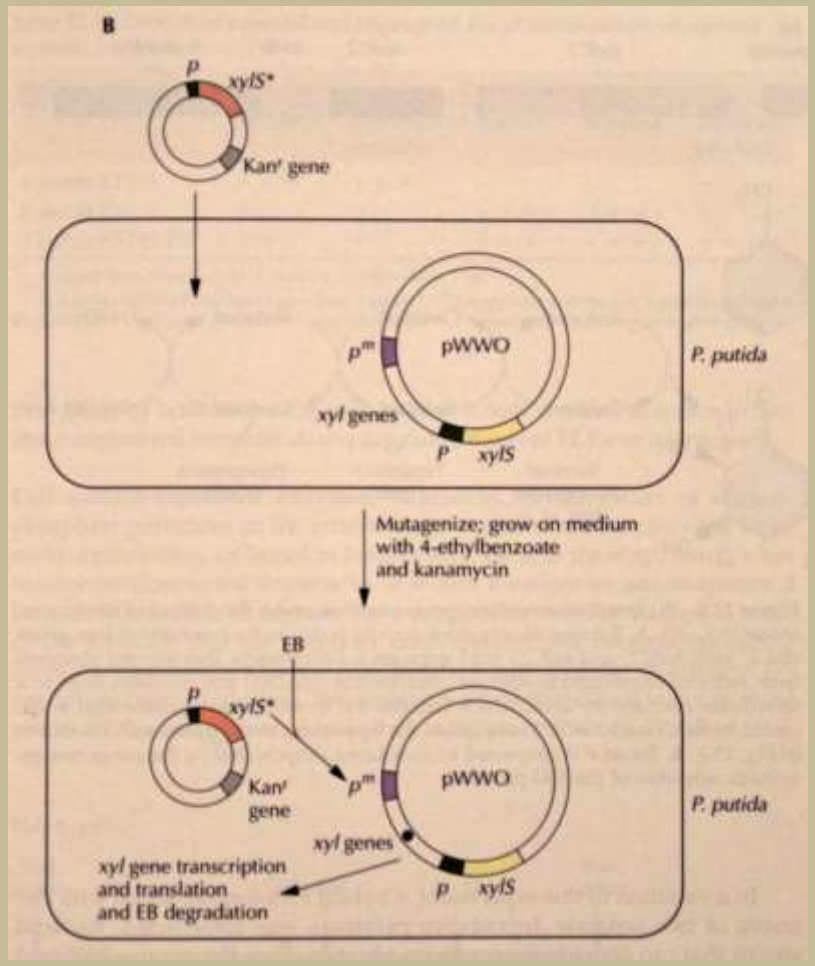


Figure 13.6 The *meta*-cleavage pathway and the *xyl* operon of the toluene- and xylene-degrading plasmid pWWO. Transcription of the *xyl* operon is controlled by the p^m promoter, which is regulated by the XylS gene product, which must be activated by one of the initial pathway substrates. The genes from *xylX* to *xylH* are under the control of the p^m promoter. The *xylS* gene, which is not part of this operon, is constitutively expressed. Some of the primary substrates are benzoate, where R and R' = H; 3-methylbenzoate, where R = H and R' = CH3; 3-ethylbenzoate, where R = H and R' = CH2CH3; and 4-methylbenzoate, where R = CH3 and R' = H. The *xylXYZ* genes encode toluene dioxygenase; *xylL* encodes dihydroxycyclohexadiene carboxylate dehydrogenase; *xylE* encodes catechol 2,3-dioxygenase; *xylF* encodes hydroxymuconic semialdehyde hydrolase; *xylG* encodes hydroxymuconic semialdehyde dehydrogenase; *xylH* encodes 4-oxalocrotonate tautomerase; *xylI* encodes 4-oxalocrotonate decarboxylase; *xylJ* encodes 2-oxopent-4-enoatehydratase; and *xylK* encodes 2-oxo-4-hydroxypentonate aldolase.


A
pWWO
Subclone
p^m promoter
Subclone
xylS gene
p
xylS
Tet^r
gene
Amp^r
gene
pJLR200
pNM185
Transform
Kan^r
gene
p^m
Tet^r gene
p
xylS
Amp^r
gene
Kan^r
gene
E. coli
Mutagenize; grow on medium
with 4-ethylbenzoate
and tetracycline
EB
S*
p^m
Tet^r
gene
product
xylS*
Amp^r
gene
Kan^r
gene
E. coli


B
p
xylS*
Kan^r gene
p^m
pWWO
xyl genes
p
xylS
P. putida
Mutagenize; grow on medium
with 4-ethylbenzoate
and kanamycin
EB
p
xylS*
Kan^r
gene
p^m
pWWO
xyl genes
xyl gene transcription
and translation
and EB degradation
p
xylS
P. putida

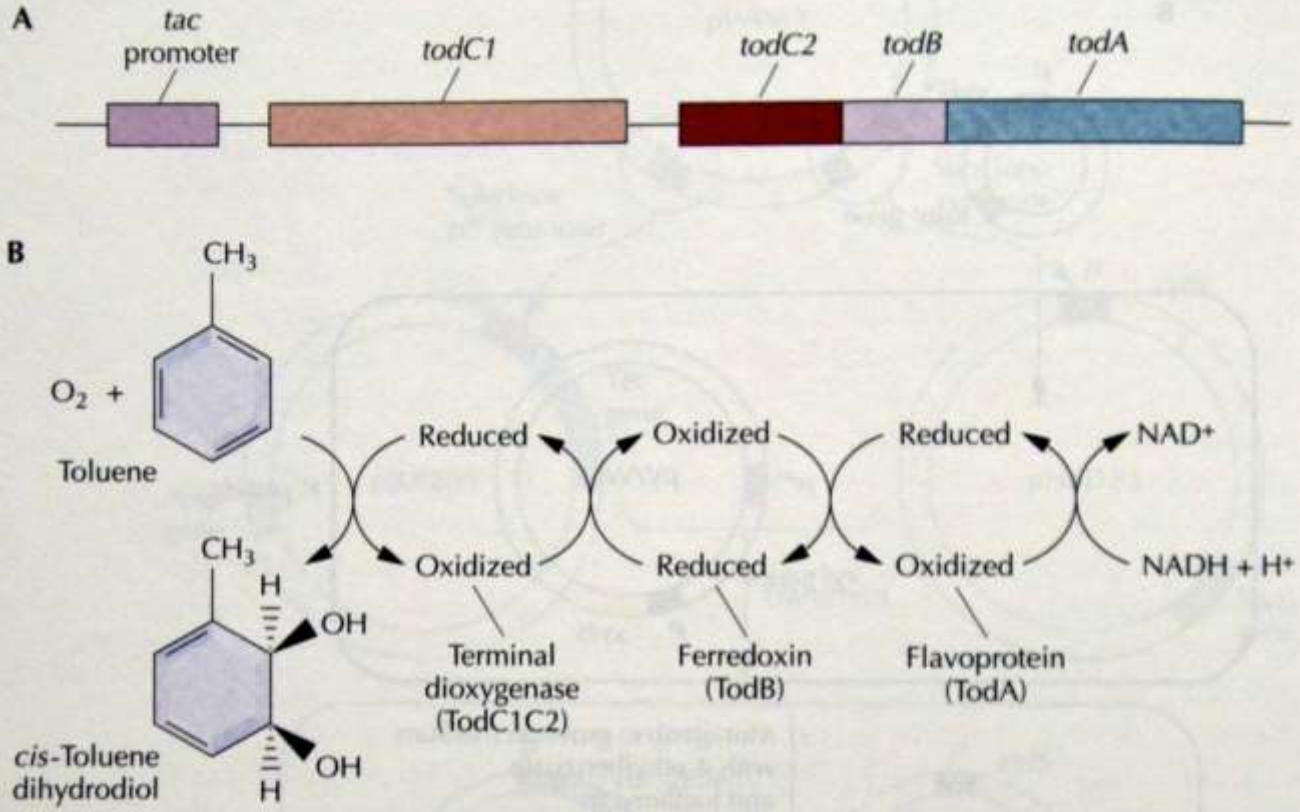


Figure 13.8 A cloned toluene dioxygenase operon under the control of the *tac* promoter in *E. coli*. **A.** Toluene dioxygenase activity is due to the products of four genes (*todA*, *todB*, *todC1*, and *todC2*). *todA* encodes a flavoprotein that accepts electrons from reduced nicotinamide adenine dinucleotide (NADH) and transfers them to a ferredoxin encoded by *todB*, which reduces the terminal dioxygenase that is encoded by *todC1* and *todC2*. These genes are equivalent to the genes *xylXYZ* shown in Fig. 13.7. **B.** Toluene is converted to *cis*-toluene dihydrodiol by the concerted enzymatic activities of the Tod proteins.

Table 13.3 Growth of parental and engineered *Pseudomonas* strains on various aromatic compounds

Strain	Growth on:				
	Biphenyl	Diphenyl-methane	Toluene	Benzene	Trichloro-ethylene
P. putida KF715	+ + +	+ + +	−	−	−
P. putida F1	−	−	+ + +	+ + +	+
P. putida KF715-D5	+ +	+	+ + +	+ + +	+ + +

Adapted from Suyama et al., *J. Bacteriol.* **178**:4039–4046, 1996.

In *P. putida* KF715-D5, the *bphA1* gene from *P. putida* KF715 is replaced with the *todC1* gene from *P. putida* F1. Symbols: + + +, good growth; + +, moderate growth; +, poor growth; −, very poor or no growth.

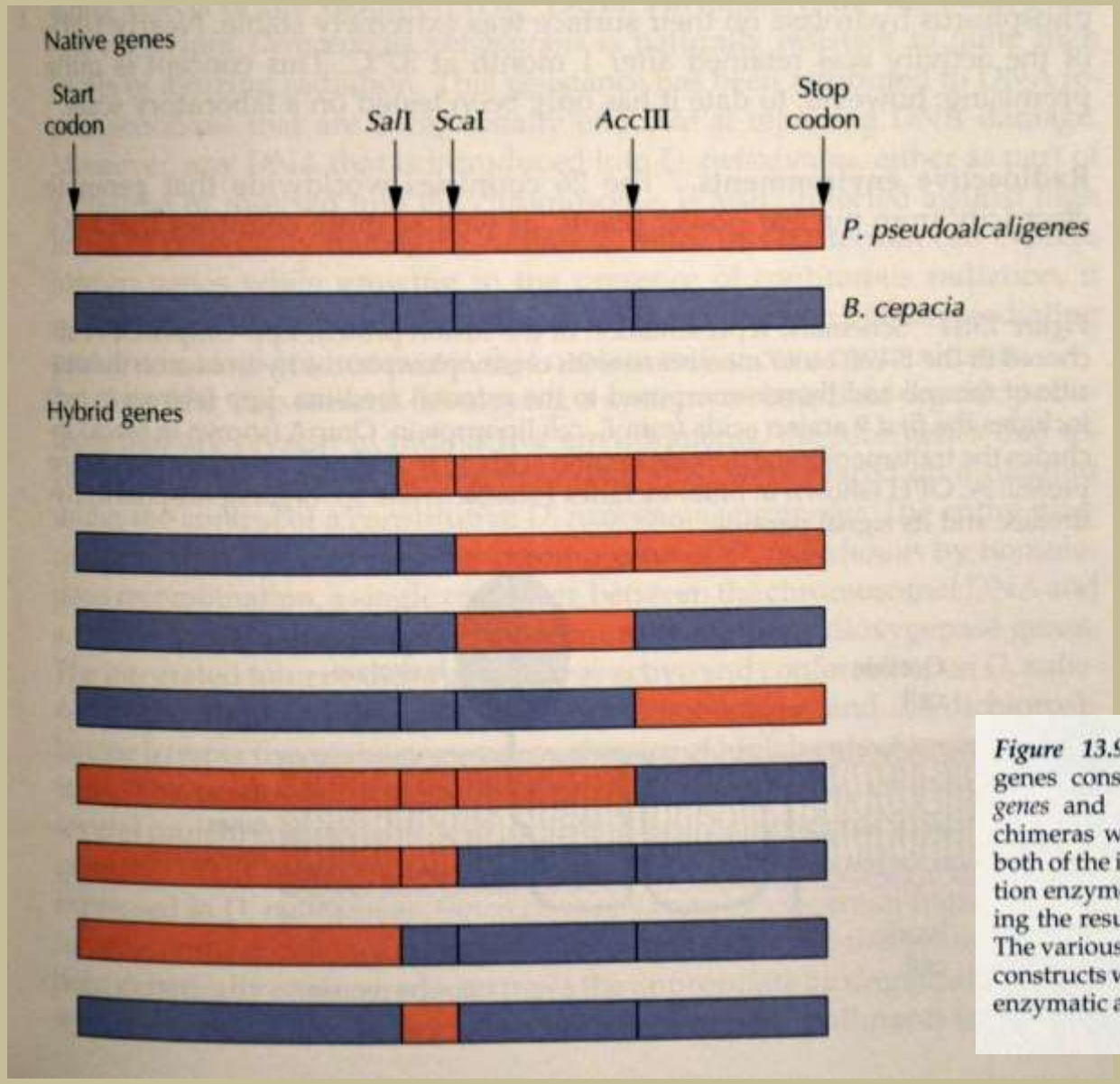


Figure 13.9 Chimeric (hybrid) *bphA1* genes constructed from *P. pseudoalcaligenes* and *B. cepacia bphA1* genes. The chimeras were constructed by digesting both of the isolated genes with the restriction enzymes shown and then recombining the resultant fragments as indicated. The various transformants carrying these constructs were subsequently assayed for enzymatic activity.

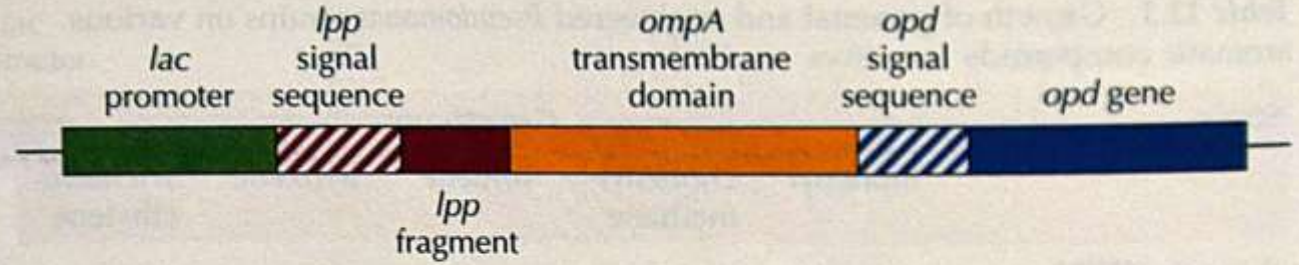
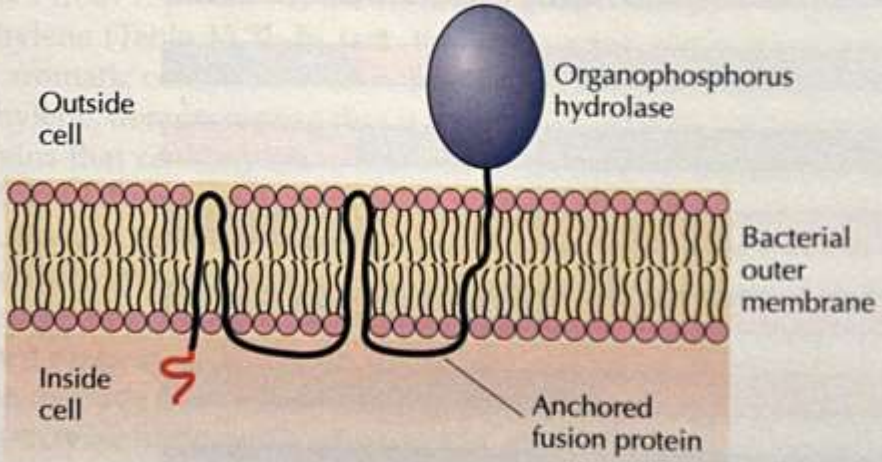


Figure 13.10 The DNA construct used to produce the fusion protein Lpp-OmpA-OPH. This construct includes the *E. coli lac* promoter, DNA encoding the *E. coli lpp* signal sequence and the first 9 amino acids of the mature *E. coli* lipoprotein, the portion of the *E. coli ompA* gene encoding the transmembrane domain, and the gene for the *Flavobacterium* sp. organophosphorus hydrolase (*opd*) and its signal peptide.

Figure 13.11 Schematic representation of the fusion protein Lpp-OmpA-OPH anchored in the *E. coli* outer membrane with organophosphorus hydrolase on the outside of the cell and therefore exposed to the external medium. Lpp (shown in red) includes the first 9 amino acids from *E. coli* lipoprotein. OmpA (shown in black) includes the transmembrane domain (amino acids 46 to 159) of *E. coli* outer membrane protein A. OPH (shown in blue) includes *Flavobacterium* sp. organophosphorus hydrolase and its signal peptide.



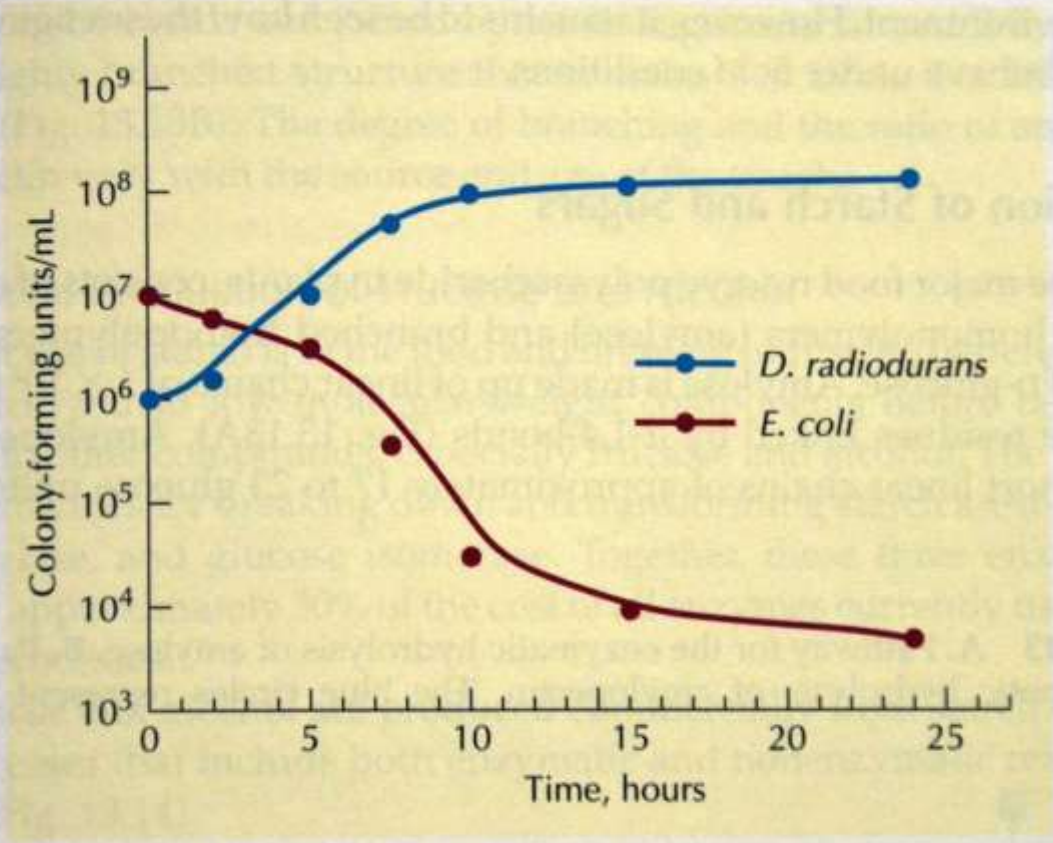


Figure 13.12 Effect of γ-irradiation on the growth of *E. coli* and *D. radiodurans*.