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Recombinant Anti-Thrombin Production from *Saccharomyces Cerevisiae*: Large Scale Trends Based on Computational Predictions

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Abstract. Anti-thrombin III is a protein performing anticoagulant function by preventing coagulation process and currently it is used in critical Covid-19 patients. In the present research, recombinant anti-thrombin production at large scale is simulated using SuperPro Designer. Special emphasis was analysed for the elucidation of required unit operation at industrial scale. *Saccharomyces cerevisiae* yeast is fed with 50 g/L carbon source together with 1.9 g/L amino acids and 6.7 g/L nitrogenous yeast base. All yields are taken from current references. The process is divided in two stages: upstream and downstream. In upward flow a total 11.2 g/L of biomass is calculated. Subsequently, anti-thrombin extraction and purification strategies are proposed. Interestingly, a purified anti-thrombin protein is obtained at 312 mg/L. Based on the latter, the large scale plant proposed in this research can reach 26 vials/hour produced at a concentration of 250 mg. Also productivity and prefeasibility are evaluated and annual production of 205,920 anti-thrombin vials is calculated. Therefore, each vial has a production cost of 180 USD. Based on the authors knowledge, information regarding anti-thrombin large scale trends are scarce. That is why this is the motivation of this research to perform a computational estimate for the large scale operations involved for the anti-thrombin production.

1. Introduction

Recombinant proteins are obtained by cloning a gene from any expression vector. This vector is induced in the host for producing the recombinant protein. Later the metabolite is recovered by extraction and purification process [1]. For some years, clinical trials have been carried out on approximately 1.300 recombinant proteins and more than 400 peptides. That is why these kind of products have been marketed with the approval of the FDA [2]. Currently the production of recombinant proteins is performed from microorganisms ensuring its quality. This technology emerged approximately 30 years ago to respond to certain needs by producing substances intended for the diagnosis, prevention and treatment of different pathologies that affect the living beings. The latter for obtaining therapeutic proteins giving rise to novel gene recombination techniques [3]. Anti-thrombin III (ATIII) is a glycoprotein which performs an anticoagulant function by preventing the action of blood clotting proteinases [4]. Thrombosis, together with cerebrovascular diseases are the cause of a large number of deaths and disabilities worldwide [5]. ATIII, in addition to being produced in the liver, is also produced by the plasma fractionation acquired from donor blood [6]. However, an unreliable blood source causes the potential risk of ATIII contamination by blood borne pathogens [7]. It is for this reason that the need arises to produce this protein through a source other than human blood.



Saccharomyces cerevisiae is the most studied eukaryotic single-celled microorganism and also the most widely used as an industrial microorganism for the production of biopharmaceuticals and recombinant proteins [8]. This yeast has the ability to make post-translational modifications, which significantly reduces the cost of purification. In addition, it also has the characteristic of being tolerant to low pH, high concentrations of sugar and ethanol, which makes it a microorganism for industrial fermentation [9]. There are many strains of *S. cerevisiae* for reference and therefore it is easy to find a suitable host based on parameters such as production, temperature, pH, yield and substrates. But many times, the target host cannot be found due to the genetic engineering of different species, and therefore it is necessary to carry out a genetic modification [10]. As in this case, it is required to work with the genetically modified strain *Saccharomyces cerevisiae* BY4741 to produce the protein of interest, in this case, the recombinant human anti-thrombin III (rhAT). Although there are currently studies related to production, extraction, purification and characterization of rhAT regarding lab scales, there is no information that specifies the industrial level rhAT production. There are not enough information regarding the large scale operating costs. Therefore, it is necessary to determine the unit processes to carry out this production, and likewise, to identify all the costs involved, from its upstream to its downstream. Motivated by the latter the goal of this paper is to perform simulations using the SuperPro Designer software. An industrial scale plant is proposed for the production of rhAT, taking substrates and other parameters from the literature. In addition, productivity and prefeasibility are evaluated for large scale trends.

2. Methodology

The rhAT simulation process is divided in two stages: upstream and downstream. In the upstream, the unit operations related to biomass of *Saccharomyces cerevisiae* production using are designed. Extraction and purification unit operations for downstream processing are also proposed in Figure 1.

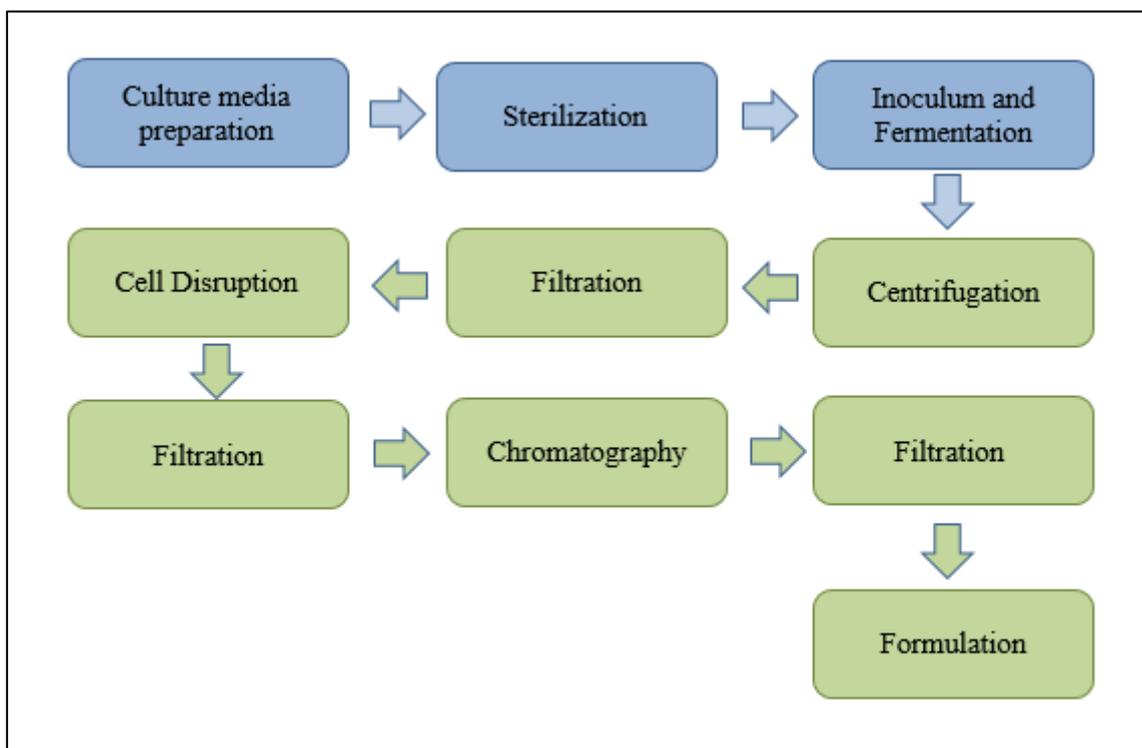


Figure 1. Unit operations implemented for the production of rhAT. The blue colour represents the upstream and green one means for downstream.

2.1. Upstream

2.1.1. Culture medium preparation and sterilization. The culture medium used for the simulations was reported from the literature [7], and is defined by the following composition: 50 g/L of carbohydrates (40% galactose, 40% glucose and 20% raffinose), 1.9 g/L of an amino acid pool (aspartic acid, histidine, isoleucine, methionine, phenylalanine, proline, serine, tyrosine and valine) and 0.67% of yeast nitrogenous base (YNB). The media mass flow was fed at 47.9 kg/h. The latter is mixed using a mixing tank with a carrying capacity of 1150 L and operates in adiabatic mode at a temperature of 88° C, pressure of 1.013 bar. The input mixing energy was set up at 3 kW/m³. Subsequently, this medium was sterilized with a sterilizer with a sterilization holding temperature of 140°C and a specific death rate of 2.44 s⁻¹.

2.1.2. Biomass fermentation and growth. The fermentation process consisted of three fermenters that operated continuously. The first fermenter with a volume of 40 L, the second one with 400 L and the third one at 4000 L. Sterile media was passed through a divider that distributes flow (47.9 kg/h) to the three fermenters as follows: 1 %, 9 % and 90 %, respectively. Based on the literature, the operating conditions of the fermenters were: 37° C, stirring speed of 500 rpm and an air flow of 2 vvm. Biomass growth reaction was determined according to equation (1) and a conversion rate of 96% was also determined.



The biomass yields from substrate $y_{x/s}$ and product yield from biomass $y_{p/x}$ of the process were found mathematically using equations (2) and (3) respectively.

$$y_{x/s} = \frac{\text{biomass}}{\text{substrate}} \quad (2)$$

$$y_{p/x} = \frac{\text{product}}{\text{biomass}} \quad (3)$$

2.2 Downstream

2.2.1 Collection and separation of biomass. The biomass produced was separated from the fermentation broth by centrifugation and later it was stored in a reservoir tank. The latter was performed with an inflow of 100 kg/h composed with EDTA 0.05% and TRIS-HCl 0.01% [11]. Subsequently, a filtration was proposed to collect the highest concentration of biomass.

2.2.2 Cell disruption. Several references report the ultrasound method for cell disruption [7]. In this study, cell lysis was performed using a bead milling is capable of grinding products in sizes from micro to nanoparticles. This process was operated at a temperature of 18°C and an input energy of 33.66 kW. For calculating protein that was being released into the medium, equation 3 was used based on references [7]. During cell lysis not only the product of interest (in this case rhAT) is obtained, but also cellular debris (remains of cellular structures such as cell walls, vacuoles, Golgi, etc.) and contaminants (proteins, lipids) are released. This process is modelled using equation (4):



2.2.3. Extraction and purification. Once the cell disruption process was carried out, it was stored in a reservoir tank, with an inflow of 100 kg/h of magnesium sulphate. Later it was filtered again with a rotating drum.

To try to remove contaminants, a chromatography tower was proposed. The latter, by using a washing phase of sodium chloride at a concentration of 0.01% and an elution phase composed of sodium chloride at 0.5% and sodium phosphate at 0.025%. Finally, the unit operation of diafiltration was used to eliminate the salts used in the chromatography. This tower operated at a temperature of

25.43°C and an input energy of 0.2 kW. The fully purified protein passed through a reservoir tank containing a feed stream of 19.13 kg/h WFI (Water for Injection).

2.2.4. *Lyophilisation and formulation.* The freeze drying unit operation was proposed to eliminate the WFI used in the previous steps. The totally pure protein finally obtained was packaged in 10 ml vials at a concentration of 250 mg.

2.3. Determination of rhAT production costs

The costs were calculated based on the prices found in the market for each substrate and reagent used in this simulation. The material prices from Sigma Aldrich web site can be seen in Table 1. The prices of the substrates (carbon source, YNB and amino acids), and reagents (EDTA, TRIS-HCl, sodium phosphate and sodium chloride) were taken from the supplier Sigma-Aldrich, which offers very high quality products.

Table 1. Raw material costs for the production of recombinant anti-thrombin.

Source type	Name	USD/Kg
Yeast nitrogenous base	YNB	618.12
Carbon source	Glucose	50.67
	Galactose	603
	Raffinose	1760
Amino acids	Aspartic acid	208
	Histidine	689
	Isoleucine	951
	Methionine	359
	Fenylalanine	557
	Proline	631
	Serine	692
	Tyrosine	712
Reactives	Valine	501
	EDTA	88.56
	TRIS-HCl	300
	Sodium phosphate	85
	Sodium chloride	76

The SuperPro Designer software was used for calculating all the costs involved in this plant, from its annual operating cost to the cost necessary for the investment. The latter based on mass and energy balance calculations. The annual production cost of rhAT was calculated by the software, and from that value the production cost was found in USD/kg according to equation (5). This price was estimated in USD/mg using equation (6). Subsequently, it was calculated the production price of 250 mg of rhAT, which is the concentration at which this protein is formulated in the market, using equation (7).

$$USD/kg = \frac{\text{Annual operating cost}}{\text{annual production}} \quad (5)$$

$$USD/mg = \frac{USD/Kg}{1000000} \quad (6)$$

$$USD/250\ mg = USD/mg * 250 \quad (7)$$

Finally, the production price of a vial with 250 mg of rhAT produced from this simulated plant was compared with the current sale price. The sale price was taken from Vademecum web site in which different drugs are marketed, from synthetics to recombinants.

3. Results and Discussions

Based on the authors knowledge, information regarding anti-thrombin large scale trends are scarce. That is why this is the motivation of this research to perform a computational estimate for the large scale operations involved for the anti-thrombin production. A plant that simulates the production of the rhAT protein from *S. cerevisiae* BY4741 yeast on an industrial scale is proposed using the SuperPro Designer v10 computer software. The latter, based on the values obtained from the literature. In addition, the viability of this production plant was evaluated, comparing the production prices with the sale prices in the market. The outline of the entire process is shown in Figure 2, where all the unit operations used for the production of rhAT on an industrial scale are found.

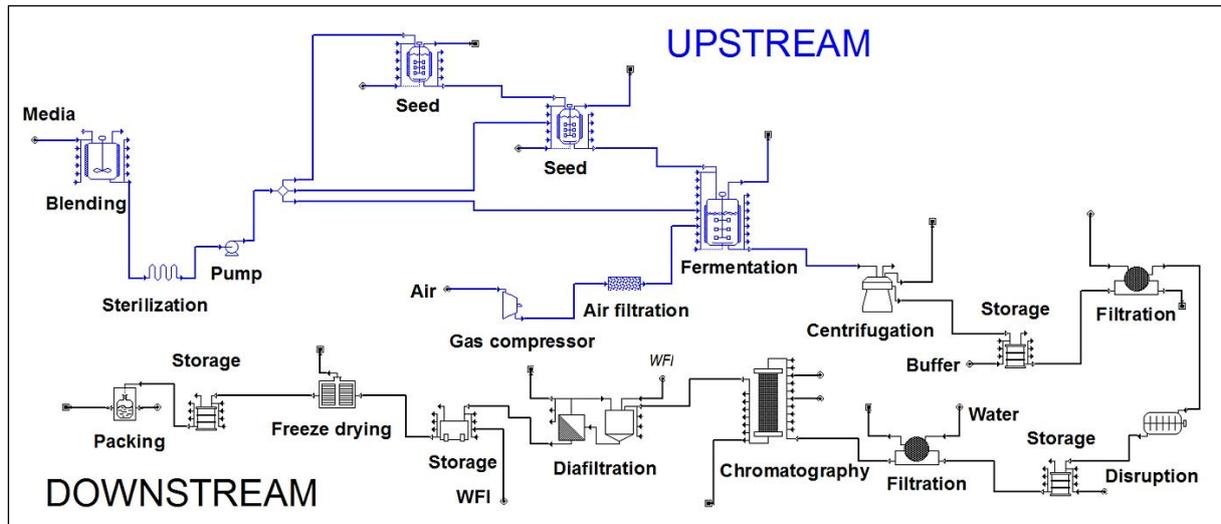


Figure 2. Industrial plant Scheme proposed for rhAT production from *S. cerevisiae*.

The culture media flow used [7] was pumped to the plant at a rate of 47 kg/h as mentioned above. Table 2 shows the inlet flow to the mixing tank (composition of carbon source was defined in materials and methods section). The mixing tank operates under conditions such that all the medium is completely mixed to subsequently carry out the sterilization process. Sterilization was used to eliminate the growth of those unwanted microorganisms that could affect biomass growth [12].

Table 2. Culture media Input flow and concentrations used for rhAT simulations.

Component	Volumetric flow (kg/h)	Mass Composition (%)	Concentration (g/L)
Amino acids	0.09	0.19	1.91
Carbon source	2.40	5.00	49.98
Water	45.09	94.12	939.16
YNB	0.32	0.67	6.69

According to equation (1), the biomass is produced from the carbon source, since it was assumed that the yeast nitrogenous base and amino acids were completely consumed. According to this equation, for each 60 g of glucose, 60 g of galactose and 30 g of raffinose, 33.6 g of biomass and 114.6 g of CO₂ are produced at a large scale rhAT production. Table 3 shows this reaction results. Table 3 shows the flow-in culture medium at 4000 L fermenter based on Carbon source composition. Also in Table 4 the output stream is observed from this same fermenter. Here the biomass produced in the fermentation is shown and a 11.2 g/L *S. cerevisiae* is reached. In addition, the exhausted carbon source medium can be observed. The flow of this stream was 46.103 kg/h.

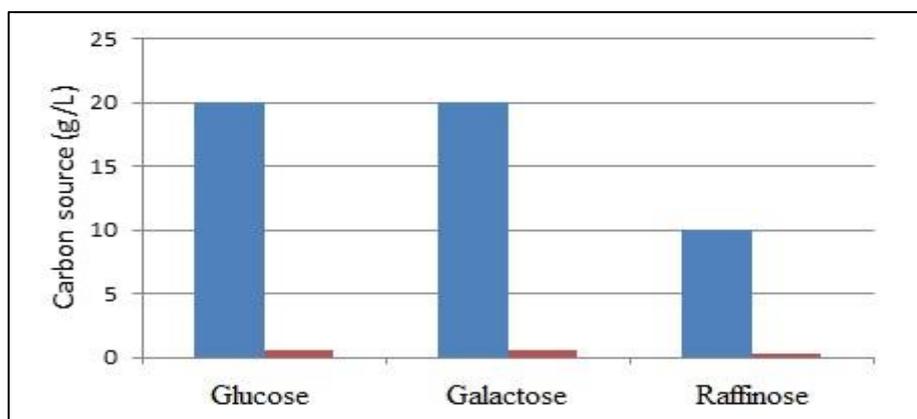
Table 3. Flow stream-in composition at 4000 liters fermentation tank.

Component	Volumetric flow (kg/h)	Mass Composition (%)	Concentration (g/L)
Galactose	0.86	2.00	19.91
Glucose	0.86	2.00	19.91
Raffinose	0.43	1.00	9.95
Water	48.58	94.12	935.66

Table 4. Flow stream-out composition at 4000 liters fermentation tank.

Component	Volumetric flow (kg/h)	Mass Composition (%)	Concentration (g/L)
Biomass	0.52	1.13	11.20
Galactose	0.03	0.06	0.63
Glucose	0.03	0.06	0.63
Raffinose	0.01	0.03	0.31
Water	45.09	97.81	969.38

In Figure 3, a comparative analysis is observed between inlet and outlet fermentation media. According to [7], the input substrate concentration was 50 g/L, the produced biomass 11.2 g/L and the protein formed (rhAT) 312 mg/L. These values were used according to equations 2 and 3 to estimate the yield coefficients. Therefore, biomass yield reached at 0.224 g biomass/g substrate, while the product yield was found with 0.028 g rhAT/ g substrate.

**Figure 3.** Carbon source concentration at in and outlet fermenter streams.

In centrifugation unit operation it was possible to concentrate the biomass stream up to 149 g/L. However, biomass must be washed to be detached from the exhausted medium. That is why EDTA, TRIS-HCl and water were used for washing procedure. Consequently, biomass was diluted a concentration of 5 g/L. The latter is low for rhAT extraction. For that reason, a rotary drum filter was proposed. Both the exhausted medium and EDTA TRIS-HCl were filtered and the biomass was retained in the filter cake. The biomass concentration in this stream was estimated at 630 g/L. Therefore, the objective of concentrating the biomass was achieved.

As mentioned above, equation (3) was typed into the cell disruptor. This indicates that each gram of biomass contains 0.028 grams of rhAT. Based on the latter a bead milling process is proposed for the cell disruption. Likewise, not only will the product of interest be released, but lipids, proteins and other intracellular compounds will also be liberated [13].

The cell disruptor output stream had a concentration of 18 g/L of rhAT, but also it contained cells that failed to be disrupted, in addition to cell debris and contaminants. For that reason, this stream is

transferred to a reserve tank with an inflow of 100 kg/h of magnesium sulphate. The latter, for precipitating rhAT protein for an easy extraction by preserving its structure and functionality [13].

For removing waste and biomass, a rotary drum filter was proposed. As expected, the contaminants and magnesium sulphate failed to leak due to their size, leaving a protein contaminated. For this reason, chromatography operation was carried out in this research. The mentioned unit operation allows to eliminate cellular contaminants and magnesium sulphate.

A concentration of 7.9 g/L of rhAT is recovered, and 4.94 g/L sodium chloride and 0.25 g/L sodium phosphate was obtained from the chromatography output stream. For releasing sodium phosphate and sodium chloride a diafiltration equipment is proposed. Consequently, a concentrated protein of 5 g/L diluted in WFI is obtained. It should be noted that from the chromatography tower the diluent is changed from water to WFI, the quality being a high purity water used for the production of drugs [14].

Required machinery for rhAT large scale is also estimated using SuperPro Designer Software (see Table 5). According to results, for reaching a rhAT productivity of 26 vial/h (10 mL/vial), a 4.500 L bioreactor is required for the fermentative process. For solid-liquid separation a Disk-Stack Centrifuge with a 46 L/h of capacity is also needed. Chromatography column and freeze drying specifications are also estimated in Table 5.

Recombinant human antithrombin is commercialized by a lyophilized type in 10 ml vials with a concentration of 1750 IU (250 mg). Therefore, the lyophilisation operation was proposed, removing volatile components using the sublimation concept. Subsequently, the totally pure protein was packaged in 10 ml vials, a concentration of 250 mg of rhAT each vial. According to the proposed large scale plant, 26 vials are capable of being produced per hour and according to the software a productivity of 205.920 vials/year is expected.

According to the software, the productivity of rhAT is estimated at 47.52 kg/year and the annual operating costs are worth 34.234.000 USD. Therefore, the highest percentage of the investment is destined to the raw material by 41%, followed by operator costs in 31%. The investment in raw materials is the highest, due to its high quality material costs as shown in Table 1.

Once the annual production costs were known, the production cost of 250 mg of rhAT (which is how it is commercialized in the market) was found. Using equations (5-7), the operating cost for one kilogram of rhAT equals to \$ 720.000 USD, cost per milligram equals \$ 0.72 USD and the production cost per 250 mg equals approximately \$ 180 USD. As mentioned above, lyophilized antithrombin is currently sold on the market in 10 ml vials with a concentration of 1750 IU (equivalent to 250 mg). A box with 10 vials of antithrombin commercial from ATryn brand costs in the market 4170.81 EUR, which is equivalent to USD 4679.5 (USD 467.5 USD/vial. Prices taken from https://www.vademecum.en/medicine-atryn_31813).

Table 5. Machinery required for large scale recombinant rhAT production for *S. cerevisiae*

Type	Units	Size (Capacity)		Material of Construction
Blending Tank	1	1.280	L	SS316
Heat Sterilizer	1	49.16	L/h	SS316
Flow Splitter	1	47.91	kg/h	CS
Seed Fermentor	1	48	L	SS316
Seed Fermentor	1	448	L	SS316
Fermentor	1	4.482	L	SS316
Centrifugal Compressor	1	48.63	kW	CS
Air Filter	1	92.298	L/h	CS
Disk-Stack Centrifuge	1	46.52	L/h	SS316
Liquids Drum	49	198.12	L	SS316
Rotary Vacuum Filter	1	0.41	m ²	CS

Bead Mill	1	0.18	L	SS316
Diafilter	1	0.06	m ²	SS316
Liquids Tote	1	1.932	L	SS304
PBA Chromatography Column	1	15.33	L	SS316
Liquids Drum	9	189.97	L	SS316
Rotary Vacuum Filter	1	0.07	m ²	CS
Centrifugal Pump	1	0.00	kW	SS316
Freeze Dryer	1	494.02	kg	SS316
Filler	1	25.84	entities/h	SS316
Liquids Drum	1	0.03	L	SS316

Operating cost are subdivided according to raw materials, facility-dependent and consumables and labor-dependent. These calculations are presented in Figure 4.

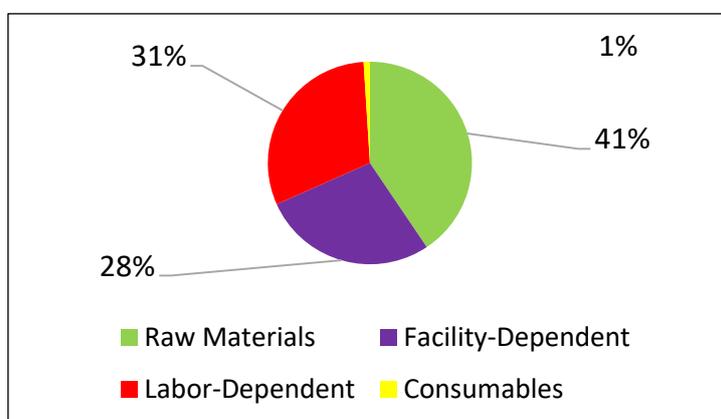


Figure 4. Annual Operating cost estimations for rhAT large scale production.

Based on Figure 4, raw material represents significant operating cost due to its influence on 41 % regarding all operating costs for producing recombinant rhAT at a large scale. The latter considering high quality raw materials for pharmaceutical applications. Better appreciation of these kind of costs is shown in Figure 5.

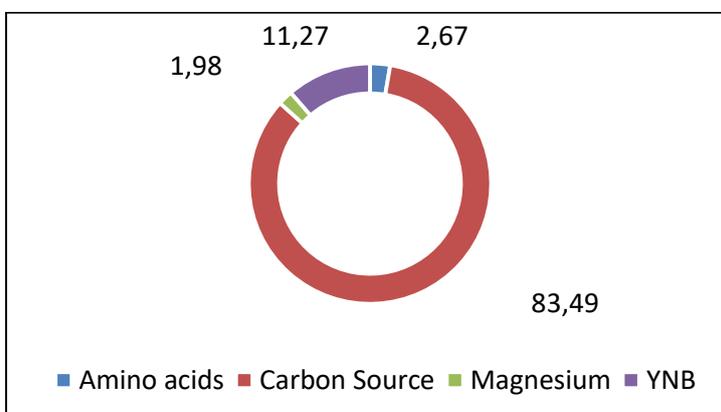


Figure 5. Annual Raw material cost estimations for rhAT large scale production (%).

Considering high carbon source demand required at large scale fermentative process, the 83,49 % of raw material costs is represented by glucose, galactose and raffinose sugars. Being the latter the most significant culture media species for considering at future optimization of rhAT large scale process. Result found in this research would be helpful for a detailed mechanical design regarding a large scale plant for recombinant rhAT from biotechnology production. Considering the currently evidence of rhAT utilization in critical covid-19 patients [15], it is evident that anti-thrombin proteins will be high demand metabolites for clinical treatments.

4. Conclusions

Industrial-scale simulation of recombinant human antithrombin (rhAT) production using SuperPro Designer v10 computer software was found to be successful in all expected settings. Both upstream and downstream unit operations turned out to be appropriate for rhAT large scale processing. Likewise, the final objective of preparing this work was to predict the viability of the plant. 26 vials of 10 ml are obtained every hour with a lyophilized protein of 250 mg. The production costs of one vial turned out to be worth USD 180, which represents 60% less than the cost of selling a single vial currently on the market (USD 467.5 a single vial).

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