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Perspective approaches with the use of biocatalysts for improving the processes of polyaspartic acid production from oil benzene fraction after oxidative desulfurization

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Abstract. Two promising approaches to greening the production of polyaspartic acid were demonstrated. The mixture of dibenzothiophene-sulfone (DBTO₂) and benzothiophene-sulfone (BTO₂) as potential products of oxidative desulfurization of petroleum products during benzene production was used for the first time for biocatalytic recovery to sulphide ions. The process was carrying out at the presence of biogenic H₂ produced during cultivation of anaerobic cells *Clostridium acetobutylicum* and *Desulfovibrio vulgaris*. It was shown that after 240 h conversion of sulfur, contained in mixture of 0.03 mM DBTO₂ and 0.03 mM BTO₂ was as minimum 32.2±2.1% using ethanol as extractant. This approach can be recommended for intensification and ecologization of oxidative desulfurization of various petroleum products. For production of stereoregular polymer of polyaspartic acid the correction of native process of chemical synthesis was done by addition of biocatalytic stage during conversion of ammonium fumarate into aspartic acid. The obtained immobilized biocatalyst on a base of *E. coli* BL-21 cell included into poly(vinyl) alcohol cryogel make it possible to accumulate 129.1±3.4 g/L aspartic acid during biotransformation of ammonium fumarate.

1. Introduction

More than forty states worldwide currently pursue explicit political strategies to expand and promote their bioeconomies. In connection with global environmental problems and the gradual depletion of resources the modern chemical production, as it is organized now, requires significant modernization. The main accent made on introduction of new ecoefficient technological decisions and realization of principles of bioeconomy [1]. Complete and deeper conversion of resources, minimization of wastes, the use of renewable raw materials, selective catalysts and biocatalysts, while the overall production efficiency should not be lower than that of existing analogues. Obviously, that achievement of all these targets is possible only through a comprehensive review of all stages, starting from the raw materials and ending with the production of a wide range of needed products that can be obtained during of a multistage conversion practically without waste formation. Increasing the market potential of such industries and processes is mainly possible if at least some of the products will be potentially with high value-added products or components for production such products.

Today such products are presented in innovative directions of medicine and pharmaceutical industry. Polymers based on lysine, aspartic (AA) and glutamic (GA) amino acids containing side functional groups that can be easily chemically modified are highly demand in dynamically developing innovative industries [2-3]. On their basis already obtained inexpensive biocompatible, non-toxic, biodegradable polymers, composite materials with desired properties, new systems of



targeted drug delivery, therapeutic peptides, enzymes, combined systems for the therapy and diagnosis of a number of diseases. A number of copolymers-ampholytes on the basis of polyAA, polyGA or polylysine can show coassociative behavior with the formation of nanoparticles, which is especially valuable for the development of stable functioning systems of targeted delivery of pharmaceutical ingredients, monitoring of their prolonged releases and operations [2, 4].

Previously the authors of this work were demonstrate new effective approaches to the use of non-toxic polyelectrolyte complexes of polyAA and polyGA with hexahistidine-tagged organophosphorus hydrolase (His₆-OPH) in the agricultural, pharmaceutical and medical areas: for 100% degradation of pesticides in soils, [5], detoxification of animal feed [6], and increase the effectiveness of antibiotics [7-9].

Thereby, polyAA, polyGA and polylysine are products with potentially high added value, and modernization of their production, from the point of view of implementing the principles of green chemistry, is perspective.

Today the wide range of biocatalytic production processes of polyGA and polylysine from renewable sources are known. PolyGA can be obtained using biocatalytic method in one stage. [10]. In industry PolyAA is obtained by chemical synthesis from maleic anhydride during of multistage conversion of oil-and-gas substrates with production a non-stereo-regular product [2].

The absence of stereoregularity practically does not affect on biodegradability index. However, at the presence of calcium ions the chelating ability of non-stereoregular polyAA polymers was decrease on relation to cations [11]. This fact overlay restrictions on possible use of such polymer, in particular, in innovative areas. For example, when developing a dendriplexes based on electrostatic interactions for gene delivery systems, it is preferable to use stereoregular polymers of polyacids [12].

Thereby, the search of opportunities to modernize the scheme of the process for stereoregular polyAA production with a glance of principles of green chemistry and the needs of actively developing innovative areas is perspective and promising. The purpose of this work is demonstrate two promising approaches to greening the production of polyAA: (I) bioutilization of wastes, obtained after oxidative desulfurization of oil Benzene fraction, using *D. vulgaris* and *C. acetobutylicum* as biocatalysts, and (ii) correction of the initial chemical synthesis (Figure 1A), including into the process the biocatalytic stage with *E. coli* cells with aspartase (aspartase ammonia lyase, EC 4.3.1.1) activity immobilized in the polyvinyl alcohol (PVA) cryogel as biocatalyst for conversion ammonium fumarate to AA – monomer for synthesis of α -polyAA or β - polyAA (Figure 1B).

2. Materials and methods

2.1 Microorganisms and cultivation conditions

The bacterial strains *Clostridium acetobutylicum* B1787 and *Desulfovibrio* were obtained from the Russian National Collection of Industrial Microorganisms (www.genetika.ru). The *Clostridium acetobutylicum* strain B1787 was cultivated in the following medium (g/L): glucose – 20; triptone – 10; yeast extract – 5 (pH 6.8). The *Desulfovibrio vulgaris* strain B4053 was cultivated in the Postgate medium [13]. Cultivation of bacterial cells was performed under anaerobic conditions in an argon atmosphere at 37°C for 20–24 h and 48 h, respectively.

For conversion of ammonium fumarate to AA bacterial cells of *E.coli* SG13009[pREP4] (Qiagen, Hilden, Germany), BL-21 (Novagen, Darmstadt, Germany) and DH5 α (Thermo Fisher Scientific, MA, USA) were used.

2.2 Methods.

2.2.1. Preparation of immobilized biocatalyst on base of *E.coli* cells for AA synthesis

Different samples of granules of immobilized biocatalyst (IBC) containing bacterial *E.coli* cells immobilized in polyvinyl alcohol cryogel were prepared by cryoimmobilization using a previously published technique [14]. IBC formation was carried out at -18°C during 30 h. After that, the granules (cylinders, $d = 5.9 \pm 0.1$ mm, $h = 5.0 \pm 0.1$ mm) were thawed at 4°C for 12 h, and the obtained samples of IBC were used in subsequent investigations.

For AA production in continuous and repeated modes at pH 8,5 and 37°C the following medium

was used (g/L): ammonium fumarate - 150, $\text{MgSO}_4 \times 7\text{H}_2\text{O}$ – 0,25.

2.2.2. Determination of concentrations sulphide ions and AA

Sulphide ions in liquid phase was monitored spectrophotometrically at 660 nm using Shimadzu UV-1202 (Japan) [15]. For the assay of AA concentration we used assay kit: Amplite™ Colorimetric (575 ± 5 nm) L-Aspartate (Aspartic Acid) Assay Kit (AAT Bioquest, Sunnyvale, CA, USA).

The data were shown as means of at least three independent experiments \pm standard deviation (\pm SD). Statistical analysis was realized using SigmaPlot 12.5 (ver. 12.5, Systat Software Inc., San Jose, CA, USA).

3. Results and Discussion

3.1. New approaches to the polyAA production scheme

Today, the most common process for polyAA production is chemical synthesis through the hydrolysis of polysuccinimide with formation of non-stereoregular polymer containing a mixture of α and β forms (Figure 1A) [2, 16].

A modified hybrid scheme of polyAA synthesis was proposed in this work using AA as initial monomer for stereoregular polyAA production (Figure 1B). This solution (Figure 2B) contains greater number of stages, but, despite of this, it's competitive and reasonable in terms of implementing the principles of green chemistry. All additional chemical stages were tested and actively used on an industrial scale in technological schemes for the production of other products. The production scheme of polyAA after the proposed modernization contains a stage of biocatalytic transformation of chemically obtained ammonium fumarate into a non-toxic monomer – AA (Figure 2B, gray area with *E.coli* immobilized in the PVA cryogel). For the first time for carrying out this reaction the use of a biocatalyst immobilized in PVA cryogel has been proposed. This carrier has been previously successfully used for production of biocatalysts based on cells of various microorganisms [17, 18].

The AA can be then transformed into a stereoregular polymer by known chemical methods. So the synthesis of α -polyAA can be realized using *N*-carboxyanhydride (NCA) method with protected pendant group [19, 20].

For this purpose AA is preliminarily transformed into aspartic acid β -benzyl ester, which then is convert by cyclization (the Leuchs method) method into the *N*-carboxyanhydride [21]. Further by the ring-opening polymerization of *N*-carboxyanhydride is obtained poly(β -benzyl-aspartate) and then hydrolyzed for conversion into α -polyAA [22]. By the ring-opening anionic assisted polymerization, poly(α -benzyl-aspartate) can be synthesized from the previously obtained aspartic β -lactam benzyl ester (3(R)-benzoxycarbonyl-2-azetidinone) by using solvents with low polarity. Poly(α -benzyl-aspartate) may be hydrolyzed for conversion into β -polyAA [23]. The price of stereoregular polyAA forms today is 6 times higher than the price of non-stereoregular forms (www.sigmaaldrich.com), in this regard, their production is more economically attractive.

The proposed process modernization is partially affects the production of benzene from hydrocarbons - the initial chemical compound in the polyAA synthesis scheme (Figure 1). Today, the production of benzene is based on the conversion of a number of raw materials: oil, toluene, the heavy pyrolysis fraction, and the coking coal tar. The main amount of benzene is obtained by catalytic reforming of a heavy oil gasoline fraction (naphtha), which is present itself a mixture of paraffins, naphthenes and aromatic hydrocarbons of the C6-C9 fraction, boiling away at 62-180°C. The reforming process is carried out in the presence of a catalysts ($\text{Al}_2\text{O}_3/\text{Pt/Re}$ and other) at a temperature of 500-530°C and at pressure of 18-35 atm (or at 2-3 atm using machineries with continuous regeneration). The resulting product (stable reformat containing benzene) is cooled and removed. Crude benzene is mainly presented as a mixture of low-boiling aromatic hydrocarbons (80-95%), unsaturated hydrocarbons (5-15%), sulfur-containing compounds (0.2-2.0%) and others. The sulfur compounds are presented in raw benzene as carbon disulfide (CS_2), thiophene ($\text{C}_4\text{H}_4\text{S}$) and its homologues. Even minor impurities of sulfur in benzene, used for organic synthesis, can cause rapid deactivation of metal catalysts.

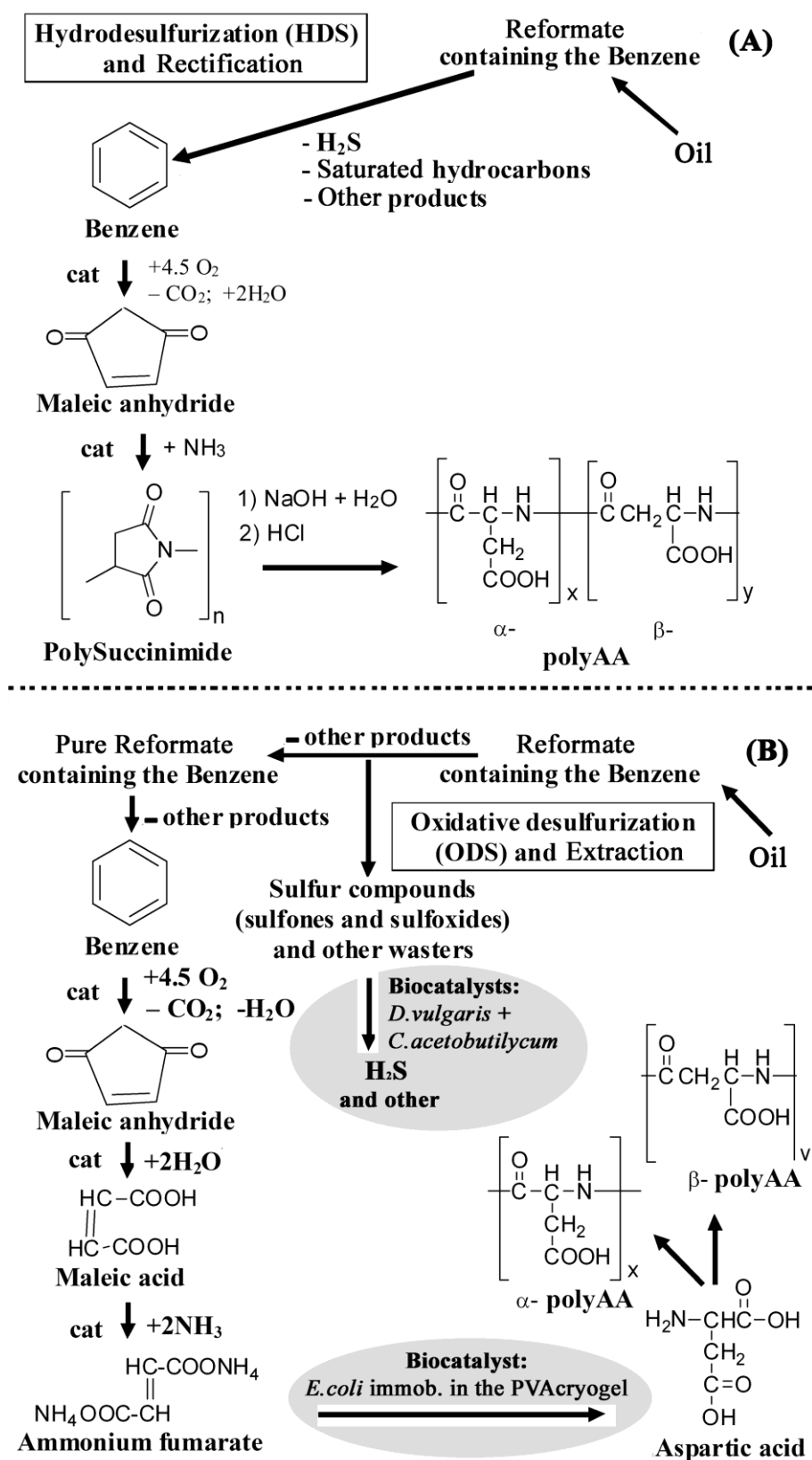


Figure 1. Scheme of non-stereoregular polyAA production from benzene, obtained from oil reformat and purified from sulfur compounds by hydrodesulfurization (HDS) (A) and Scheme of stereoregular polyAA forms production from benzene obtained, from oil reformat and purified from sulfur compounds by oxidative desulfurisation (ODS).

In this regard, it is necessary to purify the reformat from sulfur-containing and unsaturated compounds before distillation of reformat (when the main part of benzene boils at a temperature below 180°C) in order to isolate benzene, toluene, xylenes and solvent (solvent naphtha). For this purpose a low-waste method of catalytic hydrotreatment is recommended, which consists in treating of crude benzene and its fractions with hydrogen or coke oven gas. The target purification reactions are hydrodesulfurization (HDS) and hydrogenation of unsaturated hydrocarbons. Under preparation of high purity benzene the hydrodesulfurization reactions has a key role, especially the hydrogenolysis (destruction) of the most thermally stable compound-thiophene. The hydrodesulfurization catalysts are presented as sulfides or oxides of molybdenum, cobalt, tungsten, nickel, vanadium.

The process of hydrogenation is carried out under pressure in the gas phase over the catalyst; for this purified products are completely transformed into a vapor state and separate from the non-evaporating residue. The resulting vapors are mixed with pure hydrogen or with coke oven gas (about 57–60% of hydrogen) and go through the catalytic hydrotreating. Under the influence of temperature and pressure, various reactions take place, including the following: hydrodesulfurization reactions, hydrogenation of unsaturated compounds, hydrogenation of aromatic hydrocarbons, hydrocracking of saturated hydrocarbons, demethylation of benzene homologues. The chemistry of the hydrotreating process is consist in the sulfur-containing impurities are hydrogenated to form the corresponding hydrocarbons and hydrogen sulfide.

Until recently, hydrodesulfurization was considered the most effective way to purify hydrocarbon feedstock from sulfur compounds, however, in connection with hardening requirements for sulfur content, hydrofining capabilities almost reached the limit, and bringing the sulfur content in petroleum products to the required standards of 0.001 wt.% and below using this method is not economically rational [24]. As an alternative to hydrotreatment, hydrogenless methods for the removal of sulfur compounds are activity considered, of which the most perspective and low-cost from an economic point of view, are presented a catalytic oxidative desulfurization (ODS) [25].

In contrast to hydrodesulfurization, this approach is involves the catalytic treatment of the sulfur-containing fraction under mild conditions with a low-cost oxidant (for example, H_2O_2) with formation of dibenzothiophene, benzothiophene, etc., which are further separated by an extraction method. Catalytic systems have been developed for the efficient oxidative desulfurization of various petroleum fractions using H_2O_2 and O_3 and the most effective methods were founded for extracting of the oxidation products of sulfur compounds from hydrocarbon fractions using the extraction method [26, 27].

This approach is generally promising, including the possibilities of modernizing the technological scheme for polyAA production at the stage of obtaining of highly purified benzene. However, at the present time the question of environmentally safe and economically viable utilization of extracts containing sulfur compounds is still not resolved. During the modernization of the scheme presented in this work for the first time was proposed to recover such extracts by anaerobic biocatalytic way with *D.vulgaris* and *C.acetobutylcum* (Figure 1B gray area). If successful, this approach to the anaerobic degradation of waste oxidative desulfurization of petroleum products can be further combined with the production of commercial products, such as biogas.

3.2. Biotransformation of model waste oxidative desulfurization of hydrocarbons used to produce benzene

As a model extract containing oxidized forms of organic sulfur-containing compounds, which can be obtained after the oxidative desulfurization of hydrocarbons during the production of benzene, solutions containing a mixture of 0.03 mM dibenzothiophene-sulfone (DBTO₂) and 0.03 mM benzothiophene- sulfone (BTO₂) in ethanol or N,N-dimethylformamide (DMFA) were used. The cells of *D. vulgaris* and *C. acetobutylcum* were tested as biocatalysts for biotransformation of these compounds. To support the effectiveness of the action of biocatalysts, glucose (3 g /L) was used as a substrate.

From the obtained data (Figure 2), it is follow that after 240 h at least 30% of sulfur contained in a mixture of 0.03 mM BTO₂ and 0.03 mM DBTO₂ can be transformed into sulfide ions using ethanol as a potential extractant.

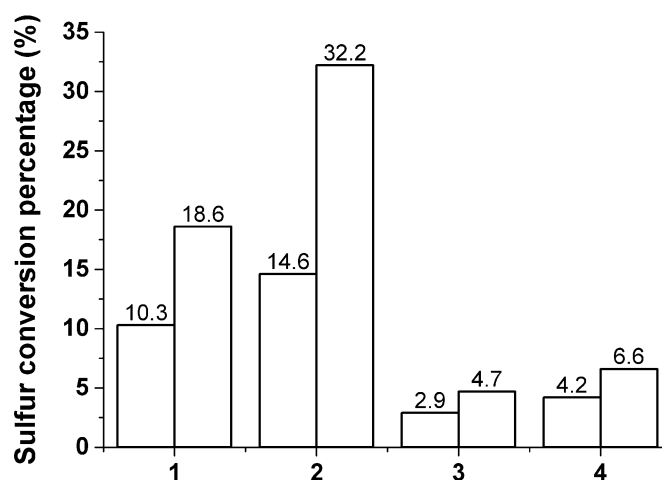


Figure 2. Biotransformation of sulphur from 0.03 mM DBTO₂ + 0.03 mM BTO₂ to S²⁻ with *D.vulgaris* (white bars) or *D.vulgaris* (95%) + *C.acetobutilycum* (5%) (shaded gray bars) after 120 h (1) and 240 h (2) with ethanol as extractant, after 120 h (3) and 240 h (4) with DMFA as extractant, respectively.

The use of DMFA as an extractant in the proposed process was not rational, since it's characterized minimal degrees of conversion of sulfur-containing compounds. This is apparently connected with toxicity of DMFA, which is showed inhibitory effect on the activity of biocatalysts.

For the most efficient run of bioconversion process of sulfones, it is preferred to additionally introduce of *C. acetobutilycum* cells to medium with sulfate reducers.

Thereby, for the first time was shown the principal possibility of biocatalytic reduction of BTO₂ and DBTO₂ mixture as potential products of oxidative desulfurization of petroleum products, during the production of benzene to hydrogen sulfide. The process is run in the presence of biogenic hydrogen produced by anaerobic bacterial cells. This approach can be proposed for the intensification and ecologization of the processes of oxidative desulfurization of petroleum products.

3.3. Investigation of the possibility of transformation of fumaric acid into aspartic acid under the action of biocatalysts based on *E. coli* bacterial cells immobilized into poly(vinyl) alcohol cryogel

The main chemical method of industrial production of AA is the condensation of maleic anhydride with ammonia under high pressure and followed hydrolysis of the resulting ammonium salt of aspartic acid and separation of the free acid. In this case, a racemic mixture of AA isomers is formed with a yield about 72-80%. Currently, pure L-AA or D-AA is obtained by biotechnological methods. It was proved that using of cells with aspartate ammonia-lyase (aspartase) activity as biocatalysts is more efficient as compared with technologies used pure enzyme. The process of enzyme separation and purification is long, laborious and accompanied by decreasing of enzyme activity.

After a comparative analysis of the possibilities of biocatalytic production of L-AA by biotransformation of ammonium fumarate using bacteria of the genus *Serratia*, *Pseudomonas*, *Bacillus*, *Brevibacterium*, *E. coli*, etc. [28] it was determined that using of *E. coli* cells is more reasonable from a perspective of process productivity and product yield [29]. At the same time, it was determined that cells immobilized into polymer matrix (polyacrylamides, alginates, carrageenans, polyurethanes, polyazetidine, etc.) were characterized enhanced resistance and good productivity [30]. The activity of free cells was reduced when used substrate concentration above 1 M, and the immobilized cells was remained active up to 1.5 M of ammonium fumarate [31].

Since in natural *E.coli* strains aspartic activity was reduced, the processes of AA production using various genetically modified bacterial strains have been proposed [32].

However, the high cost of these recombinant strains, the hardness of their cultivation and the problems in utilization of biomass are limits use of such producers in practice. Thus, the development of a new efficient and stable biocatalyst based on natural *E. coli* strains is highly urgent.

In this regard, screening among strains of *E. coli* was done to identify the possibility of carrying out biotransformation of ammonium fumarate to AA (Table 1). Ammonium fumarate can be obtained by known chemical methods from benzene according to the proposed in Figure 1B. Also, ammonium fumarate can be obtained from renewable raw materials through the furfural and fumaric acid [33, 34].

The highest transformation efficiency of ammonium fumarate into AA was observed when *E. coli* BL-21 strain was used (Table 1). It is known that the best results of obtaining different products using *E. coli* cells can be achieved by using producers in immobilized form [35] and when PVA cryogel used as a carrier can be obtained stable and high effective biocatalysts [36].

Table 1. Efficiency of transformation of ammonium fumarate (1 M) into AA by *E. coli* cells.

<i>E.coli</i> strain	Concentration of AA, g/L	Conversion, %
SG13009[pREP4]	34.2±1.7	77.2±3.9
DH5α	38.7±1.9	87.4±4.3
BL-21	41.2±1.9	93.1±4.6

So, cells of the *E. coli* BL-21 were immobilized into PVA cryogel. During the formation of immobilized biocatalyst (IBC), the concentration of bacteria biomass introduced into the mixture with PVA solution was varied from 10 to 30 wt%. Maximal concentration of AA was accumulated when IBC with a cell concentration of 25–30 wt% was used (Table 2).

Under conditions of a flow reactor of column type, the period of IBC semi-inactivation with 25 wt% cell biomass was exceeded 180 days. Under periodic conditions such biocatalyst can be used more than 10 times. The characteristics of development IBCs were better or comparable with similar characteristics of known analogues [37, 38].

Table 2. Influence of cell concentration in the composition of IBC on conversion of ammonium fumarate (1 M) into AA.

Cell concentration, wt %	AA, g/L	Conversion of ammonium fumarate, %
10	119.1±3.9	89.5±1.5
15	123.5±3.1	92.9±1.3
20	125.2±3.2	94.1±1.4
25	127.3±3.3	98.2±1.5
30	129.1±3.4	97.1±1.5

Thus, the use of cells with aspartase activity immobilized into PVA cryogel can be perspective from the point of view of possibilities of further use in the processes of AA production and its polymers.

4. Conclusion

Two promising approaches to greening the production of polyAA were demonstrated. The mixture of dibenzothiophene-sulfone (DBTO₂) and benzothiophene-sulfone (BTO₂) as potential products of oxidative desulfurization of petroleum products during benzene production was used for the first time for biocatalytic recovery to S²⁻ in anaerobic conditions. This approach can be proposed for the intensification and ecologization of oxidative desulfurization processes of various petroleum products. To obtain stereoregular polyAA was suggest a correction of the polymer initial chemical synthesis by introduction of biocatalytic stage during conversion of ammonium fumarate to AA - monomer. The high conversion (98,2±1,5%) of 1M ammonium fumarate to AA was shown under action of biocatalyst with *E. coli* BL-21 cells immobilized into PVA cryogel. Immobilization of cells with aspartic activity in PVA cryogel is promising way from the point of view of the development of IBC for the production of AA and its polymers.

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