**Lactic acid production by immobilized fungus cells *Rhizopus oryzae* scaled up to the complex experimental system**

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## Introduction

The common scheme of microbiological process of lactic acid production supposes the use of bacterial cultures and usually contains two main parts: 1 – lactic acid fermentation, catalyzed by free cells [Narayanan, 2004; Shamtsyan, 2002]; 2 – isolation of lactic acid from cultural medium with its further, purification and concentration [Wasewar, 2002; Cao, 2002; Gonzales, 2006].
The purified and highly concentrated lactic acid can be used in various fields of industry including production of polymers based on poly(lactic acid) [Hofvendahl, 2000].
It should be noted that further improvement of the process organization and its intensification can be realized via modernization and advancement of each mentioned technological parts.
The use of immobilized fungus cells at first stage of the process should allow obtaining of cultural media with less amounts of various ingredients including free cells, metabolites nutrition components as compared to process with suspended bacterial cells [Oda, 2002]. This approach should simplify the next stage, namely, isolation of lactate-ions.
A new technological solution for production of purified and concentrated lactic acid and sodium lactate was offered due to the use of previously developed immobilized biocatalyst with filamentous fungus cell entrapped into cryogel of poly(vinyl)alcohol (PVA) [Efremenko, 2005].

## Material and methods

The *Rhizopus oryzae* NRRL-395 cells were taken from VKPM (Moscow, Russia). Fungus spores were grown up on potato-dextrose medium containing agar (2%) and stored in refrigerator at 4$^{o}$C.
The biocatalyst on the base of immobilized fungus cells entrapped in PVA cryogel was prepared in accordance with proposed procedure [Efremenko, 2005].
The following medium was used for the lactic acid production (g/L): $(NH_4)_2SO_4$ – 3.02, $MgSO_4$x7H2O – 0.25, $ZnSO_4$x7$H_2O$ – 0.04, $K_2HPO_4$x3 $H_2O$ – 3.0, pH 5.0-6.0. Concentration of glucose was varied in the range 80-150 g/L.
The pH value was controlled using pH-meter model PBL. To maintain pH at the optimal level, calcium carbonate (20 g/L) was added into medium at the beginning of cultivation of immobilized cells. The nutritional medium was sterilized directly in the reactor (108$^{o}$C, 30 min).
During the continuous process the sterile medium was pumped (Cole Parmer L/S® Economy digital pump, USA) into reactor (Biostat, Germany) under aseptic conditions The duration of each batch cycle was 25 h.
To reduce the foam formation, 0.1% (v/v) antifoam C emulsion (Sigma, USA) was introduced into the medium during fermentation process. The cultivation of immobilized cell was organized in reactor with constant agitation, realized with mechanical mixer (500 rpm) at 30°C and air injection. The experiments were carried out in reactors with different constructions, characterizing by the presence and absence of vertical flow breakers.
The concentration of glucose in the cultivation medium was analyzed spectrophotometrically by common glucose-peroxidase methods. The concentration of lactic acid was concurrently estimated by HPLC and enzymatic method with application of L(+)-lactate oxidase kit. The intracellular ATP

concentration in immobilized fungus cells was analyzed by bioluminescent method with luciferine-luciferase reagent (Lumtek, Russia).
The cultural broth was forwarded to the step of isolation and purification lactate-ions. The spent medium was filtered by filter FD-293-3 (Biotest, Russia) and loaded onto chromatographic column (Pharmacia, Sweden) with anionic carrier. Several anionic carriers were tried in the work for isolation of lactate-ions: AB-17-8 (Azot, Ukraine), Silasorb DEA (Lachema, Chech), Aminex A-7 (BioRad, USA), Amberlite IRA-400 (Purolite, USA), Amberlite IRA-401 (Purolite, USA), Amberlite IRA-410 (Purolite, USA).
The velocity f loading of cultural medium onto anionic carrier was varied in the range 0.03-0.6 m/h (1-20 mL/min). The elution of lactate-ions from the carrier was realized using HCl or NaOH or NaCl or the mixture of 0.5 M NaOH and 0.5 M NaCl (1:1).
The concentration of lactate-ions in the eluates was constantly controlled and then the probes were combined into one and concentrated using vacuum evaporating system Laborota 4000 (Heidolph, Germany). The latter procedure was carried out for 1-2 h at 30-40ºC. The final concentration of the target product was 80-90%.

**Results and Discussion**

New technological scheme of lactic acid production based on application of immobilized cells of filamentous fungi was developed. The main stages of the process are present in Fig.1.

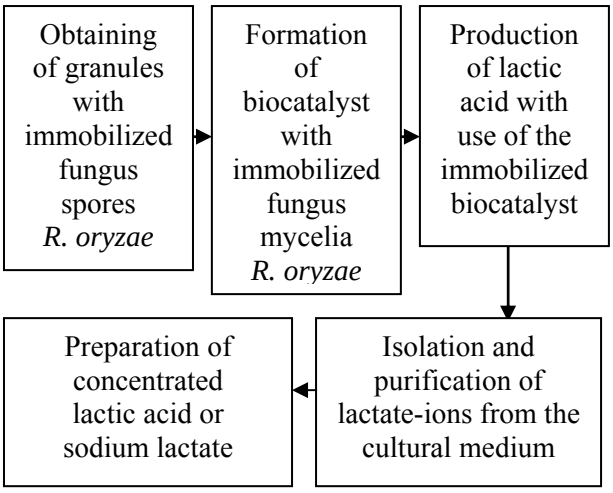


**Fig.1. The scheme of developed process of lactic acid production with immobilized fungus cells**

To realize the technological scheme of lactic acid production, the investigation of individual stages was concurrently carried out with scaling up of the process.
The comparative analysis of efficiency of application of immobilized biocatalyst under batch conditions with and without additives of carbon source was carried out. The additives were introduced into medium after each 8-12 h of cultivation of immobilized cells.
It was established (Table 1), that productivity of batch process with glucose additives appeared to be 1.5-2 times less as compared to batch process with constantly reduced glucose concentration. The 20-30% decrease in yield of target product was also revealed, when the additives were used. At the same time, the final concentration of required product was 1.5-1.7 times higher in the cultural medium than in the case of process without substrate additives.

It was shown that the process of lactic acid production could be continuously conducted in the 5L-reactor with initial concentration of glucose equal to 100 g/L and 65 g/L immobilized biocatalyst for 10 days under batch conditions. The high enough concentration of intracellular ATP in the granules with immobilized fungi was established at the end of their use in the batch process both with and without additives.

| Initial glucose concentration, g/L | LA max, g/L | LA yield, % |
|---|---|---|
| 100[a] | 80±1 | 80±1 |
| 120[a] | 89±2 | 74±2 |
| 100 (270)[b] | 145±2 | 54±1 |
| 120 (300)[b] | 151±6 | 50±2 |

**Table 1. Characteristics of batch process of lactic acid production by immobilized cells under batch conditions with and without glucose additives.** The letters "a" and "b" correspond to batch process without and with glucose additives, respectively. The totally introduced concentration of glucose is given in brackets.

The cultural medium, obtained after exploitation of immobilized cells in the frame of complex biotechnological laboratory plant, was filtered to remove the residue of calcium carbonate, and loaded to the column with anionic carrier, allowing concurrent isolation and purification of lactate-ions.

It was established that various anionic carriers used at this stage of process possessed lactate-ion capacity considerably differed from theoretical level of anionic capacity declared by their producers. The cheapest anionic carrier AB-17-8 was preferred for further experiments. It should be noted someone did not previously use carrier chosen in this work for isolation lactate-ions.

It was shown that increase in concentration of lactate ions in the cultural medium loaded onto the carrier allowed to obtain more concentrated solutions of the target product from the column.

After optimization of conditions applied at this stage (velocity of loading of cultural medium onto carrier, velocity of elution of lactate ions, temperature of the process, type of eluent) the yield of lactate ions reached 95% level and was 25% higher as compared to known analogue [Mantovani, 1993].

Since the effluent being lactic acid or sodium lactate, obtained after anionic chromatography, was characterized by slightly yellow color, its additional treatment by activated charcoal was applied. It was shown that activated charcoal of various shapes (granules, tablets, fiber, etc.) could be used for product decolorization. The maximal capacity of the carrier in relation to lactate ions was determined to be 5 g/L carrier. Since then, the yield of product at the stage was 97-98%.

The concentrate obtained lactic acid or sodium lactate, the vacuum evaporating system was used. The procedure was carried out at 30-40$^{o}$C. The increase in temperature of evaporation resulted in slight polymerization of lactic acid. The losses of required product were not exceeded 3% at the stage of process.

Finally, two main products, 800 g/L lactic acid and 940 g/L sodium lactate, was obtained. The chromatography analysis of target products allowed to reveal, that concentration of byproducts was close to 1%. The concentration of L(+)-isomer was 98%.

## Conclusions

The laboratory system, enabling realization of multistage process of lactic acid production by the use of immobilized cells of filamentous fungi was tried in the work. The production of two highly

concentrated products from cultural medium, obtained as result of application of immobilized fungus biocatalyst was established. The conditions of functioning of technological system under batch conditions were optimized, when glucose was used as main substrate.

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