CZU 615.357.012:582.282.232(478)

CULTIVATION OF RAW MATERIAL BASED ON *PICHIA PASTORIS* FOR BIOSIMILAR GROWTH HORMONE MANUFACTURE IN REPUBLIC OF MOLDOVA

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Rezumat

CULTIVAREA MATERIEI PRIME PE BAZĂ DE *PICHIA PASTORIS* PENTRU PRODUCEREA HORMONULUI DE CREȘTERE BIOSIMILAR ÎN REPUBLICA MOLDOVA

Pichia pastoris este o specie de drojdie metilotrofică. Rata de creștere ridicată pe un mediu ieftin și aproape fără proteine, calea secretorie care prezintă o mare parte din structura și funcția sistemului secretor al mamiferelor ca capacitate de pliere, de glicozilare și secretare a proteinelor, face ca această specie să fie potrivită pentru utilizare biotehnologică. Gena modificată a somatropinei umane recombinate GH1 este inserată în genomul culturii *Pichia pastoris* prin restricție în apropierea promotorului genei AOX1 în plasmida vectorului de expresie pPIC9K. Gena GH1 este activată de promotorul genei AOX1 care poate fi indusă de prezența metanolului în mediul de cultură. Ulterior, secvența „Prepro-alfa Factor Leader" din *Saccharomyces cerevisiae* este adăugată la secvența modificată astfel încât somatropina obținută să poată fi secretată în mediul de cultură.

Cuvinte cheie: fabricarea biosimilare, hormon de creștere uman recombinant, Pichia pastoris.

Abstract

Pichia pastoris is a methylotrophic yeast. High growth rate on an inexpensive and almost protein-free medium, the secretory pathway that exhibits much of the structure and function of the mammalian secretory system as the capacity to fold, to glycosylate and to secrete proteins makes this system suitable for biotechnological use. The altered GH1 recombinant human somatropin gene is directionally cloned into the *Pichia pastoris* culture genome by a restriction near the AOX1 gene promoter in the expression vector plasmid pPIC9K. The GH1 gene is activated by the AOX1 gene promoter which may be induced by the presence of methanol in the culture medium. Subsequently, the "Prepro-alpha Factor Leader" sequence from *Saccharomyces cerevisiae* is added to the modified sequence so that the obtained somatropin could be secreted into the culture medium.

Keywords: biosimilar manufacture, recombinant human growth hormone, *Pichia pastoris*.

INTRODUCTION

Currently, only two medicinal products from the group of hormones of the anterior pituitary lobe and analogues are authorized on the market of Republic of Moldova, manufactured namely by Biosidus S.A., Argentina and Changchun GeneScience Pharmaceutical Co., Ltd., China. Thus, we can say that the domestic pharmaceutical market does not face the phenomenon of over-competition in terms of medicinal products from the ATC H01AC01 group. Given that the recombinant human growth hormone rhGH therapy usually has a recommended duration of several years and an individualized dosing regimen with a dose calculation based on the patient's body weight, a priority issue is increasing patient adherence and compliance to treatment. The benefit to the national health system of competition between biosimilar medicines is to improve patient access to safe and effective biological medicines of proven quality and at lower price.

In Moldova there is no domestic production of biosimilar products. The global rhGH market size was estimated at USD 2,840.70 million in 2018 and is expected to reach USD 5,563.60 million by 2026, registering a compound annual growth rate (CAGR) of 8.6% from 2019 to 2026. In

Moldova, a centralized public procurement is held annually with the object of purchasing medicines for the treatment of patients with pituitary insufficiency/pituitary dwarfism. For the 2022 budget period, in order to implement the National Program "Combating Rare Diseases", the contracting authority, the Center for Centralized Public Procurement in Health, launched the invitation for interested economic agents to submit offers for 21827.00 units of Somatropinum 1.33 mg / 3.33 mg / 8 mg / 10 mg.

The domestic drug manufacturer Balkan Pharmaceuticals SRL set out to manufacture the first biosimilar in the Republic of Moldova – an exhaustive goal in itself, as well as the unprecedented domestic production of recombinant human growth hormone (rhGH). This fact provides empirical exploration of the most promising segment of the global pharmaceutical industry with the fastest growth rate. Per se the field of biosimilars is an eminently boundless one for various local and international scientific researches in the vast range of medicine and pharmacy disciplines. Increasing awareness of the effectiveness of growth hormone, demand for cost-effective growth hormone therapies; increasing prevalence of serious chronic diseases such as chronic kidney disease and growth retardation and pituitary dysfunctions; and increasing compliance for growth hormone formulations are the major factors driving its global market expansion.

AIM OF THE STUDY

To obtain the recombinant growth hormone, *Pichia pastoris* was selected as an expression system, which has many advantages over other organisms. We can list the following advantages: the processing, protein folding and post-translational modifications of proteins, as well as the easy manipulation comparable with *E. coli*. All the risks of maintaining a cell line in continuous or extended culture were minimized and practically excluded by using the working cell bank of *P. pastoris*. In collaboration of Scientific Center of Drug Research of *Nicolae Testemițanu* State University of Medicine and Pharmacy, the pharmaceutical company Balkan Pharmaceuticals and ICGEB, the technology transfer during scale-up from the laboratory phase to the pilot phase of the processes of creating the working cell bank and

growing *Pichia pastoris* strains in the Erlenmeyer flask were carried out.

The recombinant human somatropin GH1 altered gene was introduced into the genome of *Pichia pastoris* culture using restriction near the AOX1 gene promoter. The pPIC9K plasmid was used as a vector. The GH1 gene was activated by the AOX1 gene promoter which can be easily induced by the presence of methanol in the culture medium. The "Prepro-alpha Factor Leader" sequence from *Saccharomyces cerevisiae* was added to the modified sequence. After that the obtained somatropin could be secreted into the culture medium. We evaluated the impact of technological processes on the obtained product, developed Technological Protocols for *Pichia pastoris* short-term and long-term storage; inoculum preparation, inoculation and growth of *P. pastoris* strains in the Erlenmeyer flask. We developed Standard Operating Procedures for working cell bank creation and *in-vitro* biological activity assay of the recombinant human growth hormone.

MATERIALS AND METHODS

Maintaining a cell line in continuous or extended culture is itself a practice with many weak points including: risk of microbial contamination, loss of characteristics of interest (e.g., surface antigen or monoclonal antibody expression), genetic drift, especially, in cells known to have an unstable karyotype, loss of the cell line due to exceeding the finite life span, risk of cross-contamination with other cell lines, etc.

All these risks are minimized or excluded by using the working cell bank. The implementation of working cell bank ensures: constant material quality, performing experiments using cultures from the same passage number range, the presence of cells in the culture only when necessary, preserving the characteristics of the original cell line. The use of working cell bank also reduces the cost of cell culture processes, providing a cost-effective alternative for constantly keeping cells in culture. Also, the frequency of cell samples that diverge from natural cell divisions over time is effectively reduced.

Picha pastoris is a methylotrophic yeast, capable of metabolizing methanol as the sole carbon source. The alcohol oxidase enzyme

catalyzes the oxidation of methanol. As result of methanol metabolism is formed formaldehyde using molecular oxygen. This reaction generates also hydrogen peroxide and for this reason the reaction takes place in peroxisomes to protect the cell from the toxic effect of hydrogen peroxide. It is necessary to mention that alcohol oxidase has a weak affinity for O2, and *Pichia pastoris* compensates by increasing the expression of alcohol oxidase enzyme. For the recombinant protein production, promoters of alcohol oxidase enzyme are vastly used. Alcohol oxidase enzyme in *Picha pastoris* strain is encoded by two genes AOX1 and AOX2. The genes AOX1 and AOX2 are 97% similar, but yeasts with active AOX2 gene only grow very slowly, those are Mut^s strains. The AOX1 gene product accounts for most of the alcohol oxidase in the cell. AOX1 gene expression is strictly regulated and induced by methanol reaching very high levels (up to 30% of the total soluble protein). The slow growth and lower methanol consumption of Mut^S strains may have some advantages at large scale processes such as a lower demand for oxygen and reduced heat formation. In Mut^S strains the force of the AOX1 promoter can be directed mainly towards recombinant protein production instead of using energy for AOX1 protein production. Nevertheless, most researchers use a wild type strain, although some researchers showed that Mut^S strains were advantageous for production. *Pichia pastoris* can express the protein of interest both intracellularly and extracellularly in the secreted form. Secretion requires the presence of a signal sequence on the expressed protein. *Pichia pastoris* as expression systems has a major advantage because it secretes very little the native protein, but mostly the recombinant protein in the culture medium. The vectors type used has influences on the site of protein concentration. If we want to have an intracellular expression, we will use the vectors pHIL-D2 and pPIC3.5, and if an extracellular one - the vectors pHIL-S1 and pPIC9. For the cultivation of *Pichia pastoris* raw material with growth hormone expression, the recombinant human somatropin gene was introduced into the *Pichia pastoris* genome. As a vector was used the plasmid pPIC9K.

Somatropin is encoded by the GH1 gene, located on chromosome 17 and has 5 exons in the human body. The GH1 gene was modified in order to be inserted into the selected vector pPIC9K. The introns were

eliminated, only the coding sequence being preserved, then, some codons were replaced with those that are more often used by *Pichia pastoris*, so as not to change the amino acid sequence of human somotropin. After that, the "Prepro-alpha Factor Leader" sequence from *S. cerevisiae* was added to the modified sequence in order for the obtained somatropin to be secreted into the culture medium. The DNA sequence containing the modified GH1 gene was inserted into the vector with the help of restrictases near the promoter of the AOX1 gene. In this way the GH1 gene will be activated by the AOX1 gene promoter which can be easily induced by the presence of methanol in the culture medium.

RESULTS

A number of pharmaceutically active recombinant proteins including human growth hormone have been obtained by fermentation at the facilities of International Center for Genetic Engineering and Biotechnology, Italy. Later, in the process of collaboration between Scientific Center of Drug Research of *Nicolae Testemițanu* State University of Medicine and Pharmacy, the pharmaceutical enterprise Balkan Pharmaceuticals and ICGEB, technological transfer works of the first stages of obtaining *Pichia pastoris* biomass for recombinant human growth hormone industrial scale manufacture were carried out. According to GMP rules the scale-up from laboratory to pilot phase allows the correct transition of a product created by the research service to the pilot phase at manufacturing site and final scale-up to the commercial batch manufacture of the biosimilar product. For the laboratory to pilot batch scale-up, activities specified below were performed and normative documents were elaborated at Scientific Center of Drug Research and Balkan Pharmaceuticals manufacturing site.

For the preparation of *P.pastoris* colonies intended for short-term and long-term storage, were used YPg agar with added geneticin, G418 poured into Petri dishes or test tubes (for short-term storage) and in YPg bullion in Erlenmeyer beaker (for long-term storage). The developed technological protocol stipulates the stages, conditions, incubation period and storage period with the concretization of the optimal temperature for the short-term storage of *P.pastoris* strains for 2-4 days at 30^{o}C or for

several weeks or months at 4°C. In the case of long-term storage, the cryovials are frozen and stored in liquid nitrogen or at -80°C, after not more than 2 years the working cell bank should be renewed. For the *in-vitro* assay of rhGH was used an appropriate lymphoma cell line, Nb2-11, whose proliferation depends on mammalian lactogens, and cell growth and density in rhGH presence is measured using a fluorescent dye or a visible reading with MTT dye or MTS dye. Were evaluated and approved the equipment, materials, reagents and media necessary to determine the biological activity assay of recombinant human growth hormone. The testing steps and testing methodology were established, identification by the MTS and MTT method was elaborated. The results reading and evaluation according to the requirements of the European Pharmacopoeia were elaborated. Thus, the rhGH estimated potency should be > 80% and <125% of the declared potency. The confidence limits (p = 0.95) of the potency should be within 64 - 156% of the declared potency. The preparation, inoculation and growth of *Pichia pastoris* strains with rhGH in the Erlenmeyer flask should be made according to the following test steps: i) thawing of *P. pastoris* colonies from working cell bank, ii) preparation of the microorganism colony growth medium, iii) the first inoculation of the colonies in the 500ml Erlenmeyer beaker on YPg medium for 24-26 hours, iv) the second inoculation of *P. pastoris* colonies, obtained at the previous stage, in the 4l Erlenmeyer beaker on YPg medium for 20 hours, v) optical density determination at 600nm. The testing methodology and conditions were developed for each step, as well as the validity term of used medium were established. During industrial scale-up process of working cells bank creation and *Pichia pastoris* strains growth in the Erlenmeyer beaker, the impact of the elaborated technological processes on the obtained products was observed, and Technological Protocols and Standard Operating Procedures were developed for each elaborated technological processes.

CONCLUSIONS

At the manufacturing site of Balkan Pharmaceuticals Ltd, Republic of Moldova, in the process of laboratory to pilot batch scale-up, was developed the working cell bank of the *Pichia pastoris* strain for short-term and long-term storage, the *Pichia pastoris* strain with the expression

of human growth hormone inoculum was prepared and grown in the Erlenmeyer flask, that being the first step for the industrial manufacture of the biosimilar preparation. The Standard Operating Procedure "Working cell bank creation" was elaborated, as well as the procedure and principles necessary for the formation of the *Pichia pastoris* working cell bank with human growth hormone expression were established for the microbiology laboratory at manufacturing site of pharmaceutical company Balkan Pharmaceuticals. The equipment, materials, reagents and media for working cell bank creation were established and the stages, conditions, duration, culture medium composition, etc. for *P.pastoris* strains inoculation process were stipulated. The Technological Protocols of *Pichia pastoris* strains with the expression of rhGH short-term and long-term storage processes were developed with the aim of the establishing the necessary steps and principles for the short-term and long-term storage of strains for the microbiology laboratory at manufacturing site of Balkan Pharmaceuticals. The equipment, materials, reagents and media necessary for storage, as well as the procedure algorithm and preliminary preparations, sanitation and equipment of the room were established. The elaborated Standard Operating Procedure "Bioassay – the biological activity of the recombinant human growth hormone preparation" aims to establish a procedure for biological activity assay of recombinant human growth hormone by *in-vitro* biotesting meaning, which is related to the quality control of the finished product requested by the European Pharmacopoeia (Monograph on Somatropin 0951), the analysis is to be carried out at the microbiology laboratory facilities of Balkan Pharmaceuticals. The elaborated Technological Protocol of *Pichia pastoris* strains preparation, inoculation and growth in the Erlenmeyer flask established the process steps, including media preparation, reagents, necessary materials, algorithm and test conditions, media term validity.

In collaboration of Scientific Center for Drug Research of *Nicolae Testemitanu* State University of Medicine and Pharmacy and the pharmaceutical company Balkan Pharmaceuticals, at manufacturer site were implemented the following normative acts: Standard Operating Procedures "Working cell bank creation" and "Bioassay – the biological

activity of the recombinant human growth hormone preparation", Technological Protocols "*Pichia pastoris* strain with the expression of human growth hormone for short-term and long-term storage" and "*Pichia pastoris* strain preparation, inoculation and growth in the Erlenmeyer flask".

For the pharmaceutical industry of Republic of Moldova, the process of creation of a biosimilar hormone working cell bank as the first step to recombinant human growth hormone manufacture is innovative.

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