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Separation and Optimization of Alpha-1 Antitrypsin Properties in Saccharomyces Yeast

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ABSTRACT

Alpha-1 Antitrypsin is one of the Serpins which in fact indicates a Glico protein with 12% carbohydrate. It contains 394 amino acids with molecular weight of 54 KDa which is synthesized in liver and secretes into plasma. Its original performance is to inhibit Neutrophil Elastase and protect the tissues, especially against destructive effects of Elastase during inflammation. Genetic (inherited) shortage of AAT results in pulmonary and hepatic illnesses. Emphysema is one of the most important one. Hence, due to their widespread application in medicine and treating the diseases in the perspective of various bacterial, plant, and yeast sources, great medical companies pay much attention to the production of recombinants in proteins. Furthermore, due to Glycosylatedness of this protein and its high molecular weight, every technique which may increase the expression output and recombinant proteins secretion in yeast can be regarded as a helpful instrument in producing this medicine. The current study is going to investigate the purity separation and determination of the properties and optimization of the alpha-1 Antitrypsin expression in Saccharomyces cerevisiae. In order to separate and purify this protein, some properties such as molecular mass, the amount and capacity of inhibiting are also measured. It is worth mentioning that the materials and performing technique in the present study have been determined in the following order: strains, plasmids, cultivation environments, transformation, protein property determination, and expression increase.

Key words: Serpins, Neutrophil Elastase, Saccharomyces, Glycosylatedness, alpha-1 Antitrypsin

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INTRODUCTION

Inhibiting activity of human plasma proteases was identified by Fermi and Pernossi in 1984, and the main inhibitor accounting for anti-proteolytic was first disintegrated by Shultz in 1955, and it was called Alpha-1 Antitrypsin. Human alpha 1-antitrypsin (AAT), a serum glycoprotein, is one of the best-known Prototypes of serine protease inhibitors (serpins) superfamily [1-2]. Laurel and Erikson introduced the reduction or lack of Alpha-protein in some patients' serum and the relation between AAT deficiency relations to pulmonary destruction chronic disease in 1963. Serpins building attracts mono chain serpins, the convertible structure appropriate to inhibiting activities need, and their structure includes a compact building and a reactive center loop (RCL). Regarding the relation to AAT

deficiency, there are various problems, including hepatic, renal, and pulmonary obstruction, which reveals the production importance and AAT production output increase. Alpha 1-proteinase is the main neutral serine proteinase in serum, especially Elastase, which enjoys three different additional Oligo-Saccharides, and it is 12% of its weight. Iso-electric pH of this protein varies between 4.4 and 7.4. An average amount of about 34g of this protein is synthesized by liver and secretes into serum [3-4]. In addition, AAT exists in the other types of cells, including plackets, pancreas megakaryocytes, langerhans islands. Its density is between1.5 to 3.5 gram/lit, and its plasmid half-life is between 4 to 8 days. AAT forms a strong complex with proteases which is an irreversible reaction. Ultimately, reduction of secretion results in this inhibitor shortage, and then leads to some health problems such as pulmonary Emphysema [5-7]. Giving pure AAT to these patients decreases the disease side effects, and due to its broad treatment application, protein exploitation process could be designed from human blood. Hence, its recombinant production through various sources such as Eukaryotic and prokaryotic micro-organisms is important for genetic manipulation facility and mass production. In this regard, since AAT is the result of mass produced Glycosylated, consistent yeast, Eukaryotic micro-organisms is preferred. After AAT expression in Saccharomyces cerevisiae yeast, expression increased through fermentor, and the properties of this protein, including molecular mass, and inhibition are measured.

Main body

The live cells ability is increased to produce protein accompanied with evolving their analysis mechanism. Hence, proteolysis is required to reconstruct the proteins of the growing cells, as supplying the Amino Acids through food is needed. A family of proteolytic enzymes is playing roles to meet these needs. There are a great number of these enzymes, but a single instrument is used to proteolize the peptide bond connected to an Amino Acid in each protein. That is why they are called Serine Protease. These enzymes have a serine in their own active region, which are related to a neighboring Histidine and Aspartate, and it can organize a nocleophilic attack for the Substrates peptide bond hydrolysis. The form and feature of protease region in which three Catalytic Amino Acids are located may vary. It has a particular performance and leads to exact Cleavage of some particular sequences in the substrate proteins [8-9]. In addition to Serine Proteases, there are also some proteins which grow in parallel and inhibit them. Organisms need to be protected against enzyme digestive attacks. This is mostly seen in the food. Many foods such as grains, potato, and other vegetables include some of the Serin Protease inhibitors in their building, which is mostly seen in glair [10]. Twenty different families of Serine Protease inhibitors action is not related to each other. Each of them has a prominent reaction center on the lobe peptide which acts as an ideal couple and a locking factor for the protease active region. 19 out of 20 families of the protease inhibitors are widely scattered in the simple species and plants, and only one family (Serpins) is dominant in the superior creatures and especially in human being. What distinguishes Serpins from the other Protease inhibiting families is the extraordinary ability to admit a variable. In addition, Serpins are bigger than other inhibitors, but they are common with them in the reaction lobe with a structure which introduces the active region for the Serine Protease. The difference is that though Cleavage lobe peptide leaves the lobe in the other lobe inhibitors, the cut end of the Serpins is replaced with the molecule different pole after Cleavage [11-12]. The building of the standard which is known by all the Serpins in a joint accurate and complete path has nine α coils and three B-sheets. One of these B-sheets, i.e. A-B-sheet is dominant to the molecule. In the first building Alpha-1 antitrypsin after Cleavage RCL by Protease compartment C of the terminal by entering the A-B-sheet middle part a new chain is made and makes the molecule imbalanced. Crystallizing the complex Alpha-1 Antitrypsin with Protease Chymotrypsin, Cleavage status was quickly clarified at the center of the reaction, but what promoted thinking about Serpins inhibiting mechanism was a new order of an unexpected structure which had been conducted as the result of this Cleavage. What had been clarified before was that the reacting lobe should be outstanding as in the other inhibiting families of Serine Proteases. The only difference in Cleavage is that lobe enters A-sheet and the Amino Acid of the reaction center which is called PI is replaced as 70 Angstrom from the other pole of the molecule. This powerful motion is carried out by Amino Acid 17 along a bend. It means that Amino Acid showed the tier classification of Amino Acids before PI reaction center, a bend was made of P10-P17 by the neighboring Amino Acids, and it similarly exists in all the Serpins with inhibiting activity [13]. Similar to all Serpins, this significant change in RCL after Cleavage is seen in Alph-1 Antitrypsin. The result of this change in RCL molecule varies from an initial changeable status with the melting point 55 centigrade to a consistent status with a melting point higher than 120 centigrade. According to all the findings, Serpins may be considered as quasi-rattrap [14]. By the initial changeable form which converts to a final consistent form by spiral movement, this similarity to the findings of the studies related to mutant serpins forms and the related diseases related to them was more empowered, especially a mutant of Alpha-1 Antitrypsin existing in 4% of European race people indicated that with the bended Amino Acid mutation p17 long chain polymers connecting reaction lobe a molecule to A-sheet of the second molecule. After a while, grading and noting the mutations similar to the agent of

many diseases, especially thrombosis to psychosis was identified in the other human serpins. As the result, it leaded to admitting rattrap as an extraordinary sensitive syndrome, because disease factors mutations intrigue the premature construction changes in a preplanned manner. The existence of structural susceptibility capacity clarifies all of the puzzle, and why it has selected the evolution of a complicated issue to inhibit the proteases [15-17]. The inhibitive serpins (I) react with proteases (E) and form a complex which may not be separated in the boiling SDS, but they are sensitive to nucleophiles. It seems that this class is particular to serpins and bring about this theory that Acyl is considered as E-I mediator and a construct very similar to inhibiting enzyme resistance. Anyway, using α2 Anti-plasine and trypsine, we can show that complex can be separated in a reversible manner, and the active inhibitor and enzyme is obtained. It indicates that E-I exists during serpins and protease reactions. It has been shown that α1pi and elastase pancreas constitute on a rectangular E=I which is identified after neighboring SDS and complex is stopped at this stage of the rectangular stage. It is probably that serpins react to proteases in two mechanisms. First, it is assumed that inhibitive serpins are balanced and they are inactive, and RCL is in a completely outstanding crescent-shape structure. Second, it is assumed that serpin is in an active status in which the lobe exists in a part of the A-sheet gate as it has entered in its natural structure. Protease forms complex only with the second status of the serpins, and this complex may occur without the structural consistency of the inhibitor. The alternative model is that it assumed that inhibition reaction with identifying the reaction region bond begins with proteinase which is a natural substrate. Anyway, the reaction of enzyme with a peptide bond results in the entrance of a part of the RCL into A-sheet. This opens the Cleavage of the reaction region and entraps the proteinase in a consistent rectangular in which RCL has a natural structure. It depicts the emergence of a new epitope on the serpins complex with proteinase in which serpin is somewhat similar to cleaved or inactive inhibitor. The reason to prefer the second model is that analyzing the crystallized construct of the reconstructed active form –α2 Chymotrypsin which is deformed as a spiral α is clearly seen. Both models accompanied with kinetic information are common in one point. Serpins may act for target proteinase only through substrata suicide, and by inserting a part of RCL into A-sheet do so to form the complex. Having formed the Michaeles complex (E-I), inhibitor (I) between rectangular complex E=I and a cleaved shape which is the inactive inhibitor (I) is divided. E-I is only formed by denaturation E=I in SDS. The relative amount of I, E, and E=I depends on complex formation conditions, serpins bended area building and the complexed proteinase. Alpha-1 Antitrypsin is a glico-protein made of a poly peptid chain with 394 Amino acids and a portion carbohydrate. The molecular weight of the protein is 52 kilo Dalton. The smallness of protein size lets it to penetrate the intermediate tissue liquids and pulmonary tissues [18-19]. The negativity of α1AT in the plasma may be important in preventing the protein to die down in the glomery membrane path which has charge. A molecule which is somewhat larger than α1AT is produced with 24 amino acids hydrophobe lipid signal in liver. CDNA emphasizes that peptide signal presence is accompanied with a metunin N terminal. Alpha1AT is not the active crystallizeable form but it is crystallized after proteolitic cleavage of the active position (RCL) and it is accompanied with forming a consistent form of Relax R. The analysis of crystal building indicates that an individual poly peptid chain has been organized in the components of the second building clearly. In the building of α1AT, the first amino acids preferably form the alpha spiral buildings. Aα1AT contains only one Cystein amino acid. There is no di sulphid bond in the building of this protein. However, vassalage group can form di sulphid bond with the other proteins such as heavy chain IgA [20-21]. Reaction center lobe (RCL) of this protein is an amino acid of structure 20-30 which is located out of the protein cluster. RCL provides a complex proteinase and α1AT around methionine 358 status quasi substrate for prteinase which helps to. It is irreversible. In this condition, α1AT is considered as a suicide molecule. Cleavage RCL frees RCL by proteinase. It is suggested they cleavage converts a part of cleavage to an additional chain in A-sheet. Hence it increases the consistency.

DISCUSSION

Table1. Fermentation of Saccharomyces cerevisiae yeast containing AAT gene without induction

pH	Air pressure by VVm	Temperature	Velocity by minute	OD 600	Time after insemination	Time by hour
7	1	30	150	0.147	0	1
7	1	30	150	0.27	1	2
7	1	30	150	0.5	2	3
7	1	30	150	0.885	3	4
7	1	30	150	1.25	4	5
7	1	30	150	1.6	5	6
7	1	30	150	2.1	6	7
7	1	30	150	2.3	7	8

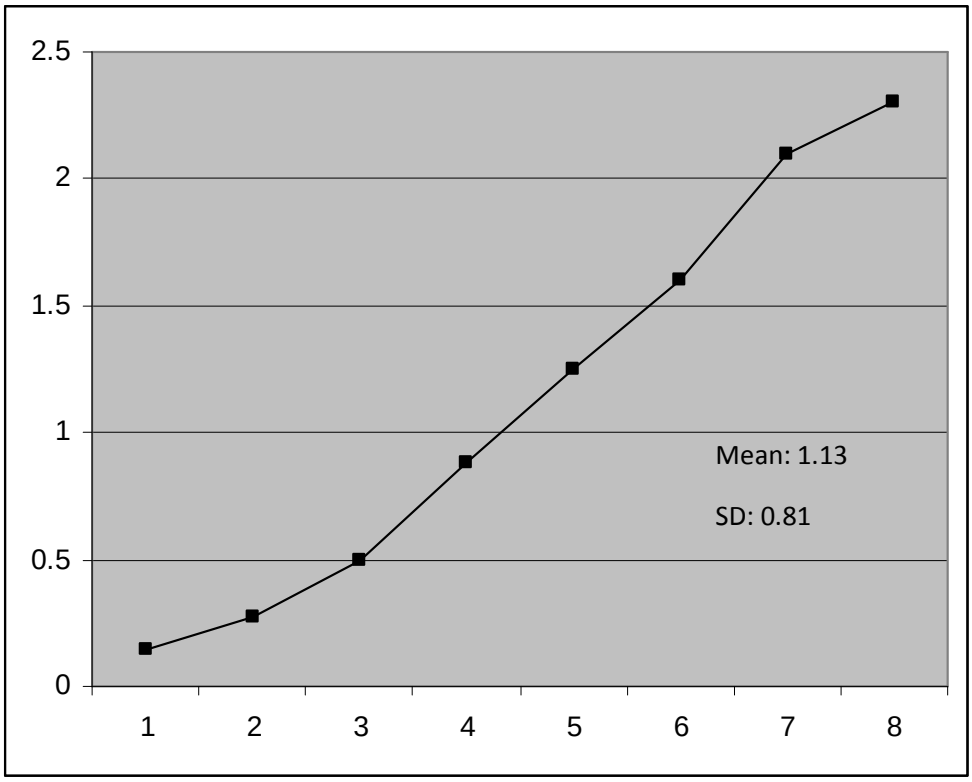

Diagram1. Fermentation of Saccharomyces cerevisiae yeast containing AAT gene without induction

According to Table 1, providing the appropriate strain which contains AAT protein gene, this fermentor strain which contains 2liter was grown. In the first experiment, revolution, air pressure, and temperature were 15 rpm, 1 VVm, and 30 centigrade respectively. In this experiment with pH=7 induction did not occur. Diagram 1-3 represents this issue.

As Table 2 indicates, mixer revolution was adjusted according to the need to the solution oxygen, hence a rising travel up to 350 rpm and OD600=2.5 was obtained, and induction did not happen. Diagram 2 represents the same issue.

Table2. Fermentation of Saccharomyces cerevisiae yeast containing AAT gene without induction by oxygen-dependent revolution

pH	Air pressure by VVm	Temperature	Velocity by minute	OD 600	Time after insemination	Time by hour
7	1	30	150	0.008	0	1
7	1	30	150	0.041	1	2
7	1	30	247	0.172	2	3
7	1	30	290	0.371	3	4
7	1	30	300	0.722	4	5
7	1	30	322	1.14	5	6
7	1	30	342	1.95	6	7
7	1	30	350	2.2	7	8
7	1	30	331	2.5	8	9

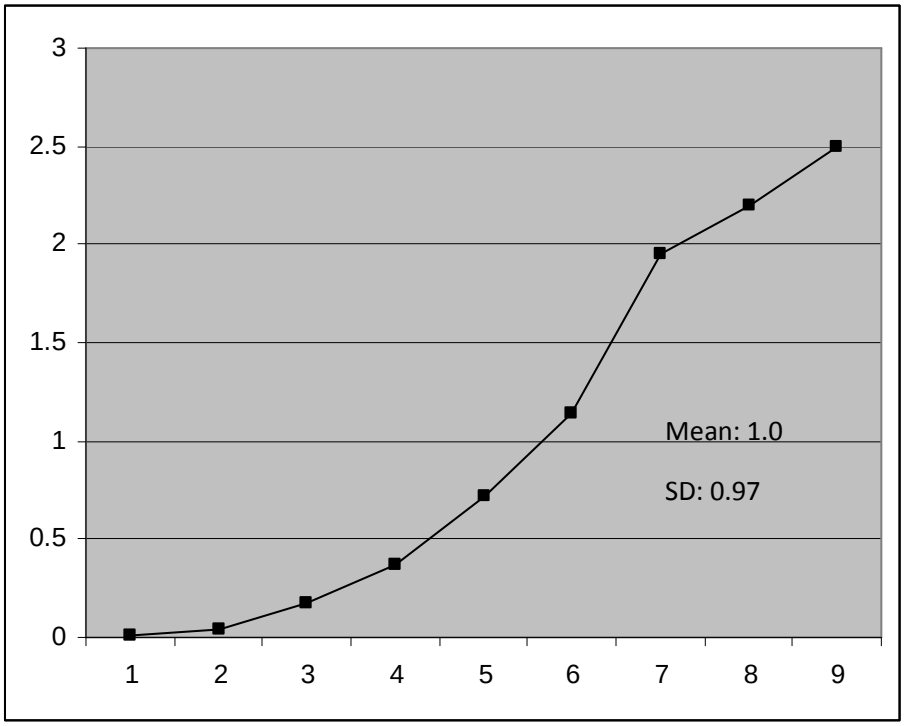

Diagram2. Fermentation of Saccharomyces cerevisiae yeast containing AAT gene without induction by oxygen-dependent revolution

According to Table 3 data, the third experiment was conducted in which mixer revolution was adjusted as oxygen-dependent, 30 centigrade, and 1 VVm was adjusted, and induction happened by IPTG 0.4 mU at OD600=0.8. The sample was collected after 5 hours.

Table3. Fermentation of Saccharomyces cerevisiae yeast containing AAT gene with induction at time 1 and oxygen-dependent revolution

pH	Air pressure by VVm	Temperature	Velocity by minute	OD 600	Time after insemination	Time by hour	
7	1	30	150	0.07	0	1	Ampicillin 100 Mg/mL
7	1	30	150	0.25	1	2	
7	1	30	200	0.38	2	3	
7	1	30	250	0.6	3	4	
7	1	30	278	0.8	4	5	mM 0.4 IPTG and Ampicillin 100 Mg/mL
7	1	30	300	1.2	5	6	
7	1	30	326	1.7	6	7	
7	1	30	350	1.9	7	8	
7	1	30	340	2.1	8	9	
7	1	30	330	2.2	9	10	

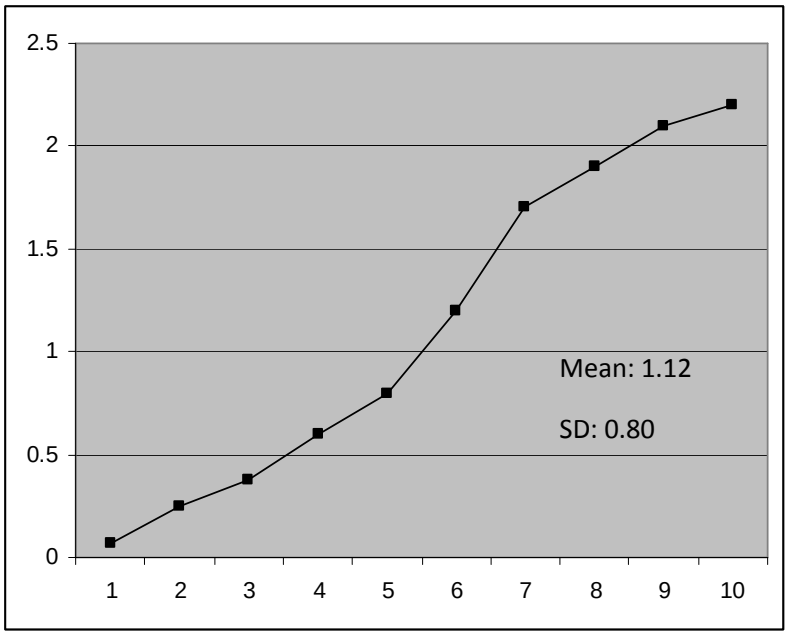

Diagram3. Fermentation of Saccharomyces cerevisiae yeast containing AAT gene with induction and oxygen-dependent revolution

Table4. Fermentation of Saccharomyces cerevisiae yeast containing AAT gene accompanied with induction start at time 2 after insemination with IPTG 0.4 mM and oxygen-dependent revolution

pH	Air pressure by VVm	Temperature	Velocity by minute	OD 600	Time after insemination	Time by hour	
7	1	30	150	0.07	0	1	Ampicillin 100 Mg/mL
7	1	30	150	0.1	1	2	
7	1	30	150	0.309	2	3	mM 0.4 IPTG and Ampicillin 100 Mg/mL
7	1	30	200	0.62	3	4	
7	1	30	210	0.71	4	5	
7	1	30	215	0.9	5	6	
7	1	30	220	1.1	6	7	

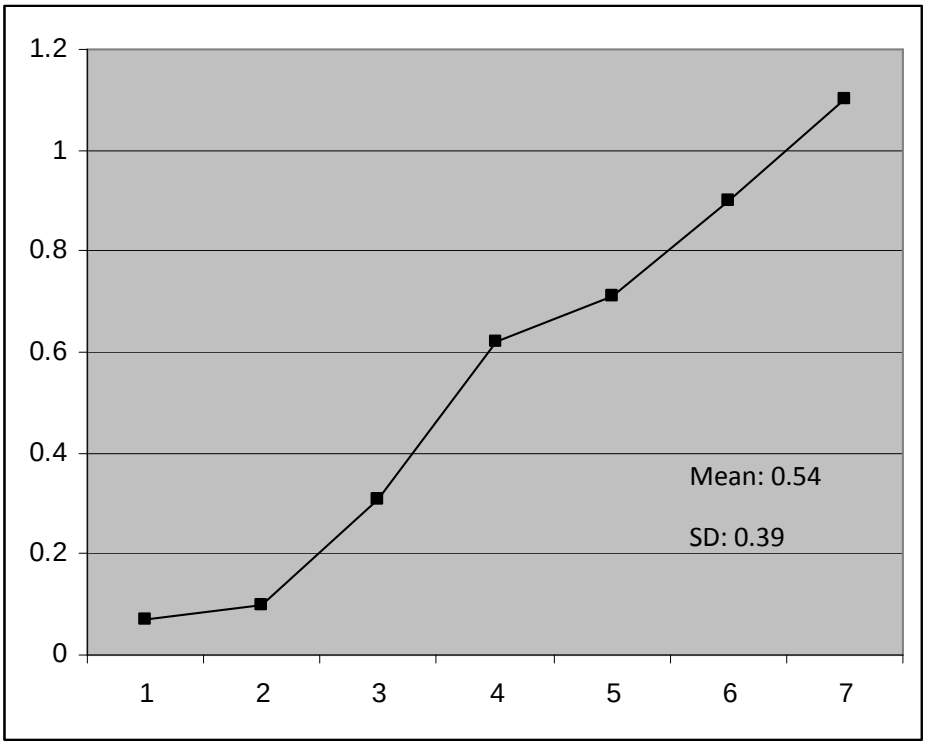

Diagram4. Fermentation of Saccharomyces cerevisiae yeast containing AAT gene accompanied with induction start at time 2 after insemination and oxygen-dependent revolution

CONCLUSION

Plasmid PYINU-AT is AAT human yeast expression vector. Having sequence of symptom INuiA is its feature. It has 23 Amino Acids. E.coil DH5α bacterium was used for its multiplication. The bacterium transformation was conducted through chemical technique. The transformed bacterium becomes resistant to Ampicillin, hence its selected environment becomes Ampicillinated. In the present study, the bacterium cultivation environment was 1LB and complete cultivation environment of YPD2, minimal sintenic (SD3) and complete (SC4) environments were used for the yeast. Since EColi DH5α does not grow at the presence of Ampicilline, and it becomes resistant to this Antibiotic after the transformation of the recombinant plasmid into it, the Ampicillinated LB cultivation environment is used when recombinant plasmid is transformed to EColi DH5α. YPD environment is used while transmitting the recombinant protein to the yeast. Providing the appropriate temperature and the optimum 30 centigrade for yeast, which is the best growth temperature for them, leads to gaining the highest expression amount. Finally, the screening of transformed cells are cultivated in the continuous environment ura SC without yovacil. They should be incubated at 30 centigrade for three 24 hours. In the completely sentinic environment (SC), the Amino Acids, which their existence is necessary with L-histirin, L-lotion, L-tryptophan, are needed. They are added with Adenine Sulphate which must have been sterilized by a 0.2 u bacteriologic filter. The production of a high amount of heterolog secretive proteins from yeast is often neutralized by a low amount of secretion. Particularly, regarding the big proteins with a molecular mass greater than 20 KDa, the secretion amount is low. The creation of natural symptom sequence is one of the determining main factors in the protein secretion efficiency. It directs the proteins for secretion. The sequence of symptom INUiA may directly affect the unglycosylated proteins secretion. Furthermore, measurement experiment was also conducted by enzymatic technique through inhibiting the trypsin. A fairly significant result was obtained from it, which is lower than AAT activity of natural serum which equals 2.1-3.5µmol/min.mol. Since producing the recombinant proteins is often accompanied with the cell growth, and obtaining protein requires increasing the cellular density in cultivation environment, fermentor is needed to increase the density, because carbon source density is efficient in the recombinant protein expression. Based on parallel growth superiority in Saccharomyces cerevisiae sauce, cultivating such a cell can be controlled in high density

(OD600=500) in fermentor environment. Yeast growth is an important characteristic for the secretive proteins, as the product density is severely appropriate to the cells density in the cultivation environment.

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