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Enzymes of Naphthalene Metabolism by *Pseudomonas fluorescens* 26K Strain

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Abstract—The ability of *Pseudomonas fluorescens* 26K strain to utilize naphthalene at concentrations up to 600 mg/liter as the sole source of carbon and energy in mineral liquid media was shown. Using HPLC, TLC, and mass-spectrometry, the intermediates of naphthalene transformation by this strain were identified as naphthalene *cis*-1,2-dihydrodiol, salicylaldehyde, salicylate, catechol, 2-hydroxymuconic semialdehyde, and 1-naphthol. Catechol 2,3-dioxygenase (a homotetramer with native molecular mass 125 kDa) and NAD^+-dependent homohexameric naphthalene *cis*-1,2-dihydrodiol dehydrogenase with native molecular mass 160 kDa were purified from crude extract of the strain and characterized. NAD^+-dependent homodimeric salicylaldehyde dehydrogenase with molecular mass 110 kDa was purified and characterized for the first time. Based on the data, a pathway of naphthalene degradation by *P. fluorescens* 26K is suggested.

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Polycyclic aromatic hydrocarbons (PAH) with two or more benzene rings in their structure are widespread hydrophobic organic compounds. Due to their wide presence in the environment, high toxicity, mutagenicity, and carcinogenicity, several PAH are included in the EPA Priority Pollutants List of the Environment Protection Agency (USA) as principal pollutants [1], including naphthalene, phenanthrene, anthracene, and fluorene. Low solubility in water and high sorption of PAH result in their accumulation in various ecosystems. To date the biodegradation of PAH by microorganisms is the most ecological and efficient method for their detoxication [1-4]. For this reason, the search for new strains of PAH degraders and their study are timely.

Naphthalene, a bicyclic aromatic hydrocarbon, enters the environment on burning of organic materials including plants (wood fires), on coal coking in industry (in coal-tar resin), and in fuel burning; it is also a constituent of creosote and cigarette smoke [1, 4]. Bacteria of the *Pseudomonas* genus, *Bacillus thermoleovorans*, *Burkholderia cepacia* 2A-12, *Polaromonas naphthalenivorans*, *Ralstonia* sp. U2, and also bacteria of *Sphingomonas* and *Rhodococcus* genera utilize naphthalene completely [1, 4-6]. The initial stage of aerobic degradation of naphthalene by most microorganisms is its dihydroxylation at the 1,2-positions with formation of naphthalene *cis*-1,2-dihydrodiol as the first intermediate [1]. An alternative pathway of naphthalene transformation via formation and subsequent degradation of 2,3-dihydroxynapthalene is found for *B. thermoleovorans* [1].

Naphthalene degradation after its dihydroxylation at positions 1 and 2 usually includes several stages with formation of salicylate [1, 4, 7], which then can be transformed by microorganisms via two pathways – by salicylate hydroxylase into catechol with its subsequent *meta*- or *ortho*-degradation or by salicylate 5-hydroxylase into gentisate with subsequent degradation to tricarboxylic acids. Although naphthalene degradation pathways and genes encoding enzymes participating in this degradation are well studied, not much is known about most of these enzymes [1].

Abbreviations: C23O, catechol 2,3-dioxygenase; NDDH, naphthalene *cis*-1,2-dihydrodiol dehydrogenase; PAH, polycyclic aromatic hydrocarbons; SADH, salicylaldehyde dehydrogenase.

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The ability of *Pseudomonas fluorescens* 26K species to actively degrade phenanthrene and anthracene was shown earlier [8].

The goal of this work was to study metabolism of naphthalene by *P. fluorescens* 26K by isolating and characterizing the enzyme systems participating in its metabolism.

MATERIALS AND METHODS

In this work we used *P. fluorescens* 26K strain from the collection of the Laboratory of Enzymatic Degradation of Organic Compounds (G. K. Skryabin Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences).

Cultivation conditions. Strain was cultivated using mineral Gorlatov (G) medium [9] and Luria–Bertani (LB) rich culture medium (g/liter): peptone, 10; yeast extract, 5; NaCl, 8. Culture (3 days) washed from LB-agar slants was used as inoculate.

To examine the degradation of naphthalene and dynamics of formation of key intermediates, culture was grown in liquid medium G (100 ml) in 750-ml flasks at 29°C at mixing rate 160 rpm.

To obtain biomass, culture was grown in medium G (200 ml), naphthalene at concentration 200 mg/liter was added at regular intervals as it disappeared from the culture medium; naphthalene was determined via disappearance of naphthalene absorbance peaks in the UV region. Cell growth was monitored by absorbance at 545 nm using a Shimadzu UV-160 spectrophotometer (Japan). Initial solution of naphthalene in dimethylformamide (400 mg/ml) from Fluka (USA) was used as substrate. Cells were harvested by centrifugation for 10-15 min at 5000*g*, washed with 50 mM Tris-HCl buffer, pH 7.2, and stored at –20°C.

Study of naphthalene degradation and dynamics of key intermediates formation. Naphthalene and its metabolites were extracted from culture medium after its acidification with HCl to pH 2.0. Extracts were concentrated using a rotary evaporator and dissolved in 400 μl of methanol. Experiments were repeated not less than thrice.

Naphthalene degradation on its transformation by *P. fluorescens* 26K cells and dynamics of formation of key metabolites were quantitatively studied by HPLC using a Waters 996 chromatograph (USA) with a Waters Spherisorb ODS-2.5 um (250 × 4.6 mm) column form Supelco (USA) and UV detector at 254 nm. Samples were analyzed in methanol–water with addition of 1% acetic acid in a methanol gradient from 20 to 100%, flow rate 0.9 ml/min at 50°C. Elution time was 15 min.

Isolation and identification of key intermediates. For preparative isolation and identification of intermediates of naphthalene transformation, extracts of culture medium of 26K strain were prepared as described earlier [10]. Extracts were analyzed and intermediates were isolated using TLC on $60F_{254}$ plates with Silica gel from Merck (Germany) as described earlier [11]. Compounds were identified by R_f value.

Metabolites were also identified by HPLC (see above), comparing retention time (R_t) of the studied compounds with those for the standard compounds.

Purified intermediate preparations were analyzed using a Finnigan MAT 8430 mass spectrometer, ionization energy 70 eV, with direct sample injection into the ionization region. The structures of intermediates were established based on mass spectra by comparison with literature data and standard compounds.

Naphthalene *cis*-1,2-dihydrodiol used as substrate for estimation of activity of naphthalene *cis*-1,2-dihydrodiol dehydrogenase (NDDH) was isolated from extracts of culture medium of 26K strain (see above) after 10 h incubation in the presence of 400 mg/liter naphthalene in 200 ml of medium G.

Isolation and characterization of enzymes. To prepare crude extracts, *P. fluorescens* 26K biomass grown on naphthalene to optical density 0.745 at 545 nm was slightly thawed at room temperature and disintegrated using a Hughes press. Crude extract obtained as described earlier [11] was used for further purification of enzymes.

Purification of catechol 2,3-dioxygenase (C23O). Crude extract was applied onto a column with Q-Sepharose (carrier volume 30 ml) pre-equilibrated with 50 mM Tris-HCl buffer, pH 7.2, containing 10% v/v ethanol and 1 mM β-mercaptoethanol (buffer A). The column was washed with two volumes of the same buffer. The enzyme was eluted with a linear gradient of 0-0.5 M NaCl in 300 ml of buffer A with the rate 1 ml/min. Active fractions were pooled, concentrated, and desalted in an ultrafiltration cell with an Amicon UM-10 membrane from Millipore (USA). The resulting preparation was applied on a Resource Q column (6 ml) equilibrated with buffer A. The enzyme was eluted with a step gradient of 1 M NaCl in buffer A: 0-20%; 20-40%; 40-100% NaCl, flow rate 1 ml/min. The desalted preparation was used in further experiments. For estimation of C23O activity at all purification stages, catechol from Sigma-Aldrich (USA) was used as the substrate.

Purification of naphthalene *cis*-1,2-dihydrodiol dehydrogenase (NDDH). Chromatography on a column with Q-Sepharose was performed analogous to that for C23O in 100 mM Tris-HCl buffer, pH 7.2, containing 5% v/v ethanol (buffer B). Ammonium sulfate was added to the desalted preparation to the final concentration 0.8 M in buffer B. The resulting solution was applied on a column with Phenyl Sepharose CL-4B (30 ml) pre-equilibrated with 0.8 M $(NH_4)_2SO_4$ in buffer B. The column was washed with two volumes of the same buffer. The enzyme was eluted with a linear gradient of 0.8-0 M $(NH_4)_2SO_4$ in 300 ml of buffer B with the rate 1.5 ml/min. Fractions with NDDH activity were pooled, desalted, and concen-

trated. Naphthalene *cis*-1,2-dihydrodiol obtained by bacterial transformation of naphthalene by *P. fluorescens* 26K (see above) was used as substrate during purification of NDDH.

Purification of salicylaldehyde dehydrogenase (SADH). Chromatography on a column with Q-Sepharose was performed as for C23O. The resulting preparation was applied on a Resource Q column (6 ml) equilibrated with buffer B. The enzyme was eluted with a step gradient of 1 M NaCl in buffer B: 0-10%; 10-100% NaCl, the flow rate 1 ml/min. The concentrated preparation was applied on a column with Superdex 200 and eluted with buffer B containing 0.1 M NaCl at flow rate 0.8 ml/min. Fractions with SADH activity were pooled, desalted, and concentrated.

Salicylaldehyde from Fluka was used as substrate for estimation of SADH activity during its purification.

Enzyme activity was estimated spectrophotometrically using a Shimadzu UV-160 spectrophotometer in a quartz cuvette with 10-mm pathlength at 25°C.

Activity of naphthalene 1,2-dioxygenase was estimated by the decrease in the absorbance of NADH at 340 nm [12]. Activity of NDDH was estimated by the increased absorbance of the reaction mixture with 1 mM NAD^+ at 340 nm [13]. Activity of 1,2-dihydroxynaphthalene dioxygenase was determined at 331 nm by the modified method described earlier [14]. Activity of 2,3-dihydroxybiphenyl dioxygenase was determined at 434 nm [15]. Activity of SADH was estimated as described in [16] but with some modifications: via increase in absorbance at 340 nm with $\varepsilon = 3.84\ mM^{-1}\cdot cm^{-1}$ in 0.1 M KH_2PO_4/NaOH buffer, pH 7.0, in the presence of 1.4 mM NAD^+ and 0.1 mM salicylaldehyde, the reaction being initiated by addition of the enzyme. Activity of C23O was determined by the rate of formation of 2-hydroxymuconic semialdehyde at 375 nm [15]. Activity of catechol 1,2-dioxygenase was determined by the rate of accumulation of muconate at 260 nm [17]. Activity of gentisate 1,2-dioxygenase was measured at 334 nm by the rate of formation of maleylpyruvate [18].

The activity unit was defined as enzyme amount catalyzing transformation of 1 μmol of substrate per minute.

Determination of kinetic characteristics of enzymes. The values of the Michaelis constant (K_m) and the maximal reaction rate (V_{max}) were determined using the Michaelis–Menten equation and also by the linearized Lineweaver–Burk equation in double reciprocal coordinates [19]. When K_m and V_{max} for C23O with various substrates were determined, the corresponding molar extinction coefficients were used [15].

For parameter calculations, experiments were repeated not less than thrice. Kinetic data were processed using the SigmaPlot 8.0 package.

Determination of pH and temperature optima. The pH optima were determined in universal 40 mM acetate–phosphate–borate buffer [20], pH range 5.0-10.0.

Temperature optima of enzymes were determined in the range 5-70°C using the following buffers: for C23O – buffer A; for SADH and NDDH – 0.1 M KH_2PO_4/NaOH buffer, pH 7.0.

Determination of native and subunit molecular masses of proteins. Molecular mass of the native enzyme was determined on a Superdex 200 prep grade column (120 ml) using proteins with known molecular mass as markers. Subunit mass was determined by SDS-PAGE in 12% polyacrylamide gel according to the modified Laemmli method [21]. Gels were stained with Coomassie G-250.

Protein concentration was determined according to a modification of the Bradford method using bovine serum albumin as the standard [22].

RESULTS AND DISCUSSION

Main characteristics of 26K strain growth on naphthalene. It was found that *P. fluorescens* 26K is capable of growing on naphthalene as a sole source of carbon and energy without preliminary long adaptation of cells to naphthalene as the growth substrate. Study of growth dynamics of *P. fluorescens* 26K on addition of various naphthalene concentrations to the culture medium showed that the culture grew actively in the presence of naphthalene (Fig. 1). Cell growth was optimal when 400 mg/liter naphthalene was added to culture medium, the highest specific cell growth rate (0.16 h^{-1}) and the minimal time of biomass doubling (4.3 h) being also observed. However, a lag phase observed during growth in the presence of 400 mg/liter naphthalene was 2 h longer than in the presence of 200 mg/liter naphthalene (8 and 6 h, respectively). Besides this, after 6 h cultivation in the

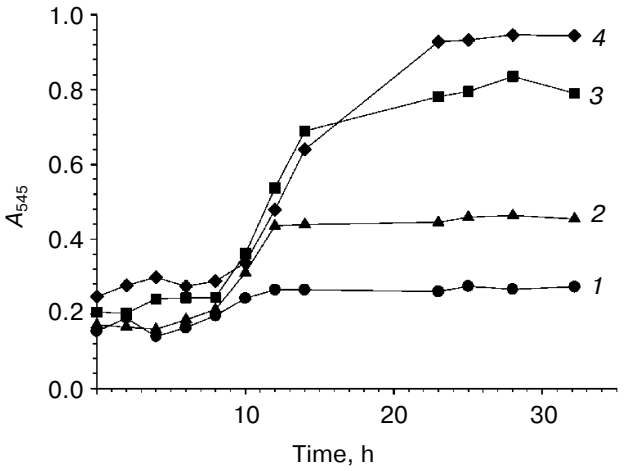


Fig. 1. Growth curves of *P. fluorescens* 26K in the presence of various naphthalene concentrations (mg/liter): *1*) 100; *2*) 200; *3*) 400; *4*) 600.

presence of 400 and 600 mg/liter naphthalene staining of culture medium was observed. With the addition of 600 mg/liter naphthalene the staining did not subsequently disappear, this possibly indicating that intermediates were accumulated.

Therefore, to obtain active biomass and isolate enzymes on subsequent cultivation, naphthalene was added into the culture medium by portions at concentration 200 mg/liter.

Identification of intermediates of naphthalene transformation by 26K strain. Studying bioconversion of 600 mg/liter naphthalene by *P. fluorescens* 26K, we found the following compounds in the culture medium using TLC, HPLC, and mass spectrometry: naphthalene *cis*-1,2-dihydrodiol with R_t (HPLC) = 0.56, R_f (TLC) = 0.22, and main characteristic peaks in mass spectrum *m/z* (%) M^+ 162 (30), 144 (40), 116 (100); salicylic acid with R_t (HPLC) = 9.9, R_f (TLC) = 0.4, and mass spectrum M^+ 138 (65), 120 (100), 92 (65); 1-naphthol with R_t (HPLC) = 12.2, R_f (TLC) = 0.63, and mass spectrum M^+ 144 (100), 116 (10), 115 (75), 89 (10), 63 (10). HPLC analysis also revealed very small quantities (<1 μM) of salicylaldehyde and 2-hydroxymuconic semialdehyde (R_t = 10.5 and 6.5 min, respectively).

Dynamics of naphthalene degradation and accumulation of intermediates of its transformation by 26K strain. Dynamics of naphthalene degradation in the culture medium of *P. fluorescens* 26K and accumulation of intermediates under the optimal conditions for cell growth (400 mg/liter naphthalene) are presented in Fig. 2. Already 3 h after incubation, salicylic acid was detected in the culture medium. As was shown for several strains of the *Pseudomonas* genus, this acid can be an inducer of expression of two operons: "upper" encoding enzymes

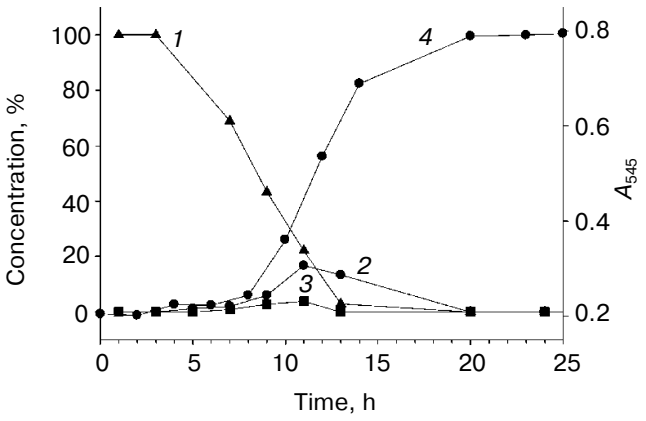


Fig. 2. Dynamics of degradation of 400 mg/liter naphthalene (*1*) and accumulation of intermediates of its transformation (salicylic acid (*2*) and naphthalene *cis*-1,2-dihydrodiol (*3*) to the initial naphthalene concentration, %) in culture medium of *P. fluorescens* 26K; *4*) curve of the strain growth in the presence of 400 mg/liter naphthalene.

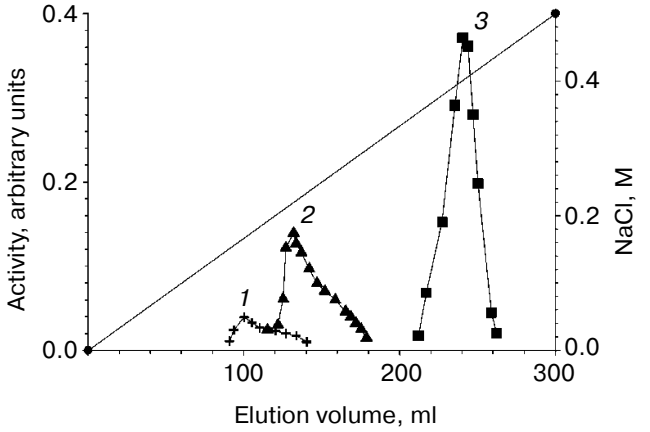


Fig. 3. Ion-exchange chromatography on Q-Sepharose of crude extract from *P. fluorescens* 26K grown on naphthalene as the sole source of carbon and energy: *1*) NDDH; *2*) SADH; *3*) C23O.

transforming naphthalene into salicylate and "lower" whose induction causes transformation of salicylate to tricarboxylic acid cycle via *meta*-degradation of catechol [1].

Slightly later naphthalene *cis*-1,2-dihydrodiol was found in the culture medium. After 11 h cultivation the concentration of naphthalene *cis*-1,2-dihydrodiol and salicylic acid attained the maximal values (11 and 67 mg/liter, respectively), whereas naphthalene almost completely disappeared from the culture medium (Fig. 2). Subsequent rapid removal of naphthalene *cis*-1,2-dihydrodiol and salicylate seems to be caused by naphthalene exhaustion and active work of enzymes of the subsequent pathway of salicylate degradation. After 24 h incubation, naphthalene and the products of its metabolism were not detected in the culture medium of *P. fluorescens* 26K, this indicating that naphthalene is completely degraded.

Enzymes participating in metabolism of naphthalene. In crude extract of *P. fluorescens* 26K grown on naphthalene as the sole source of carbon and energy, we found activities of the following enzymes (U/mg): naphthalene dioxygenase, 0.0346; NDDH, 0.098; 1,2-dihydroxynaphthalene dioxygenase, 0.0825; SADH, 0.538; C23O, 0.126; 2,3-dihydroxybiphenyl dioxygenase, 0.011.

Activities of catechol 1,2-dioxygenase and gentisate 1,2-dioxygenase were not detected in crude extract of 26K strain; this supports existence of *meta*-degradation of catechol as the key stage in naphthalene degradation.

After the first stage of purification using Q-Sepharose as a carrier, we detected activities of only three of the abovementioned enzymes in the elution profile: NDDH, SADH, and C23O (Fig. 3). The activity peaks of C23O and 2,3-dihydroxybiphenyl dioxygenase coincided.

Catechol 2,3-dioxygenase. During further purification of C23O, a peak with 2,3-dihydroxybiphenyl dioxygenase was not separated, and the final electrophoretical-

Table 1. Schemes of purification of enzymes participating in naphthalene degradation by *P. fluorescens* 26K

Stage of purification	Total protein, mg	Specific activity, U/mg	Yield, %
		C23O	
Crude extract	308	0.13	100
Q-Sepharose	18.8	0.69	33.2
Resource Q	0.14	35	12.2
		SADH	
Crude extract	280	0.66	100
Q-Sepharose	38.7	5.56	26.3
Resource Q	2.2	9.4	11.3
		NDDH	
Crude extract	280	0.29	100
Q-Sepharose	15.9	3.1	70
Phenyl Sepharose	3.16	5.1	19.6

Table 2. Screening of conditions for reactivation and storage of C23O (all buffers had pH 7.2)

Conditions	Relative activity, %		
	1 h	18 h	72 h
Control, 50 mM Tris-HCl	100	48	15
10% ethanol, 50 mM Tris-HCl	111	90	80
10% glycerol, 50 mM Tris-HCl	80	50	22
0.5 mM dithiothreitol, 50 mM Tris-HCl	100.4	132	8.3
1 mM β-mercaptoethanol, 50 mM Tris-HCl	176	61	2.6
5 mM ascorbic acid, 50 mM Tris-HCl	1420	1145	12.5
100 mM Tris-HCl	145	100	37.5
100 mM K-Na phosphate buffer	123	65	7.7
2 mM Mohr's salt, 50 mM Tris-HCl	1502	754	47
2 mM $MnCl_2 \cdot 4H_2O$, 50 mM Tris-HCl	92	27	0
2 mM Mohr's salt, 5 mM ascorbic acid, 50 mM Tris-HCl	3890	794	11
10% ethanol, 1 mM β-mercaptoethanol, 50 mM Tris-HCl	190	258	241
10% ethanol, 1 mM β-mercaptoethanol, 50 mM K-Na phosphate buffer	132	133	55.5

ly homogeneous C23O preparation (Table 1) cleaved 2,3-dihydroxybiphenyl as extradiol dioxygenase, giving a product absorbing at 434 nm [15].

Analogously to most known extradiol dioxygenases, the enzyme was very unstable and rapidly lost activity. Screening the literature data on possible preservation of activity by addition of various organic solvents and thiols to buffer and possible reactivation of inactivated enzyme [23], we chose the best conditions for retention of activity of C23O preparation obtained after the first stage of purification with Q-Sepharose (Table 2). During screening we found the best conditions for fast reactivation of the enzyme: it should be placed into 50 mM Tris-HCl buffer, pH 7.2, containing 2 mM Mohr's salt and 5 mM ascorbic acid. However, this effect was very short. The best medium for C23O isolation and storage was 50 mM Tris-HCl buffer, pH 7.2, containing 10% ethanol and 1 mM β-mercaptoethanol.

Table 3. Properties of enzymes isolated from *P. fluorescens* 26K grown on naphthalene as the sole source of carbon and energy

Enzyme	Molecular mass of native enzyme	Subunit mass	pH optimum	Temperature optimum, °C	Catechol		3-Methylcatechol		4-Methylcatechol		2,3-Dihydroxybiphenyl		Salicylaldehyde		Naphthalene *cis*-1,2-dihydrodiol		NAD^+	
					K_m	V_{max}	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}
C23O	125	31	7.6	37	91.7	37	44	4.1	66.7	15.2	0.2	3.2	–	–	–	–	–	–
SADH	110	50	6.9	53	–	–	–	–	–	–	–	–	3	11.7	–	0	480	17
NDDH	160	27	7.2	45	–	–	–	–	–	–	–	–	–	0	66	5.3	130	5.4

Note: "–", is not a physiological substrate; K_m and V_{max} values are given in μM and μmol/min per mg, respectively.

To avoid activity loss during isolation and purification of C23O, we chose methods including anion-exchange chromatography on QAE-Toyopearl and Resource Q carriers (Table 3). Gel filtration on Sephadex 75 resulted in drastic loss of enzyme activity, and subsequent enzyme reactivation appeared to be impossible. Therefore, this purification stage was used only for determination of the molecular mass of the native enzyme.

Using SDS-PAGE (12% polyacrylamide gel) and gel filtration on Superdex 75, it was established that C23O from *P. fluorescens* 26K is a homotetramer with molecular mass of the native enzyme 125 kDa and subunit mass 31 kDa (Fig. 4), which is typical of some C23O isolated from other microorganisms grown on various substrates [7, 24-26].

Analogously to most classical C23O [7, 25, 26], the isolated enzyme exhibited activity against methyl-substituted catechol substrates and 2,3-dihydroxybiphenyl, the maximal activity being exhibited against catechol (Table 3). C23O was inactive against 1,2- and 2,3-dihydroxynaphthalenes.

The enzyme was active in temperature range 5-60°C. The temperature optimum for C23O was 37°C, further heating resulting in drastic activity loss.

The studied C23O was active in the pH range 5.5-10.0, the pH optimum being at pH 7.6.

Naphthalene *cis*-1,2-dihydrodiol dehydrogenase (NDDH). Electrophoretically homogeneous NDDH was isolated from biomass of *P. fluorescens* 26K grown on naphthalene as the sole source of carbon and energy (Table

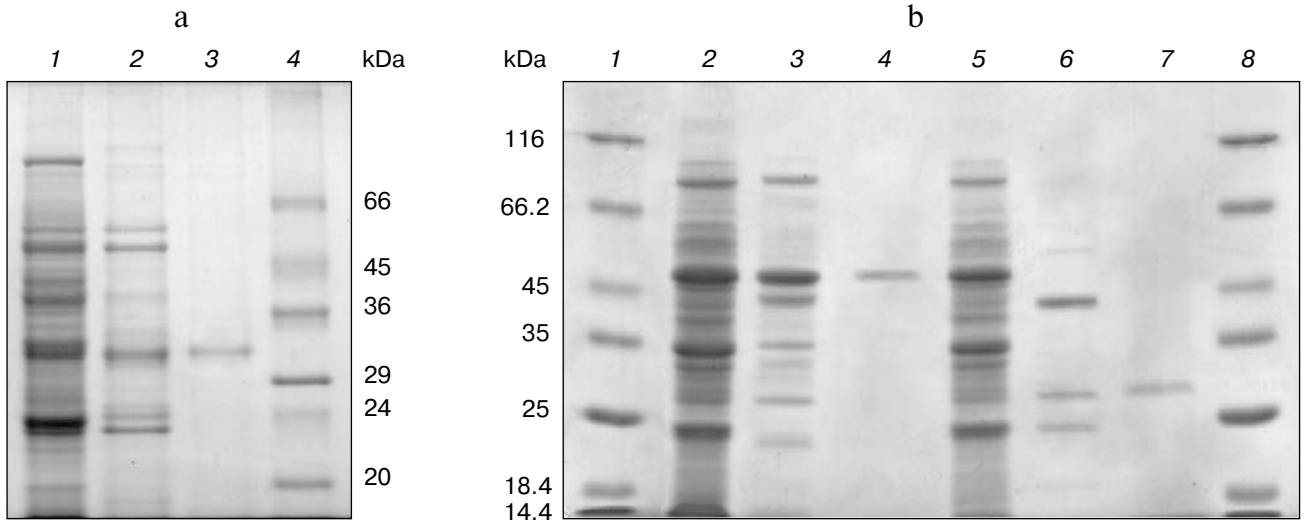
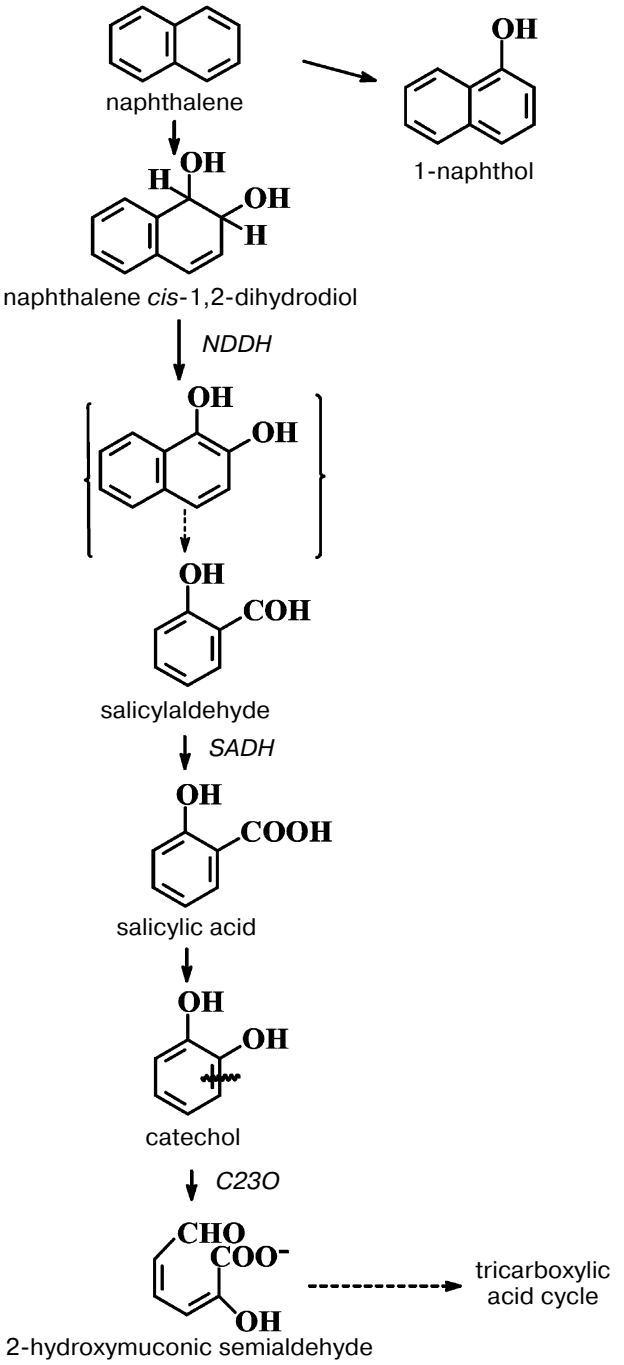


Fig. 4. SDS-PAGE (12% polyacrylamide gel) of enzymes isolated from *P. fluorescens* 26K grown on naphthalene as the sole source of carbon and energy. a) Stages of purification of C23O. Lanes: *1*) crude extract; *2*) Q-Sepharose; *3*) Resource Q; *4*) protein markers. b) Stages of purification of SADH (protein markers (*1*), crude extract (*2*), Q-Sepharose (*3*), Resource Q (*4*)) and NDDH (crude extract (*5*), Q-Sepharose (*6*), Phenyl Sepharose (*7*), protein markers (*8*)).



Possible pathways of naphthalene transformation by *P. fluorescens* 26K

1 and Fig. 4). To retain enzyme activity during purification and storage, 100 mM Tris-HCl buffer, pH 7.2, containing 5% ethanol was used. The presence of β-mercaptoethanol in the medium and also the absence of ethanol or its increased content (to 10%) decreased enzyme activity during purification and storage. A homogeneous protein preparation was obtained after purification of the enzyme on Q-Sepharose and Phenyl Sepharose CL-4B.

Using gel filtration on Superdex 200 and SDS-PAGE, it was established that NDDH is a homohexamer with molecular mass of the native enzyme ~160 kDa and subunit molecular mass 27 kDa (Fig. 4). The isolated NDDH has significantly higher molecular mass and is a hexamer, in contrast to most homotetrameric NDDHs known to date (with molecular mass 92-119 kDa) isolated from other microorganisms degrading naphthalene [13, 27, 28].

The studied NDDH was inactive against *cis*-1,2-dihydronaphthalene and salicylaldehyde but used NAD^+ as a cofactor (Table 3), which is typical of all known NDDHs [13, 27, 28].

The enzyme was active in the temperature range 5-60°C. The temperature optimum was 45°C, and further heating resulted in decrease in its activity. The NDDH was active in pH range 5.0-9.0, the optimum being at pH 7.2. This value is significantly lower than for the known NDDHs, which have pH optima in the range 8.4-9.6 [13, 29].

Salicylaldehyde dehydrogenase (SADH) was isolated and purified to electrophoretic homogeneity for the first time (Table 1 and Fig. 4). The presence of β-mercaptoethanol in the buffer also decreased enzyme activity. Therefore, during further purification and storage of SADH 100 mM Tris-HCl buffer, pH 7.2, containing 5% ethanol was used.

The molecular mass of the native SADH and subunit mass determined by gel filtration on Superdex 75 and SDS-PAGE (12% polyacrylamide gel) were 110 and 50 kDa, respectively (Fig. 4). Thus, the isolated SADH is a homodimer. Unfortunately, there is no information about a similar enzyme in the literature; however, benzaldehyde dehydrogenases and also 2-hydroxymuconic semialdehyde dehydrogenase, which are NAD^+-dependent enzymes and are mainly tetramers with similar subunit molecular mass 52-57 kDa, have been described [30-32]. Exceptions are 2-aminomuconic 6-semialdehyde dehydrogenase from *Pseudomonas pseudoalcaligenes* JS45, which is a trimer [33], and homodimeric benzaldehyde dehydrogenase from *Pseudomonas putida* [34].

The SADH was active in the temperature range 5-70°C with temperature optimum 53°C. Heating SADH above 53°C resulted in rapid activity loss. The pH activity range appeared to be 5.5-8.0 with the maximal activity against salicylaldehyde at pH 6.9.

The SADH was inactive against naphthalene *cis*-1,2-dihydrodiol but analogously to NDDH used NAD^+ as a cofactor (Table 3). The SADH activity was maximal in the presence of 1.4 mM NAD^+ in the reaction mixture, whereas for most dehydrogenases the usual NAD^+ concentration is 1 mM [13, 16].

Possible pathway for metabolism of naphthalene by *P. fluorescens* 26K. Based on the detected and characterized intermediates of naphthalene transformation and enzymes catalyzing them, we suggest a pathway of naph-

thalene degradation by *P. fluorescens* 26K via formation and further transformation of the following compounds: naphthalene *cis*-1,2-dihydrodiol, salicylaldehyde, salicylic acid, catechol, and 2-hydroxymuconic semialdehyde (Scheme). This pathway of naphthalene degradation was established for microorganisms of the *Pseudomonas* genus [1]. The presence of a new intermediate, 1-naphthol, in the culture medium of *P. fluorescens* 26K during naphthalene degradation suggests a possibility of monohydroxylation of the initial substrate by a monooxygenase. Appearance of very small amounts of this intermediate and its subsequent disappearance suggests its further utilization by the strain during degradation.

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