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A new concept of biocatalytic synthesis of acrylic monomers for obtaining water-soluble acrylic heteropolymers

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ABSTRACT

Rhodococcus strains were designed as model biocatalysts (BCs) for the production of acrylic acid and mixtures of acrylic monomers consisting of acrylamide, acrylic acid, and N-alkylacrylamide (N-isopropylacrylamide). To obtain BC strains, we used, among other approaches, adaptive laboratory evolution (ALE), based on the use of the metabolic pathway of amide utilization. Whole genome sequencing of the strains obtained after ALE, as well as subsequent targeted gene disruption, identified candidate genes for three new amidases that are promising for the development of BCs for the production of acrylic acid from acrylamide. New BCs had two types of amidase activities, acrylamide-hydrolyzing and acrylamide-transferring, and by varying the ratio of these activities in BCs, it is possible to influence the ratio of monomers in the resulting mixtures. Based on these strains, a prototype of a new technological concept for the biocatalytic synthesis of acrylic monomers was developed for the production of water-soluble acrylic heteropolymers containing valuable N-alkylacrylamide units. In addition to the possibility of obtaining mixtures of different compositions, the advantages of the concept are a single starting reagent (acrylamide), more unification of processes (all processes are based on the same type of biocatalyst), and potentially greater safety for personnel and the environment compared to existing chemical technologies.

1. Introduction

Water-soluble acrylic polymers (hereinafter WSAPs) are used in various areas of the industry, for example, for municipal wastewater treatment (Mohammadzadeh Pakdel and Peighambardoust, 2018), in the mining industry for ore enrichment (Filippov et al., 2016; Marion et al., 2020) and for the intensification of oil and gas recovery (Raffa et al., 2016; Su and Feng, 2018), in agriculture to improve soil properties (Krasnopeeva et al., 2022), as well as in the manufacture of paints, paper, fabrics, in the cosmetics industry. WSAPs occupy an important place in scientific research, where they are used for preparing gels for various tasks of molecular biology and chemistry, and for the immobilization of biocatalysts (hereinafter BCs). Promising applications of WSAPs are the development of smart polymers for medicine and biotechnology (Galaev and Mattiasson, 1999; Huang et al., 2019).

For the practical applications listed above, such properties of WSAPs in aqueous solutions as solubility, viscosity, ability and nature of interactions with the suspension phase, and resistance to elevated temperatures are important. Modern WSAPs are mostly heteropolymers, that is, their macromolecular chain consists of monomers with various side groups. For example, double copolymers of acrylamide and acrylic acid, or ternary copolymers of acrylamide, acrylic acid, and functionalized monomers (for WSAPs, these are most often N-alkyl-acrylamides) are widely used. Various properties of the side groups (hydrophobicity/hydrophilicity, ionogenicity, charge, size) make it possible to obtain WSAPs with a wide range of the above properties by varying the ratio of monomers (for example see (Lv et al., 2023; Wu et al., 2021)).

The most convenient way to obtain hetero-polymers is polymerization

Abbreviations: (ALE), Adaptive laboratory evolution; (BC), Biocatalyst; (WSAP), water-soluble acrylic polymer; (AH activity), acrylamide-hydrolyzing activity; (AT activity), acrylamide-transferring activity; (NH activity), nitrile hydrolyzing activity; (NIPAM), N-isopropylacrylamide; (MS medium), minimal salt medium; (LB medium), Luria-Bertani medium; (OD), optical density; (Cl-AcA^R), Cl-acetamide resistant.

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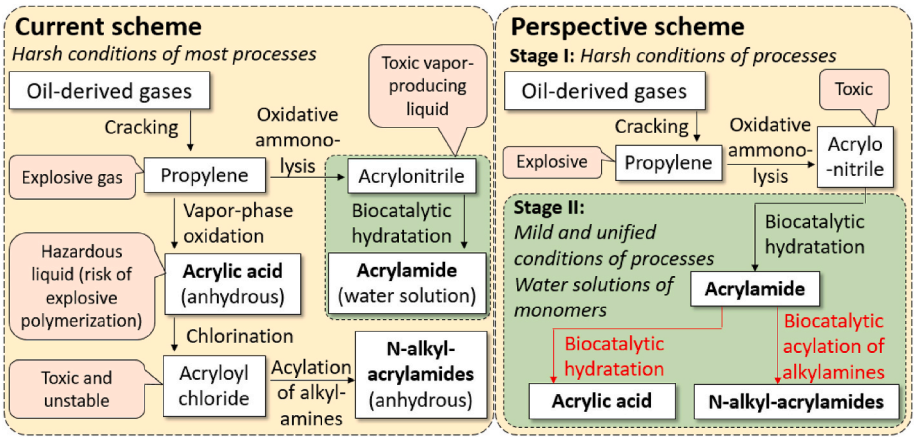

Fig. 1. The current (left) and perspective (right) schemes for the production of three types of acrylic monomers, acrylamide, acrylic acid, and N-alkylacrylamides. Chemical synthesis processes are shown on a beige background, and biocatalytic production processes are shown on a green background. The black font and arrows indicate the processes implemented in the industry, and the undeveloped processes are indicated by red font and arrows. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

from mixtures of various pre-synthesized monomers. To date, the synthesis of individual monomers for such mixtures (Fig. 1, left) is associated with a number of technological difficulties.

The starting reagent for the synthesis is propylene (obtained from refinery gases), from which acrylic acid and acrylonitrile are obtained by chemical synthesis. N-alkyl-acrylamides are obtained chemically from the former, and acrylamide is obtained from the latter biocatalytically, with the help of microorganism cells. Technological difficulties lie, firstly, in the fact that most of the processes for obtaining monomers operate with hazardous reagents - flammable and explosive (propylene), toxic (acryloyl chloride, anhydrous acrylic acid), poorly stored (anhydrous acrylic acid). Secondly, the conditions of the processes, due to differences in the phase state of the key reagents and their technological properties, differ significantly. Such processes are difficult to combine into a single integrated production.

Our work is aimed at developing a new concept for obtaining a bouquet of acrylic monomers. The idea is to make technologies for obtaining individual monomers both safer and more unified. To achieve these goals, it is proposed to transfer as many processes as possible to whole-cell biocatalysis in an aqueous medium, similar to that used to obtain acrylamide from acrylonitrile. Biocatalytic processes are usually safer than chemical ones, therefore, this approach can expand the zone of safer processes in the acrylic monomer production chain. Also, biocatalytic processes can be more unified in terms of instrumentation, because BCs with different enzyme activities do not differ in terms of the commercial form of the catalyst. A possible scheme for obtaining monomers, which illustrates the implementation of the new concept, is shown in Fig. 1, right.

Within the framework of this scheme, acrylonitrile is obtained chemically at Stage I. Further, at Stage II, only biocatalytic processes should work - acrylamide can be obtained from acrylonitrile (this process was implemented in industry (Debabov and Yanenko, 2011; Kobayashi and Shimizu, 1998; Leonova et al., 2000; Yamada et al., 2001; Yanenko and Osswald, 2012)), and both acrylate and N-alkyl-acrylamides can be obtained from acrylamide (these processes have not been implemented yet). Potentially greater safety and uniformity of this group of biocatalytic processes is due to the fact that all Stage II reagents are aqueous solutions, non-vaporous, and non-flammable. A promising advantage of this scheme is that BCs and biocatalytic methods for obtaining monomers are a convenient platform for development, i.e., for creating new compatible processes and integrating them into the existing scheme.

For the new concept, two types of BCs need to be developed - for obtaining acrylic acid and for obtaining N-alkyl-acrylamides. To create such BCs, cells with two types of enzymatic activities are required, which carry out the hydrolysis of acrylamide (hereafter referred to as acrylamide-hydrolyzing, AH activity) and transfer the acyl residue of acrylamide to alkyl-amine (hereafter referred to as acrylamide-transferring, AT activity), as shown at Fig. 2. A.

The hydrolytic activity towards low molecular weight amides, including AH activity, is characteristic for amidases from different families (nitrilase superfamily, AS amidases, for review see (Wu et al., 2020)). At the same time, amidases from different families exhibit significantly reduced activity towards acrylamide compared to its saturated analog, propionamide, and other saturated amides, see (Ciskanik et al., 1995; Doran et al., 2005; Kobayashi et al., 1993; Nel et al., 2011; Park et al., 2008; Sharma et al., 2013; Shen et al., 2012; Xue et al., 2011) and refs in the review (Wu et al., 2020). Therefore, despite the

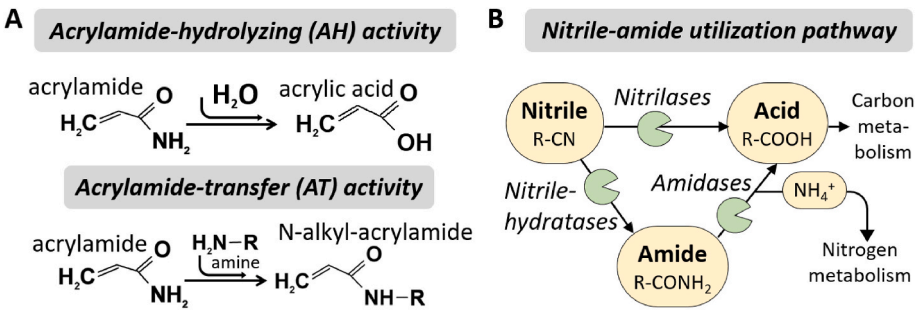

Fig. 2. A: Two types of reactions needed to create BCs for the new concept. **B:** The scheme of nitrile-amide utilization pathway and the compounds and enzymes involved in it.

Table 1
Strains and plasmids used in this work.

Strain or plasmid	Characteristic	Source
Strains		
R. rhodochrous M8	natural soil isolate, possesses AH and nitrile hydrolyzing (NH) activities	(Pogorelova et al., 1996), genome sequence NCBI GenBank accession number GCA_015654185
R. rhodochrous M8-35	derivative of *R. rhodochrous* M8 obtained during the first step of ALE, Cl-Ac^r NH$^-$ AH$^-$	This work
R. rhodochrous M8-33	Δ*amiE* Δ*fmdA1* derivative of *R. rhodochrous* M8-35, obtained during the second step of ALE, possesses decreased AH activity, NH$^+$	This work
R. rhodochrous M8-50	Δ*nhmBAG* Δ*cblA* derivative of *R. rhodochrous* M8-35, obtained during the second step of ALE, possesses increased AH activity, NH$^-$	This work
R. rhodochrous M8-20	derivative of *R. rhodochrous* M8-35, obtained during the second step of ALE, NH$^+$ AH$^+$	This work
R. rhodochrous M8-33 NH$^-$	Δ*amiE* Δ*fmdA1* Δ*nhmBAG* Δ*cblA*, derivative of *R. rhodochrous* M8-33, in which genes of NHase cluster are deleted, possesses decreased AH activity, NH$^-$	This work
R. rhodochrous M8-33 NH$^-$ FmdA2$^-$	Δ*amiE* Δ*fmdA1* Δ*fmdA2* Δ*nhmBAG* Δ*cblA,* derivative of *R. rhodochrous* M8-33 NH$^-$, in which formamidase FmdA2 gene is knocked out, AH$^-$ NH$^-$	This work
R. rhodochrous M8-2	Δ*nhmBAG cblA aam,* derivative of *R. rhodochrous* M8-50, contains P$_{nh}$-*aam-cblA* cassette, possesses AT activity, increased AH activity, NH$^-$	This work
R. rhodochrous M8-3	Δ*amiE* Δ*fmdA1* Δ*nhmBAG cblA aam,* derivative of *R. rhodochrous* M8-33 NH$^-$, contains P$_{nh}$-*aam-cblA* cassette, possesses decreased AH activity, NH$^-$ AT$^+$	This work
R. rhodochrous M8-4	Δ*amiE* Δ*fmdA1* Δ*fmdA2* Δ*nhmBAG cblA aam,* derivative of *R. rhodochrous* M8-33 NH$^-$ FmdA2$^-$, contains P$_{nh}$-*aam-cblA* cassette, NH$^-$ AT$^+$	This work
R. qingshengii TA37	natural soil isolate, possesses AT activity, was used as source of *aam* gene	(Lavrov et al., 2013), genome shotgun sequence NCBI GenBank accession number JAHREK000000000.1
E. coli XL1-Blue	*recA1 endA1 gyrA 96 thi-1 hsdR17 supE 44 relA1 lac* [F′ *proAB lacI*^{q} ZΔ*M15* Tn10 (Tet^r)], was used as a host for gene cloning procedures	Stratagen
E. coli S17−1	TpR SmR *recA thi pro hsdR-M* + *RP4*:2-TcMu:KmTn7 λpir, was used for conjugative transfer of plasmids to *R. rhodochrous* strains	Simon et al. (1983)
E. coli BLR (DE3)	F$^-$*ompT hsdS*$_B$(r$_B^-$ m$_B^-$) *gal lac ile dcm* Δ(*srl-recA)i306*::Tn*10* (tet^R) (DE3), was used for recombinant expression of acylamidase from *R. rhodochrous* M8	Novagen
Plasmids		
pET16b	Expression *E. coli* vector, contains promoter of the gene φ 10 of phage T7 and operator of lactose operon, Amp^r	Novagen
pET16b-AamM8	Derivative of pET16b containing acylamidase gene *aamM8*, was used for recombinant expression in *E. coli* cells, Amp^r	This work
pRY1	pTZ19R-based vector, which is replication-incompetent in *R. rhodochrous* (contains single pMB1 replicon for replication in *E. coli*), Amp^r, Tsr^r	Ryabchenko et al. (2006)
pRY1-NHdel	Derivative of pRY1 containing S1 and S2 fragments, was used for *R. rhodochrous* M8-33 NH$^-$ strain construction, Amp^r, Tsr^r	This work
pRY1-fmdA2del	Derivative of pRY1 containing S3 fragment, was used for *R. rhodochrous* M8-33 NH$^-$ FmdA2$^-$ strain construction, Amp^r, Tsr^r	This work
pRY3-Rho	*E. coli-Rhodococcus* shuttle vector, contains replicon from pRC4 for replication in *Rhodococcus*, and pMB1 replicon for replication in *E. coli, Apr*^{r}	This work
pRY3-P$_{nh}$-*aam-cblA*	Derivative of pRY3-Rho containing P$_{nh}$-*aam-cblA* cassette, Apr^r	This work

existing idea of obtaining acrylic acid from acrylamide using amidases (Maksimova et al., 2019; Raghavan et al., 2019; Thakur et al., 2016), the search and characterization of new enzymes with AH activity is still relevant. Acyl-transferring (including AT) activity is also characteristic for amidases when low molecular weight acceptors such as hydroxylamine are used (Fournand and Arnaud, 2001; Wu et al., 2020). However, the ability to transfer the acrylamide residue to larger alkylamine molecules has only been found for acylamidase from *R. qingshengii* TA37 (Lavrov et al., 2013).

Rhodococcus bacteria are among the most promising for developing the whole-cell BCs with AH and AT activities, because (1) they have a significant diversity of enzymes with both types of amidase activity (Azza et al., 1994; Hirrlinger et al., 1996; Lavrov et al., 2010; Soubrier et al., 1992) and (2) their cells are resistant to toxic components of the synthesis of acrylic monomers (acrylonitrile, acrylamide, etc.) (Lavrov et al., 2019; Tian et al., 2016). In rhodococci amidases are likely involved in amide-utilization pathway, which is, in turn, a part of nitrile-utilization pathway (also called "aldoxime-nitrile pathway" (Bhalla et al., 2018), "acetaldoxime degradation pathway" (see on KEGG database (https://www.kegg.jp),), "nitrile pathway" (Hashimoto et al., 2005)). This nitrile- and amide utilization pathway also includes nitrile hydratases "upstream" of amidases (Xie et al., 2003), and nitrilases, as depicted at Fig. 2. B.

In this study, we decided to obtain the BC strains for the implementation of a new concept based on the *Rhodococcus rhodochrous* M8 strain. This strain is convenient because it grows on a simple media and does not require growth factors, and its genome has been fully sequenced (Novikov et al., 2021). The tasks of the work were (1) obtaining derivatives of this strain, overexpressing amidase genes with AH (for the synthesis of acrylic acid from acrylamide) and AT (for the synthesis of N-alkyl acrylamides from acrylamide) activities, (2) testing the biocatalytic production of acrylic acid and one of the N-alkyl acrylamides, N-isopropylacrylamide (NIPAM), and (3) obtaining water-soluble acrylic heteropolymer.

2. Material and methods

2.1. Bacterial strains, plasmids, and growth conditions

Bacterial strains and plasmids, used in this work are listed in Table 1.

Bacterial strains were cultured on liquid/solid minimal salt (MS) medium and liquid/solid rich medium (Luria-Bertani, LB) with the following compositions:

1. MS medium: $Na_2HPO_4{\cdot}12H_2O$, 2.8 g/l; KH_2PO_4, 0.8 g/l; $MgSO_4{\cdot}7H_2O$, 0.5 g/l; $FeSO_4{\cdot}7H_2O$, 0.005 g/l, agar, 20 g/l (for solid medium). If indicated, the following additions were made: glucose, 5 g/l; NH_4Cl, 2 g/l; $CoCl_2{\cdot}6H_2O$, 0.01 g/l; acrylamide, 5 g/l; NIPAM, 5 g/l; propionamide, 5 g/l; acetamide, 5 g/l; Cl-acetamide, 1 g/l; urea, 6 g/l.

2. LB medium: yeast extract, 5 g/l; tryptone, 10 g/l; NaCl, 5 g/l; agar, 20 g/l (for solid medium).

Selection of recombinant *R. rhodochrous* strains after conjugation was conducted on LB medium containing thiostrepton (25 mg/l) and nalidixic acid (10 mg/l). Cured clones after the second cross of homologous recombination were selected on LB medium by thiostrepton sensitivity. *E. coli* strains with plasmids were grown on LB medium containing ampicillin (100 mg/l). *R. rhodochrous* strains with plasmids were grown the medium containing apramycine (100 mg/l).

R. rhodochrous and *E. coli* were grown at 30 °C and 37 °C, respectively. Liquid cultures were grown with constant mixing (300 rpm).

2.2. Whole-genome sequencing and annotation

Whole-genome sequencing of *R. rhodochrous* M8-35, M8-33 and M8-50 strains was carried out as described previously for *R. rhodochrous* M8 strain (Novikov et al., 2021). In brief, Illumina reads (obtained on Illumina MiSeq at NRC Kurchatov Institute, Moscow, Russia) and Nanopore reads (obtained on MinION R9 flow cell at Genotek, Moscow, Russia) were generated for each genome and used to obtain high-quality genome sequences. The genome of M8-35 strain consists of 6 149 108-nt circle chromosome and two linear contigs, 174 325-nt and 155 452-nt long, M8-33 genome consists of 6 148 942-nt circle chromosome and 121 763-nt linear contig, and M8-50 genome consists of 6 146 215-nt circle chromosome and 152 247-nt linear contig. Genomes sequences were deposited to NCBI Genbank under project numbers PRJNA989265 (strain M8-50), PRJNA989267 (M8-33), RJNA989179 (M8-35), and also available as Sequence 1-6 in the dataset (Grechishnikova et al., 2023). In order to trace the enzymes of nitrile-amide utilization pathway, genome annotations for M8-35, M8-33 and M8-50 were transferred from that of M8 strain using SnapGene® software (www.snapgene.com).

2.3. Whole-genome analysis of gene transcription levels (RNA-seq)

Total RNA for the RNA-seq approach was obtained from the cells of *R. rhodochrous* M8, grown on minimal MS media with glucose-urea and glucose-ammonium supplementation. Total RNA was extracted from frozen *Rhodococcus* cells using the RNeasy Mini Kit (QIAGEN, Germany) with a cell lysis step using liquid nitrogen, and the concentration and integrity of RNAs were quality controlled spectrophotometrically and electrophoretically. RNA was treated with DNase I (Thermo-Fisher Scientific, USA) to thoroughly remove residual genomic DNA, additional control of concentration and integrity was carried out *via* measurement on Qubit 3.0 with Qubit RNA Broad Range Assay Kit (Thermo Fisher Scientific, USA) and capillary electrophoresis on Bioanalyzer 2100 (Agilent, USA), respectively (Table S1). cDNA libraries from two biological replicates per medium variant were prepared using the Ribo-Zero rRNA Removal Kit (Illumina, USA) and NEBNext Ultra II Directional RNA Library Prep Kit (NEB, USA), according to the manufacturer's protocols. The quality of cDNA libraries was checked on LabChip GX Touch (PerkinElmer, USA), according to the manufacturer's protocol. The libraries were denatured using Denature and Dilute Libraries for HiSeq Clustering protocol and sequenced in Rapid Run mode by Genotek (Moscow, Russia) using Illumina HiSeq 2500 (Illumina, USA) at a read length of 150 paired-end bp. Adaptors and nucleotides with a Phred score <30 were removed using Cutadapt v2.10 (Erdem et al., 2011) (error-rate = 0.1). Clean reads were mapped to the genome of *R. rhodochrous* M8 (GCF_001890475.2 in RefSeq database) using BWA-MEM v 0.7.17 (Li, 2013) (−k = 30). Transcription levels were measured using the DESeq2 package (Love et al., 2014) and expressed in RPKM units (reads per kilobase per million reads).

The results of RNA-seq analysis are available in Table S2.

2.4. Two-step adaptive laboratory evolution (ALE)

The first step of the ALE of *R. rhodochrous* M8 strain was a cultivation of the microbial culture on the liquid MS medium containing urea and glucose, lasting for approx. 50 h. Then cell culture was spread on Petri dishes (1 ml/dish, approx. 10^9 cells) over solid MS medium containing urea, glucose, and Cl-acetamide. Colonies appeared on the plates after 3–4 days of incubation were selected and used to determine AH and NH activities.

The second step of ALE was to repeat the actions of the first step with the following changes: strain *R. rhodochrous* M8-35 obtained after the first step of ALE was used as a parent strain, acetamide, 5 g/l, was instead of urea and glucose in the solid MS medium, and Cl-acetamide was excluded from the composition of the medium. Colonies appeared on the plates after 3–4 days of incubation were selected and used to determine AH and NH activities.

2.5. Determination of amide hydrolyzing (including acrylamide-hydrolyzing) activity of cells

The cells were washed twice with 0.01 M Tris-HCl buffer (pH8.0) and resuspended in the same buffer. 100 μL of the cell suspension was mixed with 100 μL of 0.2 M acrylamide or 0.2 M propionamide solution and incubated for 20 min at 37 °C and stopped by the addition of 2 μL of conc. HCl. Thereafter, reaction mixture was centrifuged at 0 °C. 20 μL of supernatant was used to determine the concentration of the produced NH_4^+ ions by Nessler method. One unit of activity corresponds to the amount (μM) of ammonia ions synthesized by 1 mg of dry cells per minute (μM per min per mg c. d.w.).

2.6. Determination of acylamidase activity of cells (hydrolytic activity towards 4′-nitroacetanilide)

The cells were washed twice with 0.01 M Tris-HCl buffer (pH8.0) and resuspended in the same buffer. To measure the activity, 150 μL of the cell suspension was mixed with 150 μL of 1 mM 4′-nitroacetanilide solution (p-nitroanilide of acetic acid) and incubated for 20 min at 37 °C. Thereafter, the reaction was stopped by centrifugation at 0 °C. The supernatant was separated from the cells, and the concentration of the produced p-nitroaniline in the supernatant was determined spectrophotometrically at 405 nm (the molar extinction coefficient of p-nitroaniline was 8900 L mol^{-1} cm^{-1}). One unit of activity corresponds to the amount (μM) of p-nitroaniline synthesized by 1 mg of dry cells per minute (μM per min per mg c. d.w.).

2.7. Determination of nitrile hydrolyzing (NH) activity of cells

The cells were washed twice with 10 mM phosphate buffer (pH7.6) and resuspended in the same buffer. NH activity was assayed in the reaction mixture (200 μL) consisting of 10 mM phosphate buffer (pH7.6), 188 mM acrylonitrile, and an appropriate cell concentration. The reaction was carried out at 20 °C for 20 min and stopped by the addition of 100 μL of 1.7% HCl. The 100 μL aliquot of the mixture was diluted with distilled water up to 27-fold, and OD at 235 nm were measured. The amount of formed acrylamide was calculated from the calibration curve set up with defined amounts of acrylamide. One unit of activity corresponds to the amount (μM) of acrylamide synthesized by 1 mg of dry cells per minute (μM per min per mg c.d.w.).

2.8. Molecular genetic methods

All plasmids were assembled in *E. coli* XL1-Blue using conventional techniques (restriction, ligation, Gibson assembly, and PCR amplification) according to the recommendations of the enzyme manufacturer (ThermoFisher Scientific, USA). Plasmid DNA isolation of *E. coli* strains was conducted using GeneJet Plasmid Miniprep Kit (ThermoFisher

Scientific, USA). Plasmids were introduced into *E. coli* cells by electrotransformation using a Gene Pulser Xcell electroporator (BioRad, USA) or by chemical transformation (Hanahan's method) (Green and Sambrook, 2018). Isolation of plasmid and chromosomal DNA from *R. rhodochrous* strains was conducted using the method of treatment with ammonium salts (Sidoruk et al., 2021). All stages of construction of plasmids and recombinant *R. rhodochrous* strains were confirmed by sequencing. DNA sequencing was performed using an automatic ABI PRISM3500 sequencer (ThermoFisher Scientific, USA) at the Resource Centers of NRC "Kurchatov Institute" (Moscow, Russia).

2.9. Recombinant expression of acylamidase from R. rhodochrous M8 in E. coli cells

Acylamidase gene *aamM8* was amplified from chromosome DNA of *R. rhodochrous* M8 strain and cloned into pET16b vector under the control of T7 promoter resulting pET16b-AamM8 plasmid (sequence is available as Sequence 7 in the dataset (Grechishnikova et al., 2023)). The plasmid was introduced into *E. coli* BLR (DE3) strain and expression was performed as described by the manufacturer of pET-expression system, Novagen. *E. coli* BLR (DE3) pET16b-AamM8 cells were cultured on liquid LB medium until an optical density of 0.5 U was achieved. Then IPTG (isopropyl-β-D-1-thiogalactopyranoside) was added to a concentration of 1 mM and the culture was incubated at 20°C for 16 h before the determination of activity.

2.10. Constructing of recombinant R. rhodochrous strains

R. rhodochrous M8-33 NH^- strain was obtained from *R. rhodochrous* M8-33 strain by deletion of genes of NHase cluster, *nhmBAG* and *cblA*, *via* double homologous recombination. DNA sequences for homologous recombination, S1 and S2, were amplified from chromosome DNA of *R. rhodochrous* M8-33 strain, PCR-joined and cloned into pRY1 vector (sequence is available as Sequence 8 in the dataset (Grechishnikova et al., 2023)) resulting pRY1-NHdel plasmid (sequence is available as Sequence 9 in the dataset (Grechishnikova et al., 2023)). pRY1-NHdel was introduced into *E. coli* S17-1 strain and then transferred into *R. rhodochrous* M8-33 strain by conjugation, as described previously (Voeykova et al., 1994). Homologous recombination was caried out in two successive stages of selection: the first stage resulted in thiostrepton resistant clones containing integrated pRY1-NHdel plasmid and the second stage led to plasmid treatment and obtaining of *R. rhodochrous* M8-33 NH^- strain.

R. rhodochrous M8-33 NH^- FmdA2$^-$ strain was obtained from *R. rhodochrous* M8-33 NH^- strain by knockout of formamidase gene *fmdA2*. Target gene *fmdA2* was disrupted by insertion of S3 fragment *via* homologous recombination. S3 sequence was amplified from chromosome DNA of *R. rhodochrous* M8-33 and cloned into pRY1 vector resulting pRY1-fmdA2del plasmid (sequence is available as Sequence 10 in the dataset (Grechishnikova et al., 2023)). pRY1-fmdA2del was introduced into *E. coli* S17-1 strain and then transferred into *R. rhodochrous* M8-33 NH^- strain by conjugation. Homologous recombination resulted in thiostrepton resistant *R. rhodochrous* M8-33 NH^- FmdA2$^-$ strain.

Recombinant strains *R. rhodochrous* M8-2, *R. rhodochrous* M8-3 and *R. rhodochrous* M8-4, were obtained from strains *R. rhodochrous* M8-50, *R. rhodochrous* M8-33 NH^- and *R. rhodochrous* M8-33 NH^- FmdA2$^-$, respectively, by introducing of P_{nh}-*aam-cblA* cassette. Fragments P_{nh} and *cblA* were amplified from chromosome DNA of *R. rhodochrous* M8-33, *aam* was amplified from *R. qingshengii* TA37. The fragments were PCR-joined and cloned into *E. coli-Rhodococcus* shuttle vector pRY3-Rho (sequence is available as Sequence 11 in the dataset (Grechishnikova et al., 2023)). Obtained plasmid pRY3-P_{nh}-*aam-cblA* (sequence is available as Sequence 12 in the dataset (Grechishnikova et al., 2023)) was introduced into *E. coli* S17-1 strain and then transferred into *Rhodococcus* strains by conjugation.

2.11. Biocatalytic synthesis of acrylic monomers

The cells were washed twice and then resuspended in distilled water. Synthesis of acrylic monomers was carried out in the reaction mixture (500 μL) of the following composition: bacterial cells, 10 g c. d.w./l; acrylamide, 0.75 M; isopropylamine, 0.75 M (pH9.0). The reaction was carried out at 30 °C and pH9.0 for 1 and 6 h with constant mixing (550 rpm). Thereafter, 250 μL of the mixture was centrifuged at 0 °C for 1 min and 200 μL of supernatant was mixed with 9.2 μL of HCl to stop the reaction. The amount of residual acrylamide, produced NIPAM and acrylic acid was determined by gas chromatography.

2.12. Biocatalytic synthesis of a monomer mixture in bioreactor

The synthesis of the monomer mixture was carried out in a thermostated bioreactor with a capacity of 2000 ml, with stirring. 400 ml of cell suspension with a concentration of 25 g/l was loaded into the reactor, then 300 ml of an aqueous solution of isopropylamine with a mass concentration of 10% and a pH10.9 was added while stirring. Then, 300 ml of an aqueous solution of acrylamide with a mass concentration of 20% was added. The reaction mixture incubated with stirring at a temperature of 37 °C for 8 h, then centrifuged, and the supernatant was concentrated on a rotary evaporator at a temperature of 40–45 °C and reduced pressure (residual pressure of 0.02–0.05 atm). The content of monomers was determined using gas chromatography. Then 42% solution of acrylamide, obtained by biocatalytic method, was added to the mixture to achieve the desired ratio of monomers in the mixture.

2.13. Obtaining and testing of acrylic polymer

The solution of the monomer mixture and the solutions of the initiating system components ($(NH_4)_2S_2O_8$ and $Na_2S_2O_5$) were purged with nitrogen for 30 min to remove O_2 and CO_2. Then, they were sequentially loaded into a 500 ml reactor, with a total volume of 400 ml of reaction solution. Polymerization was carried out for 30 min, with the moment of temperature increase considered as the start. The temperature before polymerization was 20–25 °C, and during polymerization, the temperature was maintained not higher than 50 °C. The total initial concentration of monomers in reaction mixture was 27–32% by mass. The obtained aqueous solution of ternary copolymer, which were hydrogel, was crushed and then dried at 70 °C for 24 h, thus obtaining polymer granules. The degree of monomer inclusion in the polymer was estimated based on the residual concentrations of the monomers in the mixture after polymerization, determined after extraction of these monomers and gas chromatography analysis.

The molecular weight was estimated based on the viscosity of a 0.1% aqueous polymer solution in a 10% NaCl at 25 °C, determined using a Ubbelohde viscometer with a capillary diameter of 0.54 mm. The viscosity index was the time taken for a flow of acrylic copolymer through the capillary. Resistance to thermal salt aggression was estimated based on changes in the viscosity properties of a 0.1% aqueous solution of the polymer after exposure at 70 °C in the presence of 2% mass NaCl for 20 days.

2.14. Bioinformatic analysis of the genomes of the mutant strains – derivatives of R. rhodochrous M8 strain

Short differences (SNPs and microindels) between the genomes were identified using Snippy (Seemann, 2015) with default settings and –contigs option. Longer differences (rearrangements) were identified using progressive Mauve algorithm (Darling et al., 2004) with default settings. In the Mauve-generated list of rearrangements short ones (<5 nt) were discarded using in-home Python scripts. Differences in the amino acid sequences of enzymes of nitrile-amide-utilizing pathway (Table S3) were additionally verified using BLAST (Camacho et al.,

Table 2
Amide-hydrolyzing, NH and AT activities in strain M8.

Amide (5 g/l) added as co-substrate to the MS + glucose + NH4Cl media	Growth on liquid media	Amide-hydrolyzing activity (units)[a] towards		NH activity[c]	AT activity
		Acrylamide (AH activity)	Propion-amide		
Acrylamide	Non detectable	–	–	–	–
NIPAM	Non detectable	–	–	–	–
Propionamide	Normal[b]	5.1 ± 0.9	5.3 ± 0.6	124 ± 12	<0.1
Acetamide	Normal[b]	4.8 ± 0.4	5.0 ± 0.3	137 ± 11	<0.1
CL-acetamide	Non detectable	–	–	–	–
Urea	Normal[b]	6.8 ± 0.6	7.2 ± 0.8	153 ± 17	<0.1
No inducer	Normal[b]	<0.1	<0.1	<1	<0.1

[a] See section 2.5 in Materials and Methods.
[b] OD of the culture after approx. 60 h of cultivation was more than 1.
[c] The cultures were cultivated with addition of $CoCl_2$.

2009) algorithm supported by in-home Python scripts.

3. Results

3.1. Search for enzyme activities and candidate genes associated with the nitrile-amide utilization pathway in R. rhodochrous M8 strain

The use of strain's own genes for constructing a BC is of interest both in terms of the possibility of more efficient overexpression compared to heterologous genes, and in terms of compliance with legislation on genetically modified organisms. Therefore, we screened the genome of M8 strain for amidases and other genes involved in nitrile-amide utilization pathway, first nitrile hydratases and nitrilases. The latter were of interest to us because due to their known hyperexpression ability (Chhiba-Govindjee et al., 2018; Kobayashi et al., 1990; Nagasawa et al., 1988; Pogorelova et al., 1996) they can create metabolic burden and impair the growth characteristics of strains.

In the genome annotation of strain M8 (released at 2020, NCBI GenBank accession number GCA_015654185.1) we identified 54 genes that are likely to have hydrolytic or transferase activity towards low molecular weight amides (hereafter referred to as amidases), as well as two nitrile hydratases, Co-type and Fe-type (listed in Table S3). The relatively large number of potential amidases in the strain (common to the overall genus, data not shown) significantly complicates the rational selection of endogenous amidase genes for overexpression. To narrow down the candidates, an *in vivo* search for such activities and their encoding genes was conducted. The search pipeline included the induction and measuring the activities (including acrylamide/propionamide hydrolysis ratio), extraction and sequencing of total mRNA, and analysis of amidases transcription changes.

The activities were induced by growing strain M8 on liquid MS medium (see Materials and Methods) with 5 g/l glucose and 2 g/l NH_4Cl. This base medium was varied by the addition of different amides, namely acrylamide and its structural analogs (Table 2).

Acrylamide, NIPAM, and Cl-acetamide completely inhibited the growth of the strain, therefore their presence did not help identify the activities. Propionamide, acetamide, and urea did not impair growth and induced AH and NH activities (NH required cobalt ions in the medium), with urea inducing the highest AH activity. The acrylamide/propionamide hydrolysis ratios in all cases were approx. 1, which is promising for biocatalytic application. AT and nitrilase activities were not detected in cells with any inducer.

Transcriptomic analysis of cells grown on MS media with glucose and urea (without NH_4Cl) revealed 90 genes which transcription was activated more than 5-fold compared to growth with NH_4Cl and without urea (listed in Table S2). Among these genes there were 5 ones most likely coding amidases (these genes are indicated both in Table S3 and Table S2), as well as several hypothetical protein genes with undefined function. The majority of the remaining induced genes with annotated function were likely not related to AH activity of cells. Thus, transcriptomic analysis helped significantly narrow down the pool of candidate genes. However, considering the remaining five candidate amidases and the large diversity and overall limited knowledge of bacterial enzymes, it was difficult to draw definitive conclusions about which gene is most promising for solving the tasks of the work. Therefore, to address these challenges, we decided to use a "smart" approach rather than trial-and-error screening of individual amidase genes (through cloning, expression, knockout). This "smart" approach included the development of adaptive laboratory evolution (ALE) approach to obtain mutant variants of strain M8 with altered AH activities, and the analysis of mutants (see subsection 3.2.).

Regarding AT activity, since it was previously known for the acylamidase Aam from *R. qingshengii* TA37 (Lavrov et al., 2013), we found unique homolog of *aam* in the M8 genome, expressed it under T7 promoter in *E. coli*, and compared its activity with Aam expressed in the same system previously (Lavrov and Yanenko, 2013). The hydrolytic activities of *E. coli* cells with *aam* from TA37 and *aam* from M8 towards 4′-nitroacetanilide (represent an AT activity, see later in the text, and also section 2.6 in Materials and Methods) revealed that *aam* from M8 possesses approx. three times lower enzymatic activity than *aam* from TA37 (0.4 ± 0.1 and 1.1 ± 0.1 units, correspondingly). So, for further construction of BCs based on M8 strain, we used the *aam* gene from *R. qingshengii* TA37.

3.2. Obtaining and analyzing the derivatives of R. rhodochrous M8 strain with altered activities of the enzymes of the amide utilization pathway

The method for obtaining strains derived from M8 strain was based on the approach described by (Clarke and Tata, 1973; Smyth and Clarke, 1975). The authors obtained derivatives of *Pseudomonas aeruginosa* with altered levels of amide hydrolyzing activity by selecting spontaneous mutants resistant to fluoracetamide (F-acetamide), structural analog of naturally occurring acetamide. This approach can be referred to as ALE, based most likely on poisoning the pathway of amide utilization (see discussion in section 4, Fig. 8). Previously, we demonstrated that chloroacetamide (Cl-acetamide), a structural analog of F-acetamide, can be hydrolyzed by amidase to form ammonia and acid (Kotlova et al., 1999). So, we decided to develop such ALE approach for *R. rhodochrous* M8, using chloroacetamide Cl-acetamide as the selection agent.

First, we confirmed the toxicity of Cl-acetamide for the strain (minimum inhibitory concentration on solid MS medium was 0.25 g/l) and confirmed that Cl-acetamide does not block AH activity of the cells. The latter means that cells do not lose AH activity after incubation with Cl-acetamide (data not shown). Next, we developed a two-step ALE scheme, which involved obtaining Cl-acetamide resistant clones, selecting among them clones not growing on acetamide and lacking AH and NH activities, and then obtaining revertant clones that regained both the ability to grow on acetamide and AH activity (Fig. 3).

At the first stage (ALE stage I on Fig. 3) Cl-acetamide resistant (Cl-AcA^R) clones were selected, among which approximately 5% of clones

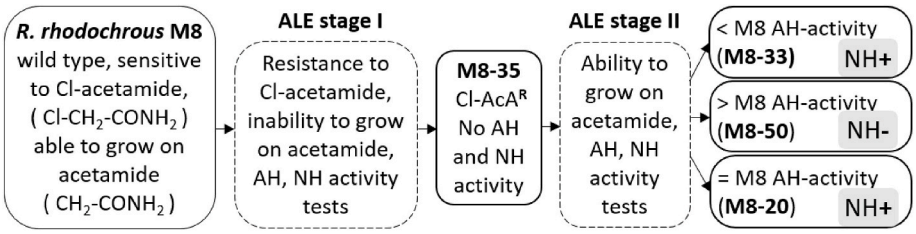

Fig. 3. Scheme of ALE used to obtain derivatives from the *R. rhodochrous* M8 strain with altered AH activities, and the resulting strains.

Table 3
Amide- and nitrile-hydrolyzing activities in mutant strains derived from M8 strain.

Strain	Amide-hydrolyzing activity (units)[a] towards		NH activity	Comment (see also Fig. 3)
	Acrylamide (AH activity)	Propion-amide		
M8	6.7 ± 0.8	7.8 ± 0.9	138 ± 16	Parent strain
M8-35	<0.1	<0.1	<1	Derivative of M8. No AH and NH activities.
M8-33	0.83 ± 0.01	0.92 ± 0.11	158 ± 18	Derivative of M8-35. AH activity is present but < M8.
M8-50	14.1 ± 2.3	13.5 ± 1.8	<1	Derivative of M8-35. AH activity is present and > M8.
M8-20	7.1 ± 1.4	8.6 ± 1.2	151 ± 13	Derivative of M8-35. Wild-type AH and NH activities

[a] See section 2.5 in Materials and Methods.

had the desired phenotype of no growth on MS medium with acetamide and no AH and NH activities. One of these clones, designated M8-35, was used for selection in the second stage (ALE stage II on Fig. 3), to obtain revertants capable of growing on MS medium with acetamide. This selection yielded three types of clones in which AH and NH activities were restored to varying degrees. The representatives of these types are indicated at Fig. 3, and their activities are shown in Table 3.

The strains - revertants to the wild type (such as M8-20) possessing both AH and NH activities were not analyzed and were not further used in the study. The history of changes in the genes of the nitrile-amide utilization pathway in the strains with modified activities (M8-35, M8-33, M8-50) was traced at the whole genome level (Table S3). It was found that when strain M8-35 was obtained from M8, one of the presumed amidases could be inactivated (two frameshift mutations), while the structural genes of the other amidases and both nitrile hydratases remained unchanged. When strain M8-33 was obtained from M8-35, the genes of two amidases (aliphatic amidase *amiE* and formamidase *fmdA1*) were deleted, within a deleted genome fragment of 52.6 kbp (Fig. 4 and Table S4), while the other amidase genes remained unchanged. When strain M8-50 was obtained from M8-35, the genes of Co-dependent nitrile hydratase were deleted (within a deleted genome fragment of 22.1 kbp), a frameshift mutation was found in *amiN*, while the genes of other amidase and Fe-nitrile hydratase remained unchanged (Fig. 4 and Table S4).

In addition, both stages of ALE yielded changes in multiple genes, presumably not encoding enzymes with AH activity, as well as changes in intergenic regions. For example, strain M8-35 had 184 point or small changes (substitutions, deletions, and insertions, see tab M8-M35 in Table S5), and 16 larger changes (>5 nucleotides), namely 9 insertions (up to 42 kbp in length) and 7 deletions (up to 5 kbp in length, see tab M8-M35 in Table S4). A comparable number of point and extended changes also occurred in strains M8-50 and M8-33 obtained from M8-35 in the second stage of ALE (represented in Fig. 4 and indicated in corresponding tabs in Table S5 and Table S4).

Based on the set of changes in the genes of the presumed amidases and nitrile hydratases, and the changes in AH and NH phenotypes during the two stages of ALE, the following assumptions can be made. It is likely that the loss of AH activity in M8-35 and its restoration in M8-50 and M8-33, as well as the loss in M8-35/restoration in M8-33 of NH activity, are associated with changes not in the structural genes of enzymes, but in the functioning of regulatory genes. Additionally, it appears that the majority of other changes did not affect the investigated activities, growth characteristics, and viability of mutant strains.

Practically important results of ALE were M8-50 strain with increased AH activity and disabled NH activity, and M8-33 strain, which had reduced but significant AH activity. Since the AH activities in both strains were insensitive to acrylamide, similar to AH activities of M8 strain (see Table 3), we decided to use both strains for obtaining BCs for monomer synthesis. Additional testing showed the genetic stability of the obtained strains, i.e., no changes or disappearance of AH activities occurred during passaging in laboratory conditions (data not shown).

To reduce the potential metabolic burden associated with the overexpression of cobalt-dependent nitrile hydratase, the genes *nhmBAGcblA* were deleted in strain M8-33 (using homologous recombination), resulting in strain M8-33 NH^-. Then, to complete the series of strains with different levels of AH activity, a derivative strain was obtained from M8-33 NH^-, in which the gene for formamidase *fmdA2* (close homolog of *fmdA1*) was disrupted, resulting in strain M8-33 NH^- $FmdA2^-$. This last strain no longer exhibited AH activity. Thus, three strains possessing different levels of AH activity (Fig. 5. A), and lacking the genes for Co-dependent nitrile hydratase were obtained.

3.3. Construction of biocatalysts with AH and AT activities

To achieve one of the tasks of this study (BCs for the production of acrylic acid), the strain M8-50 is suitable due to its increased AH activity. For another task (BCs with AT activity for the production of N-alkyl-acrylamides), we decided to express the acylamidase gene *aam* in M8-33 NH^- $FmdA2^-$ strain, which lacks AH activity. Additionally, we decided to express *aam* in the strains with different levels of AH activity (M8-50 and M8-33 NH^-), using strong cobalt-tunable promoter P_{nh} (Lavrov et al., 2018). Tunability of P_{nh} and different levels of AH activity could help us to create a "matrix" of strains with combinations of different levels of AH and AT activities. Such strains could potentially synthesize mixtures of acrylic monomers with variable ratios of monomers.

The cobalt-tunable expression cassette P_{nh}-*aam-cblA* was introduced into strains M8-50, M8-33 NH^-, and M8-33 NH^- $FmdA2^-$ on the

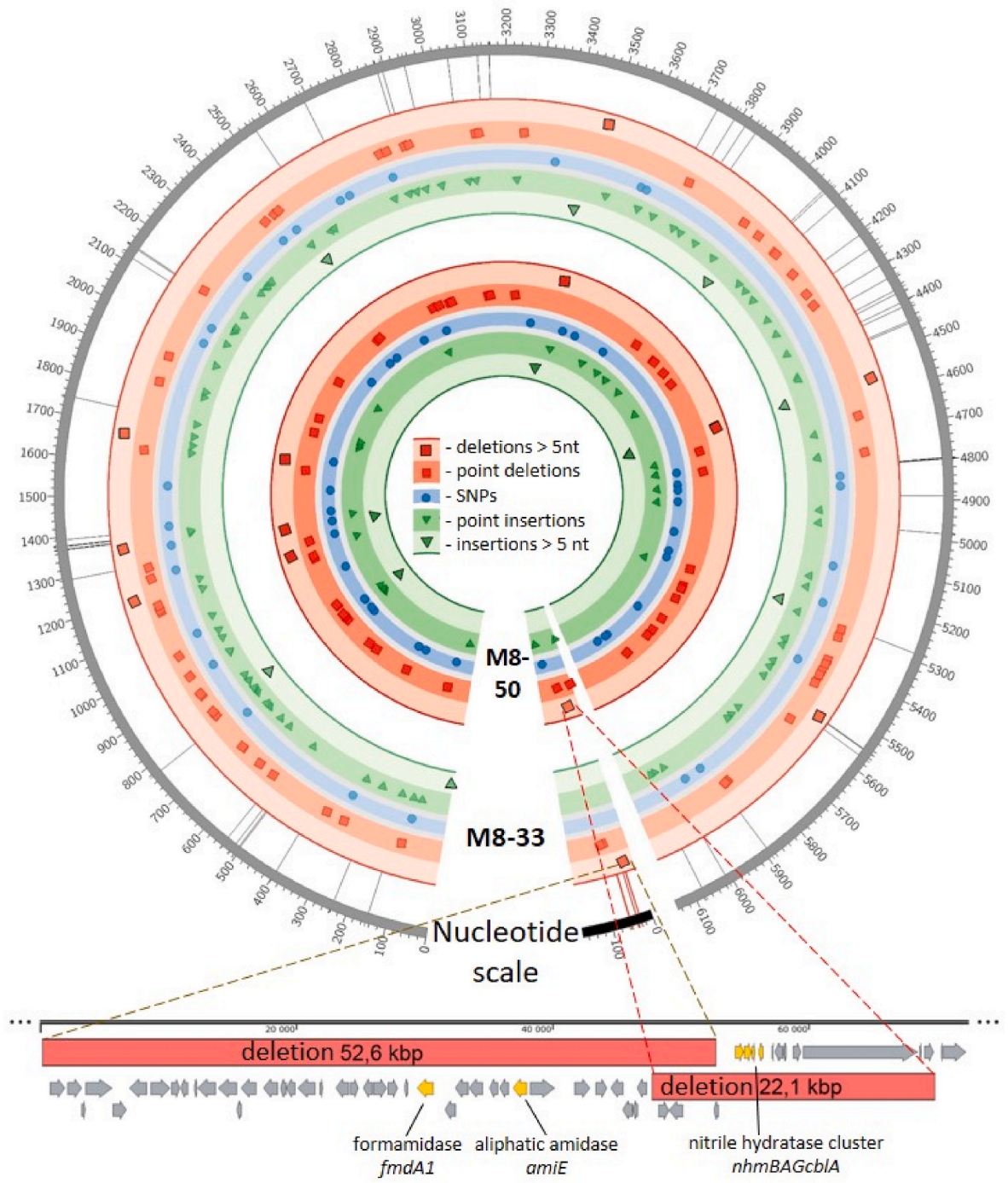

Fig. 4. Graphic presentation (obtained using Circos (http://www.circos.ca)) of rearrangements in the genomes of strains M8-33 and M8-50 compared to the genome of their parent *R. rhodochrous* M8-35 strain (see also Fig. 3). The layers of the circle representation are, from the edge to the center: nucleotide scale (gray part - chromosome, black part - plasmid), radial lines point the genes of nitrile-amide utilization pathway (red ones indicate genes deleted in M8-33 or M8-50), colored zones of M8-33 and M8-50 rearrangements. Zone of each strain is subdivided into three parts as shown on the legend at the center: large/point deletions (red rectangles on light/dark pink background), SNPs (blue circles on violet background), point/large insertions (green triangles on dark/light green background). Under the circles the two largest deletions in M8-33 and M8-50 are outlined, in which amidases and cluster of nitrile hydratase are highlighted in gold. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

autonomously replicating vector pRY3-Rho, and expression of acylamidase in the strains was quantified by measuring hydrolytic activity of cells towards 4′-nitroacetanilide. This latter activity will be further represented as AT activity, because both the hydrolysis of 4′-nitroacetanilide and the ability to transfer acrylamide moiety to acyl-amine are unique properties of this acylamidase (Lavrov et al., 2010, 2013). AH and AT activities of resulting strains (designated M8-2 – M8-4, and also M8-50 strain) after growth on MS medium with urea and glucose, in the presence and absence of cobalt ions, are presented at Fig. 5. C.

In strains M8-50 and M8-2 AH activity did not depend on the presence of the *aam* gene or the level of its expression, and was approx. 14 units. In strain M8-3, AH activity increased with increased expression of *aam* (in the presence of cobalt ions), from approx. 1.3 to 2 units. Unexpectedly, M8-4 strain showed 0.3 units of AH activity (compared to

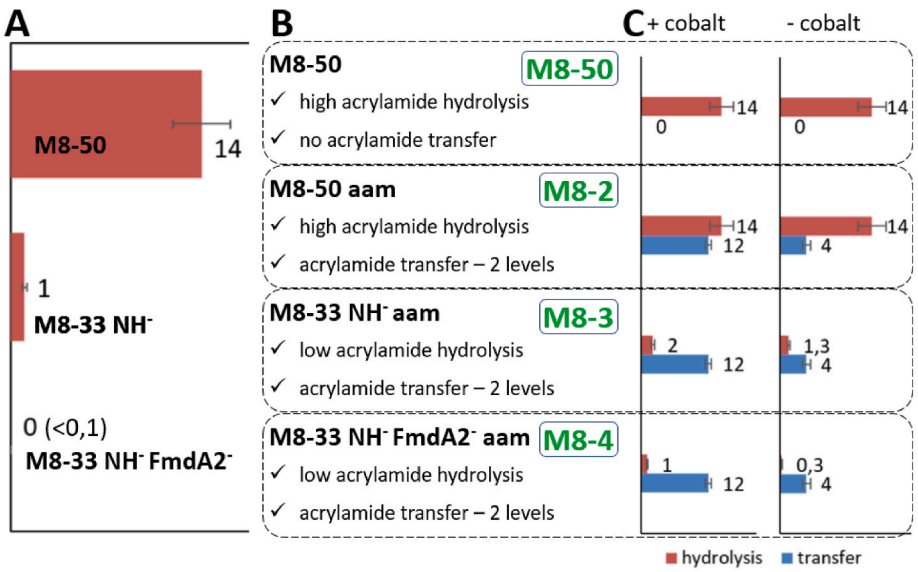

Fig. 5. Acrylamide-hydrolyzing and 4′-nitroacetanilide-hydrolyzing activities (units) (the latter is hereinafter "AT activity") of a series of strains with different combinations of amidase genes and acylamidase gene, and with different levels of acylamidase gene expression. **A:** AH activities of the M8-33, M8-50, M8-33 NH⁻ strains, in which the genes for Co-dependent nitrile hydratase were deleted (see subsection 3.2). **B:** Derivatives of M8-33, M8-50, M8-33 NH⁻ strains with *aam* expression cassette. **C:** activities of *aam*-expressing strains after growth in the presence and absence of cobalt ions. SDs were calculated using at least three independent experiments.

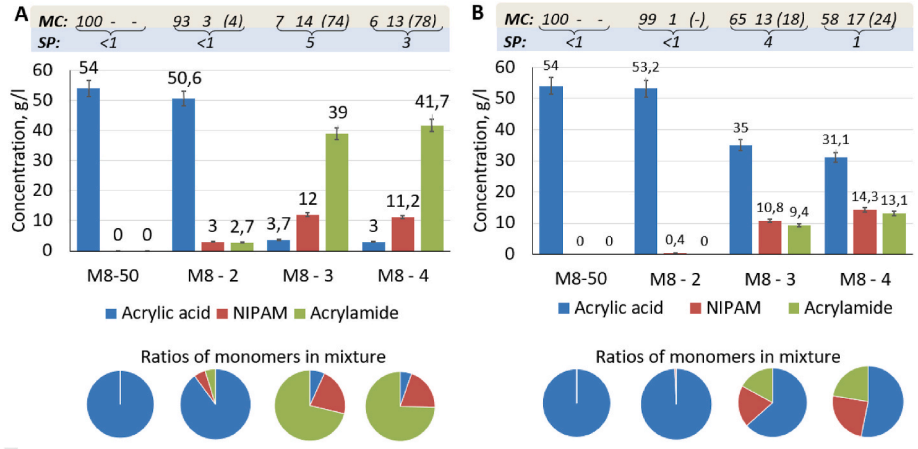

Fig. 6. Results of syntheses of acrylic monomers using BCs with different ratios of AH and AT activities. Concentrations (chart columns) and ratios (pie diagrams) of monomers in mixtures after 1 h (**A**) and 6 h (**B**) synthesis are presented. Above the columns, % of molar conversions of acrylamide to monomers (MC) and molar % of sum of possible side products (SP) are presented. The MC of acrylamide is in parentheses because this monomer in mixtures is the unhydrolyzed starting monomer. SPs were calculated using the molar concentrations of three obtained acrylic monomers. SDs were calculated using at least three independent experiments.

the zero activity of its parent strain M8-33 NH⁻ FmdA2⁻). AH activity of M8-3 strain also increased up to 1 unit (see sec. 2.5 in Materials and Methods) with increased expression of *aam*. It is suggested that in the case of strains M8-3 and M8-4, the side low level AH activity of acylamidase manifests itself. The AT activity of acylamidase was similar in all M8-2 – M8-4 strains, differing approx. threefold depending on the presence of cobalt ions (4 and 12 units).

3.4. Biocatalytic synthesis of acrylic monomers

The synthesis of acrylic monomers was tested using both BC with single AH activity (M8-50) and BCs with AH and AT activities in different ratios, M8-2 to M8-4. The latter, containing the acylamidase gene, were grown with maximum AT activity (in the presence of cobalt ions, see Fig. 5. B). The concentrations of resulting monomers (determined using endpoint GC) and their ratios in the mixtures are shown in Fig. 6, for syntheses conducted over 1 h and 6 h.

BC M8-50 synthesized only acrylic acid, completely converting it from acrylamide, both in 1 h and 6 h. However, in a shorter time, it produced mixtures of acrylamide and acrylic acid in different ratios, and the amount of acrylamide in the mixture directly depended on the reaction time (data not provided). BC M8-2 in 6 h synthesized a mixture of acrylic acid with a minor portion of NIPAM. Other BCs and M8-2 in 1 h synthesized triple mixtures of acrylic acid, NIPAM, and acrylamide in different ratios. Decreased AH activity in the strains M8-2 to M8-4 led to a decrease in the proportion of acrylic acid in the mixture, resulting in an increase in both the absolute amount and proportion of acrylamide and NIPAM. Decreased AT activity (by using strains grown without induction of the cobalt acylamidase promoter, see Fig. 5. B) resulted in a proportional decrease in the amount of NIPAM in all variants and did not significantly affect the ratio of acrylamide to acrylic acid (data not provided).

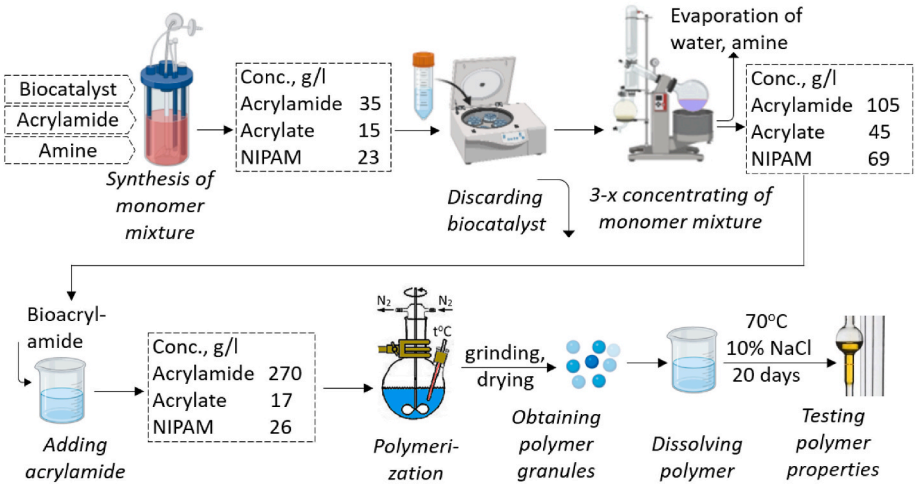

Fig. 7. Scheme of obtaining of a triple copolymer from acrylamide, acrylic acid, and N-isopropylacrylamide, and testing its properties.

In the case of BCs M8-2 - M8-4, the increased reaction time (comparing 6 h with 1 h) decreased the amount of acrylamide, and increased the amount of acrylic acid, as a result of continued hydrolysis of acrylamide through AH activity. As for NIPAM, most of it was synthesized within 1 h of the reaction, and then its amount did not change significantly, possibly due to depletion of the second substrate required for the synthesis of NIPAM – isopropylamine (due to its high evaporation capacity). Thus, the time of synthesis is also a factor regulating the ratio of monomers in the mixture.

In the conducted syntheses, neither pure NIPAM nor a double mixture of acrylamide and NIPAM was obtained. In all mixtures with BCs containing acylamidase (except for the 1-h synthesis with M8-2), the absolute amount of NIPAM was similar, ranging from 10 to 14 g/l. Obviously, obtaining pure NIPAM or at least a mixture with its predominance requires reducing the side AH activity of acylamidase. Nevertheless, NIPAM is a minor modifying additive in polymers, so the obtained mixtures may be suitable for obtaining heteropolymers containing NIPAM units.

Thus, using a series of obtained strains, we have demonstrated the ability to influence the composition and ratios of monomers in a mixture through two pathways: by regulating the synthesis time and adjusting the levels and ratios of AH and AT activities. We believe it is possible to create a flexible biocatalytic platform consisting of a strain with two types of activities, which levels can be regulated during the BC cultivation stage. Such an acrylamide-starting platform will be suitable for synthesizing not only acrylic acid but also double and triple mixtures of monomers with different compositions.

3.5. *Obtaining and testing of acrylic polymer*

In the synthesis of the most demanded high molecular weight acrylic polymers, the suitability of monomers and their mixtures for efficient

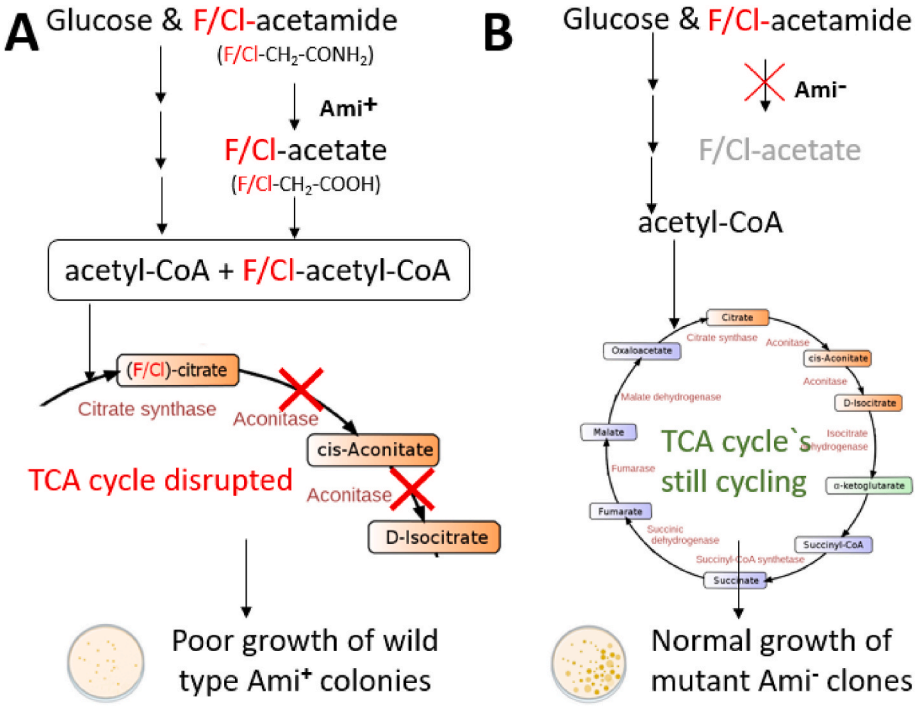

Fig. 8. Presumable scheme of F-acetamide and Cl-acetamide toxicity in wild-type strains possessing active amidases (A) and mutant strains with inactivated amidases (B)..

polymerization is important. Such suitability depends, among other things, on the so-called "polymer purity" of the monomers, i.e., the absence of inhibitors of radical polymerization. The simplest way to comprehensively assess polymer purity under laboratory conditions is to obtain the polymer and evaluate its molecular weight and other properties.

The synthesis of a triple heteropolymer was conducted using a monomer mixture obtained in the bioreactor using BC M8-4 (see subsections 2.11. and 2.12.). A mixture containing 35 g/l of acrylic acid, 15 g/l of acrylamide, and 23 g/l of NIPAM was concentrated three-fold using a vacuum evaporator, resulting in a mixture containing 105 g/l of acrylic acid, 45 g/l of acrylamide, and 69 g/l of NIPAM. After addition of bio-acrylamide, the synthesis of the heteropolymer was conducted through radical copolymerization, in the presence of an oxidizing-reducing initiating system, followed by drying and grinding (see the synthetic steps at Fig. 7) and Section 2.13.

The quality of polymerization was evaluated based on the content of residual free monomers in the polymer and the characteristic viscosity of the heteropolymer solution (the latter indicate its molecular weight). Additionally, the stability of the polymer solution's properties under thermal-salt aggression was tested.

The content of monomers in the polymer did not exceed 0.3%, indicating a high degree of monomer inclusion in the polymer. The characteristic viscosity of a 0.1% solution, calculated based on the flow rate in an Ubbelohde viscometer, was 6–7 dl/g, indicating a high molecular weight of the polymer. The dynamic viscosity measured on a rotational viscometer did not change after heating the solution for 20 days at 70 °C (including in the presence of 0.2% NaCl), indicating a good level of resistance of the heteropolymer to thermo-oxidative degradation and thermal-salt aggression.

4. Discussion

The main goal of this work was to develop BCs for the production of two types of acrylic monomers, acrylic acid and N-alkyl acrylamides, from acrylamide. Such BCs should possess two types of amidase activity: AH and AT.

Since *Rhodococcus* bacteria have a large number of amidases, most of which are unexplored, we developed and applied ALE based on the sensitivity of *R. rhodochrous* M8 to Cl-acetamide. We found that Cl-acetamide inhibits the growth of *R. rhodochrous* M8 but does not block the activity of amidases capable of hydrolyzing it. The mechanism of Cl-acetamide toxicity is likely similar to that of F-acetamide, where (in the presence of amide-hydrolyzing activity in cells) F-acetamide is cleaved to form F-acetic acid, which is converted in the TCA cycle to F-citrate, a strong inhibitor of aconitase, leading to growth arrest (Fig. 8. A). In the absence of amide-hydrolyzing activity, halogen-acetamides do not hinder growth at the concentrations used (Fig. 8. B).

The developed two-step ALE methodology significantly improves the approach of (Clarke and Tata, 1973; Smyth and Clarke, 1975). First, Cl-acetamide is less toxic to personnel than F-acetamide but achieves similar results in selecting clones with reduced AH activity. Second, we utilized information that nitrile hydratases and amidases in *R. rhodochrous* M8 may be co-regulated (Table 2). This allowed us to obtain clones in the first stage of ALE in which both amidases and nitrile hydratases were disabled. Obtaining Ami^{+} revertants in the second stage demonstrated that it was possible not only to achieve constitutive synthesis, as it was shown earlier (Clarke and Tata, 1973; Smyth and Clarke, 1975), but also to obtain different levels of AH activity (including increased activity) and disable interfering genes. The increased amide-hydrolyzing activity (in strain M8-50) was not due to mutations in amidase genes but most likely due to changes in regulation and the disabling of hyper-expression of nitrile hydratase genes.

The discovered AH activities were found to be tolerant to acrylamide, distinguishing them from the activities of several described amidases (see section 1). Furthermore, this approach allowed us to point three candidate amidase genes that are promising for rational construction of such BCs. These include the aliphatic amidase *amiE* and the formamidases *fmdA1* and *fmdA2*. Deletion of DNA fragment containing *amiE* and *fmdA1* resulted in a several-fold reduction in AH activity of strain M8, and further deletion of DNA fragment containing *fmdA2* led to a complete loss of AH activity. The aliphatic amidase, based on sequence homology and the presence of conserved domains belonging to the nitrilase/cyanidhydratase family (IPR000132 at InterPro database (https://www.ebi.ac.uk/interpro/)), differs from its closest experimentally studied homolog, the amidase from *Brevibacterium* sp. R312 (Soubrier et al., 1992), by 8.4% in amino acids. Both formamidases, belonging to the acetamidase/formamidase family (IPR004304 at InterPro database (https://www.ebi.ac.uk/interpro/)), are similar to each other by 94% and differ from their closest experimentally studied homolog, the formamidase from *Methylophilus methylotrophus* (Wyborn et al., 1996), by 52%. Additionally, for the first time, functional activity of the acylamidase gene product from *R. rhodochrous* M8 was experimentally demonstrated. This acylamidase differs from its closest experimentally described homolog, the acylamidase from *R. qingshengii* TA37, by 32%.

As for the ALE methodology used and its results, it is interesting to note two points. Firstly, unlike several other examples of ALE application (for example (Lenski, 2023),), the desired changes in the genomes were relatively easily obtained. We did not use chemical or other mutagenesis, and did not conduct long adaptation of cultures on selective media. It should also be added that similar relatively high frequencies of appearance of Ami^{-} mutants and Ami^{+} revertants were easily obtained by us when conducting similar ALE with the known strain *R. rhodochrous* J1 (Nagasawa et al., 1988), which also possesses the discussed pathway of amide utilization (data not shown). Secondly, as a result of ALE, not only SNPs (short nucleotide substitutions, microdeletions/insertions) appeared in the derived M8 strains, but also substantial genome rearrangements, including insertions and deletions up to 52 kbp. We assume that these rearrangements were induced by stress that occurred in cells upon exposure to the toxicity of chloroacetamide (for example, see review (Foster, 2007)), and could be associated with the activity of mobile elements (for example, see (Darmon and Leach, 2014)).

When discussing whole-genome comparisons of changes during ALE, it is important to consider the possibility and reliability of drawing conclusions from such comparisons. It is known that due to the high variability of bacterial genomes, bioinformatics tools for detecting changes (primarily SNPs) are not sufficiently accurate (Bush, 2021; Bush et al., 2020). Additionally, sequencing errors can occur even with high-quality sequencing using different platforms (Illumina and Oxford Nanopore (Heo et al., 2021),). Therefore, experimental verification of SNPs found using Snippy will be required to make assumptions about their role in the phenotypes observed in our study. As for larger rearrangements (found using Mauve), they are presumably detected with greater reliability (although we could not find an analysis in the scientific literature).

The fundamental question of whether the activity of mobile elements increases under stress conditions in *Rhodococcus* bacteria or remains constant, is of great interest, but an additional research is needed to answer this question. However, we showed that simple ALE scheme led to significant self-editing of the genome, demonstrating the plasticity of *Rhodococcus rhodochrous* and the potential of ALE to identify and enhance useful properties of *Rhodococcus* cells for biotechnology. Our results contribute to the development of ALE methods (Dragosits and Mattanovich, 2013; Maeda et al., 2020; Sandberg et al., 2019), which are gaining popularity due to the high genetic diversity of bacteria and the limited knowledge even of the most well-studied ones.

In conclusion to the discussion of scientific relevance of our results, it should be noted that *Rhodococcus* possess significantly more amidases than other industrially relevant bacteria, first *E. coli*, *Bacillus*, *Corynebacterium* (Table S6). This particularity of the genus is possibly the result of its specific ecological niche of degraders of recalcitrant compounds

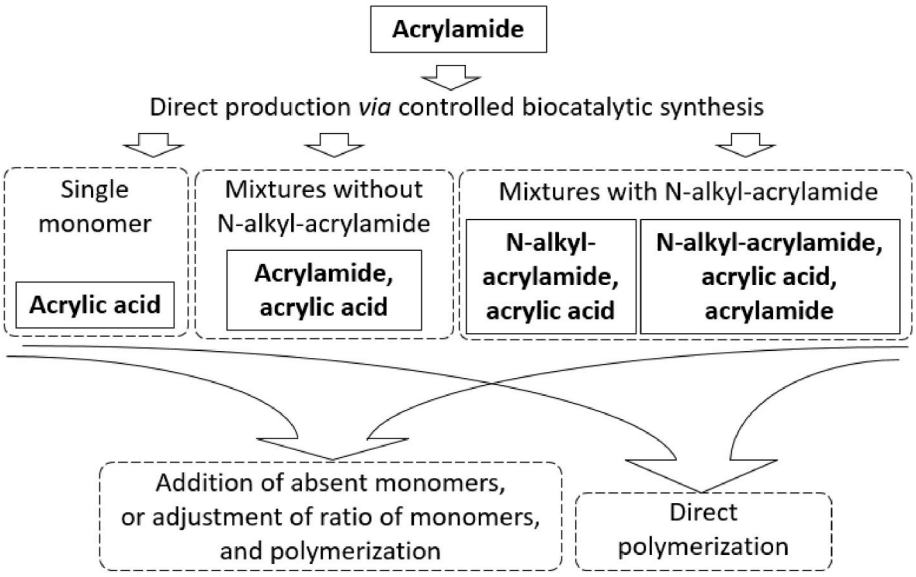

Fig. 9. Acrylonitrile-free scheme for biocatalytic synthesis of three types of monomers for the synthesis of water-soluble acrylic heteropolymers.

(Martínková et al., 2009). Thus, our results additionally highlight the significance of the genus as a powerful source of new amide-hydrolyzing enzymes for biotechnology.

5. Conclusions

As a result of using the AH activities found in M8, and combining them with the AT activity of acylamidase from *R. qingshengii* TA37, the BCs were created for the production of acrylic acid, binary acrylic acid + acrylamide mixtures, and ternary acrylic acid + acrylamide + NIPAM mixtures. For BCs with two types of activity, AH and AT, the ratio of these activities affected the ratio of monomers in the mixture. It can be said that (1) the higher the proportion of AT activity, the more N-alkylacrylamide in the mixture and (2) the higher the proportion of AH activity/longer the reaction time, the higher the proportion of acrylic acid. The proportion of acrylamide, which is a residual monomer, depends on the ratio of the above factors. It is obvious that the dependence of the ratio of monomers on the ratio of activities is not directly proportional, and the available results do not offer a mathematical apparatus for predicting the composition of the mixture. Despite this, in the practical application of BC, the leading role for the controlled synthesis of mixtures will be played by the accumulation of experience in obtaining monomers and their mixtures under industrial conditions.

The advantage of the new concept is that acrylic monomers and their mixtures can be obtained based on acrylamide, cheap and available biotechnological product, without using acrylonitrile. Based on the obtained results, and taking in account the ability of acylamidase to synthesize another N-alkyl-acrylamide, N,N-dimethylaminopropylacrylamide (Lavrov et al., 2013), an acrylonitrile-free scheme for obtaining three types of monomers for the synthesis of water-soluble acrylic heteropolymers can be proposed (Fig. 9).

Thus, the initial concept (Fig. 1) is feasible with the modification that N-alkylacrylamide can be obtained directly as a mixture with other monomers. The obtained products are water solutions of mixtures of monomers in adjustable ratios, which can be directly used for polymerization. In addition to reducing mixing operations, direct synthesis of mixtures, compared to obtaining individual monomers, allows for the reduction of technological difficulties associated with storing and transporting highly reactive acrylic acid. The obtained results provide a basis for developing a unified technological chain for obtaining three types of monomers for the synthesis of water-soluble polymers for a wide range of applications.

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Author contributions

Leonova T.E., Gerasimova T.V. – laboratory evolution and evaluation of strains; Shemyakina A.O., Grechishnikova E.G., Ryabchenko L.E. – strain construction and evaluation; Bayburdov T.A., Shemyakina A.O. – synthesis and testing of acrylic monomers and polymer; Lavrov K.V., Shemyakina A.O. – visualization, bioinformatics methods, data curation; Novikov A.D., Kalinina T.I. – obtaining and curation of genomic and transcriptomic data; Lavrov K.V. - writing original draft; Shemyakina A. O., Grechishnikova E.G., Leonova T.E., Yanenko A.S., Lavrov K.V. - review & editing; Yanenko A.S., Lavrov K.V. – conceptualization, project administration; Yanenko A.S. - funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data is available in Mendeley Data; doi: 10.17632/b9fz39cbn3.1

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mec.2023.e00231.

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