UNIVERSIDADE DA BEIRA INTERIOR

Ciências da Saúde

Biosynthesis of human membrane-bound Catechol-*O*-methyltransferase: optimization using Plackett-Burman and Central Composite Design

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Master degree thesis in

Biomedical Sciences

(2nd cycle of studies)

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Covilhã, June 2012

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Biossíntese da proteína Catecol-*O*-metiltransferase membranar humana: optimização com recurso a desenho experimental Plackett-Burman e Composto Central

Rui Filipe Lopes Soares

Dissertação para obtenção do Grau de Mestre em
Ciências Biomédicas
(2º ciclo de estudos)

Orientador: Professor Doutor Luís António Paulino Passarinha

Covilhã, Junho de 2012

Just have a little of faith.

Acknowledgments

Em primeiro lugar, quero deixar uma forte palavra de agradecimento ao Professor Doutor Luís Passarinha pela orientação deste trabalho, confiança demonstrada no meu trabalho, por todo o incondicional apoio, respeito e consideração cruciais para a concretização deste trabalho. Esta forma de tratamento que recebi marcará, certamente, toda a minha vida profissional e pessoal. Foi para mim um verdadeiro privilégio e enorme gosto poder trabalhar soba a sua orientação.

Agradecer à Susana por toda a disponibilidade e pelo enorme conhecimento que trouxe a este trabalho. Agradecer ao Augusto, pelo apoio e por tudo o que me ensinou durante este ano. Sem vocês, tudo seria muito mais complicado.

A todos os membros e colaboradores do CICS, particularmente do grupo "Biotechnology and Biomolecular Sciences", quero agradecer pelo bom ambiente proporcionado durante a realização do meu trabalho, bem como por todos os ensinamentos que uns e outros me ministraram.

À Sofia e à Margarida agradeço a vossa contribuição para o bom desempenho do meu trabalho.

Aos meus verdadeiros amigos que desde sempre têm estado a meu lado, em todos os momentos da minha vida, o meu enorme agradecimento.

Por último, à minha adorada família, especialmente aos meus pais e ao meu irmão, bem como à Diana por todo o amor, sacrifício e apoio, quero agradecer-vos do fundo do meu coração. Devo-vos tudo.

A todos aqui mencionados, a minha eterna gratidão.

Abstract

Catechol-*O*-methyltransferase (COMT, EC 2.1.1.6) is an S-adenosyl-L-methionine-dependent methyltransferase enzyme that catalyzes the methylation of catechol substrates (catecholamines, catecholestrogens). Physiologically, it is responsible for the elimination of biologically active or toxic catechols, making it a protein with great clinical relevance as therapeutic target in serious disorders, like schizophrenia and Parkinson´s disease. To fulfill pharmaceuticals requirements, new strategies of optimization and large-scale production of COMT enzyme are crucial. Statistical optimization approaches have demonstrated their enormous value in laboratory and industrial scale, namely in biotechnological production processes, in which an incremental enhancement can be a perpetual improvement.

In this work, we aimed the optimization of recombinant human membrane-bound COMT (hMBCOMT) enzymatic activity yields following a statistical optimization as a solving approach. *Plackett-Burman* design was used as a first optimization step to identify which factors have a significant effect in hMBCOMT productivity and activity levels, and Response Surface Methodology (RSM), by a Central Composite Design (CCD), to optimize the process.

We applied *Brevibacillus choshinensis* cells for the biosynthesis of hMBCOMT and a semi-defined medium for cell growth. This medium was subjected to a first screening using the *Plackett-Burman design* to evaluate the influence of the culture parameters (chemicals and physicals) in hMBCOMT enzymatic activity levels. Enzymatic activity were measured in a high performance liquid chromatography (HPLC) coupled to a coulochemical detector. Among the eleven variables tested, polypeptone, ammonium sulfate, glucose and temperature were selected owing to their significant effect on human MBCOMT enzymatic activity. The biological human MBCOMT activity obtained with the semi-defined medium in *Plackett-Burman design* were very promising, while were higher than the obtained with 2SYNm medium, a traditional growth medium for *Brevibacillus* cells of this work. Typically, we obtained values of 93nmol/h for hMBCOMT total enzymatic activity and 30 nmol/h/mg of specific activity with protein in its native form, without the use of any kind of detergents on protein solubilization step. Based on the results of *Plackett-Burman* design, a CCD was adopted to define optimal components concentration and temperature in order to maximize our response. The CCD model presented a multiple correlation coefficient value of 0.635 and a significant lack of fit, showing the lack aptness of the model to the process optimization and the failure to attain the optimal concentration of each variable.

Keywords

Human MBCOMT; Parkinson's disease; Plackett-Burman Design; Response Surface Methodology.

Resumo

Catecol-O-metiltransferase (COMT, CE 2.1.1.6) é uma enzima metiltransferase dependente de S-adenosil-L-metionina (SAM) que catalisa a metilação de substratos catecóis (catecolaminas, catecolestrogénios). Fisiologicamente, é responsável pela eliminação de catecóis biologicamente activos ou tóxicos, tornando-a uma proteína de elevado interesse clínico e utilizada como alvo terapêutico em doenças graves, como a esquizofrenia e a doença de Parkinson. Para suprir as necessidades farmacêuticas, novas estratégias de otimização e produção em larga escala desta enzima são fundamentais. Abordagens de otimização estatística têm demonstrado o seu enorme valor à escala laboratorial e industrial, nomeadamente nos processos de produção biotecnológicos, em que um pequeno detalhe melhorado pode significar um grande passo para o sucesso.

Neste trabalho, objetivou-se a otimização do nível de atividade enzimática da proteína recombinante COMT, na sua forma membranar, através do recurso a modelos de otimização estatística como uma abordagem resolutiva. Numa primeira fase de otimização e de seleção dos fatores mais significativos para a atividade enzimática da proteína em estudo foi utilizada a técnica de desenho experimental Plackett-Burman. Após esta seleção foi aplicada a Metodologia de Superfície de Resposta (RSM), através de desenho composto central (DCC), para otimização da concentração dos fatores que revelaram ser mais significativos e, consequentemente, do processo.

Foi utilizado o sistema de expressão *Brevibacillus choshinensis* para a biossíntese da proteína membranar COMT e um meio semi-definido para o seu crescimento. Este meio foi submetido a uma primeira triagem através do desenho experimental Plackett-Burman, avaliando-se desta forma a influência dos parâmetros de cultura (produtos químicos e físicos) nos níveis de actividade enzimática da COMT membranar. Os níveis de actividade enzimática foram medidos num sistema de cromatografia líquida de alta eficiência acoplado a um detector amperométrico. Entre as onze variáveis testadas, a polipeptona, sulfato de amónio, glucose e temperatura foram as variáveis selecionadas dado o seu significativo efeito na actividade enzimática da COMT membranar. Os níveis de atividade enzimática obtidos nesta primeira triagem revelaram-se bastante promissores, sendo mais elevados do que os obtidos com o meio 2SYNm, meio de crescimento mais comum para as células usadas neste trabalho. Foram obtidos valores de 93nmol/h para a actividade enzimática total e cerca 30 nmol/h/mg de actividade enzimática específica com a proteína na sua forma nativa, sem o uso de qualquer tipo de detergentes no processo de solubilização. Com base nos resultados do desenho Plackett Burman foi aplicado o desenho Composto Central para a otimização dos quatro fatores em causa a fim de maximizar a nossa resposta. Este apresentou um valor do coeficiente de correlação múltipla de 0,635 e uma falta de ajuste significativa, demonstrando a falta de adequação do modelo para a otimização do processo.

Palavras-Chave

COMT membranar humana; Doença de Parkinson; Desenho Plackett-Burman; Metodologia Resposta de Superfície.

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List of Symbols

°C	Celsius
CCD	central composite design
G	gravitational force
kDa	kiloDalton
*K*m	Michaelis–Menten affinity constant
M	mol per liter
RPM	rotations per minute
V_{max}	maximum rate
µm	micrometer
OD660	Cell density at 660 nm

List of Acronyms

CCD	Central composite design
B. choshinensis	Brevibacillus choshinensis
COMT	Catechol-O-methyltransferase
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DTT	Dithiotreitol
EDTA	Disodium Ethylenediamine tetraacetic acid
HPLC	High Performance Liquid Chromatography
MB-COMT	Membrane bound catechol-O-methyltransferase
Met	Methionine
OSA	Octil sulfate sodium
PFC	Prefrontal Cortex
RNase	Reoxyribonuclease
RSM	Response surface methodology
S-COMT	Soluble catechol-O-methyltransferase
Val	Valine

CHAPTER I: INTRODUCTION

1.1. Catechol-O-methyltransferase Protein: An Overview

Proteins are the key operators and driving force of all cellular activity due to its extraordinary capabilities that underlie the dynamic processes in living cells [1]. Biological properties of proteins are determined by its physicals interactions with others molecules [1]. The ability to bind to other molecules enables proteins to act as signal receptors, switches, tiny pumps or catalysts [1].

Catechol-*O*-methyltransferase (COMT; EC 2.1.1.6), first described by Axelrod and Tomchick (1958), is an intracellular S-adenosyl-l-methionine-dependent methyltransferase enzyme that catalyzes the methylation of catechol substrates (catecholamines, catecholestrogens) in the presence of magnesium [2, 3]. COMT is present in prokaryotes, plants, yeast, invertebrates, and vertebrates [4]. In mammals, COMT exists in two different molecular forms and plays important roles with great clinical interest [5].

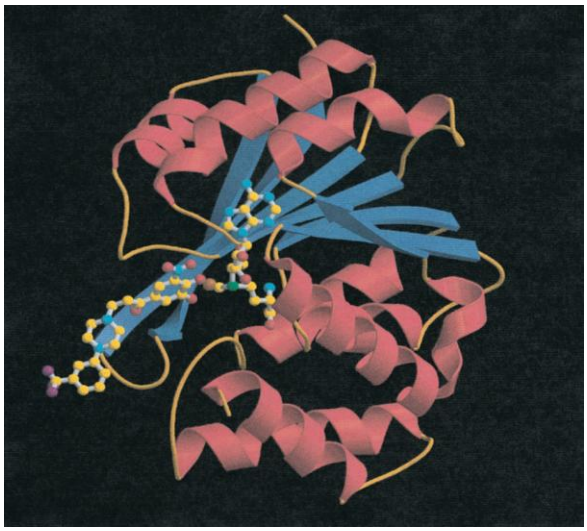

Figure 1 – Three-dimensional structure of catechol-*O*-methyltransferase complexed with the cofactor SAM and the Mg^{2+} ion. α-helices are red, ß-strands are blue, and the Mg^{2+} ion is brown. SAM is shown as ball-and-stick models (adapted from [6])

1.1.1. Physiological Functions

Physiologically, is one of the major enzymes responsible for the inactivation of catecholamine neurotransmitters, with special importance to the methylation of levodopa to 3-*O*-methyldopa in levodopa/aromatic amino acid decarboxylase inhibitor-treated Parkinson's disease patients [2, 7]. The enzyme COMT acts as an enzymatic detoxifying barrier between the blood and other tissues, shielding them from the negative effects of hydroxylated xenobiotics [8]. COMT also modulates some excretory functions in the kidney and intestinal tract by modulating the dopaminergic tone, as in the brain, where COMT activity regulates

the levels of dopamine, namely in prefrontal cortex (PFC) areas (figure 2) [9, 10]. Recently, COMT has been suggested to be associated with the modulation of several behavioral and cognitive processes [11, 12].

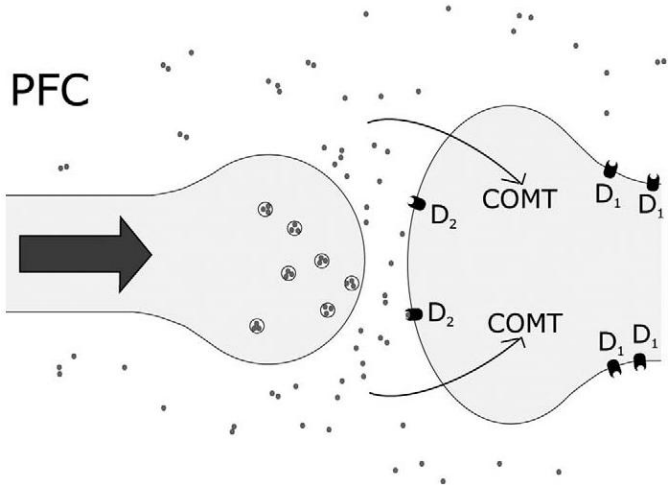

Figure 2 - Dopaminergic transmission in prefrontal cortex. Lack of the dopamine transporter in the synapse means that COMT plays a more prominent role in inactivating dopamine (adapted from [12]).

1.1.2. COMT Gene and Mammals Tissue Distribution

In humans, COMT is expressed in two molecular forms by one single gene, that contains six exons with the two first exons being noncoding, located in chromosome 22, band q11.2, which codes for both forms (figure 3) [3]. The expression of the COMT gene is controlled by two distinct promoters located in exon 3, where the distal 59 promoter (P2) regulates synthesis of 1.9-kb (rat) and 1.5-kb (human) mRNA species [3].

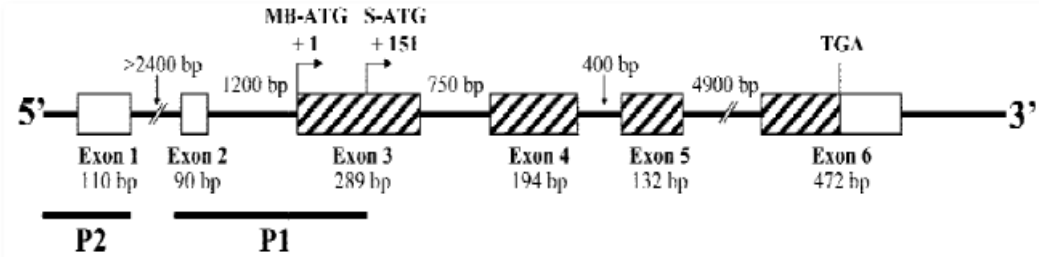

Figure 3 – Human COMT gene structure [13].

The two COMT isoforms are a soluble form (S-COMT) and a form associated with the rough endoplasmic reticulum membrane, called membrane bound catechol-*O*-methyltransferase (MB-COMT) [5]. The primary structures of both forms are identical, however, in humans, S-COMT contains 221 amino acid residues and a molecular weight of 24.7

kiloDalton (kDa) whereas MB-COMT contains the 221 amino acids from the soluble form and an additional peptide in its amino terminal of 50 amino acid residues corresponding to a molecular weight of 30 kDa [5, 6]. The stretch of 21 hydrophobic amino acid residues contained in this peptide, constitute the membrane anchor region [5].

In mammals, these two molecular isoforms that are widely distributed throughout several organs of the human body, namely in brain and peripheral tissues such as liver, kidney and gastrointestinal tract, where the highest activities are found (Table 1) [4, 8]. In the tissues where COMT is present, S-COMT is the predominant form, except in human brain, where MB-COMT predominates, reflecting its higher affinity for the catecholamine neurotransmitters [3, 6]. The enzyme S-COMT is particularly abundant in liver, kidneys, mammary glands and, in a very small proportion, in human brain. On the other hand, MB-COMT is found at higher levels in human liver, brain, kidneys, adrenals, and lungs [2].

It has been proposed that this differential expression of MB-COMT and S-COMT transcripts in several tissues of the human body can be explained by a number of putative regulatory elements discovered in the COMT gene [12].

Table 1 - Quantification of S-COMT and MB-COMT proteins in human tissues and cells expressed as % of total COMT in immunoblot assays [14].

Tissue	*SCOMT*	*MBCOMT*
Liver	85	15
Kidney	77	23
Adrenal	74	26
Duodenum	89	11
Brain	30	70

1.1.3. Genetic Polymorphisms

The level of COMT enzyme activity in human tissues is characterized by a trimodal distribution of low (*COMT*^{LL}), intermediate (*COMT*^{LH}), and high (*COMT*^{HH}) activities, which are determined primarily by the presence of a functional polymorphism in the coding sequence [8],[12].

The COMT gene contains a relative number of target polymorphisms, including A-287G, rs737865, Val^{158}Met, and rs165599 (figure 4). The rs737865, Val^{158}Met, and rs165599 polymorphisms comprise the haplotype shown to be highly significantly associated with schizophrenia [12].

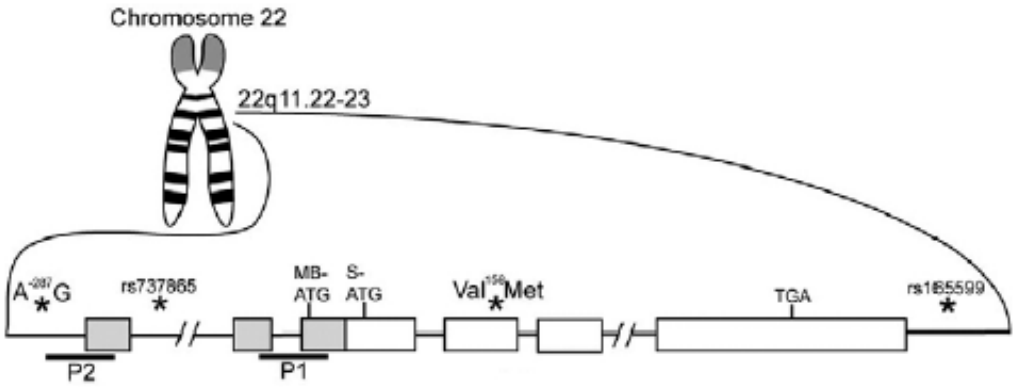

Figure 4 – COMT gene polymorphisms (adapted from [12]).

The Val^{158}Met polymorphism is the result of a G-A substitution, which leads to a valine (Val) to methionine (Met) substitution at position 158 in MB-COMT and 108 in S-COMT, promoting changes in COMT thermostability and functional COMT alterations in all tissues [8]. Typically, Met158 form of COMT has a lower thermostability and therefore a lower activity at physiologic temperature, while Val^{158} has a greater COMT activity [12]. On the other hand, heterozygotes individuals reveal intermediate levels of COMT enzyme activity, because the alleles are codominant [12].

Although it is not entirely related, the COMT genotype can lead to clinical implications. It has been proposed in some studies the existence of a relationship with neuropsychiatric disorders [8]. Low COMT activity allele appears to be associated to an obsessive-compulsive disorder and with an aggressive and highly antisocial impulsive schizophrenia [8]. Other studies were ineffective in establishing a considerable relationship between the polymorphism of COMT activity and bipolar disorder, attention deficit hyperactivity disorder or Parkinson's disease, however this polymorphism it seems to influence prefrontal cortex physiology [8].

1.1.4. Membrane-bound Catechol-O-methyltransferase

As stated before, MB-COMT is the major transcript of COMT isoforms and the predominant form in the brain [12].

Kinetically, S-COMT and MB-COMT forms have identical mechanisms (Ca^{2+} inhibition, Mg^{2+} requirement, pH optimum, a similar *K*m value for SAM), but they have significantly different affinities for substrates, as shown in Table 2 [8].

Table 2 – Affinities of S- and MB-COMT for several substrates, from distinct sources (adapted from [2]).

			S-COMT	MB-COMT
Enzyme source		Substrate	K_m (μM)	
Human	Brain	Dopamine	280	3.3
	Recombinant *E.coli*	Catechol	108	10
Rat	Liver & Brain	Epinephrine	168–345	0.9–3
	Liver & Brain & Kidney	Norepinephrine	304–464	5.5–11.4
Pig	Brain	R-Salsolinol	156	43
Mouse	Liver	Epinephrine	242	12
Rabbit	Aorta	2-Hydroxyestradiol	0.27	0.15
		Isoproterenol	121	0.91

The enzyme MB-COMT is involved in the termination of dopaminergic and noradrenergic synaptic neurotransmission at physiologically relevant low concentrations of catecholamines [7]. MB-COMT has been shown to have a higher affinity for catechol substrates and a lower *K*m value for dopamine than S-COMT, a common characteristic to different species [11, 15]. On the other hand, S-COMT is an enzyme with higher V_{max} values than those reported for MB-COMT [11, 15]. This distinct kinetic behavior suggests that both forms are definitely different enzymes and that MB-COMT predominates at low physiological concentrations of catecholamines, contrary to S-COMT that acts at high catecholamines concentrations (MB-COMT saturation) [7]. It has been proposed, that this differences in kinetic behavior could be related to the additional interactions between the substrate and the extra N-terminal residues of MB-COMT (absent in S-COMT), or a change in the conformation of the active site, due to membrane interactions [2, 4].

1.2. Production of Membrane Proteins: A Pharmaceutical Perspective

The production of specific proteins with desired biological activities has been a growing interest in the last years for pharmaceuticals applications [16].

Membrane proteins have crucial roles in cellular processes, making them interesting subjects for fundamental scientific research on their structure-function relationships. Furthermore, they have a great importance for pharmaceutical industry, because these proteins play important roles in diseases and represent the majority of the all known drug targets. Unfortunately, membrane proteins have low a natural abundance in the cells comprising the development of functional and structural studies [17, 18] Furthermore, there is a poor knowledge about membrane proteins due to the lack of appropriate strategies for

optimal overexpression [19]. Thus, novel approaches to produce these kind of proteins, are crucial to improve membrane proteins research as well as for further scientific development of pharmaceutical industry [5, 19].

The use of microorganisms as cell based factories for commercial production of membrane proteins is an excellent option, due to low cost and the absence of technical limitations, compared to chemical synthesis [16]. The choice of production host is determined according to the specific characteristics of protein being produced, since every microorganisms have a specific track record for protein production [16].

1.2.1. Brevibacillus Choshinensis Expression System

Gram-positive bacteria have the upper hand of gram negative ones, due to the fact that the former bacteria have only one cell wall layer for the secretory proteins to cross [20].

Among gram-positive bacteria, *Bacillus* species are attractive industrial organisms and have been frequently used in a large range of industries (food, pharmaceutical) [20]. *Brevibacillus Choshinensis*, a related species to *Bacillus*, has a remarkable track record of successful secretory production of heterologous proteins with high efficiency [20, 21].

Brevibacillus Choshinensis (B. choshinensis) referring to Choshi, in Japan, where it was isolated are strictly aerobic, Gram positive, motile, rod-shaped cells, with cell diameters greater than 0,5µm and cell lengths greater than 3.0 µm [22]. It is a bacterium with an exceptional ability for the expression of heterologous proteins extracellularly [21]. Grows on routine media such as nutrient agar and trypticase soy agar among others, producing pale yellow colonies [22]. Typically, growth occurs at 15ºC, but not at 50ºC as well as at pH 5.5 and pH 9.0 or in presence of 2% sodium chloride [22]. Sometimes, amino acids, citrate, and some organic acids may be used as carbon and energy sources [22].

The *Brevibacillus* Expression System based on *B. choshinensis* cells presents numerous advantages namely, a very efficient protein secretion mechanism of the host bacterium, with high yield and nearly free of extracellular proteases maintaining protein products intact in the culture medium [20, 21]. *B. choshinensis* is well-suited for expression of eukaryotic proteins because facilitates disulfide bond formation, so proteins from eukaryotic and prokaryotic organisms have been successfully produced in this system at high levels and with native biological activity [21].

B. choshinensis cells favor the construction of expression clones due to his high transformation efficiency by electroporation [21]. Several expression vectors can be used for the expression system, which includes the pNI or pNI-His DNA (intracellular protein expression) or the pNCMO2 and pNY326 (extracellular protein expression) [21]. For the construction of expression system used in this work, the pNCMO2 vector (figure 5) was used, which is a shuttle vector between *B. choshinensis* and *Escherichia coli* [21].

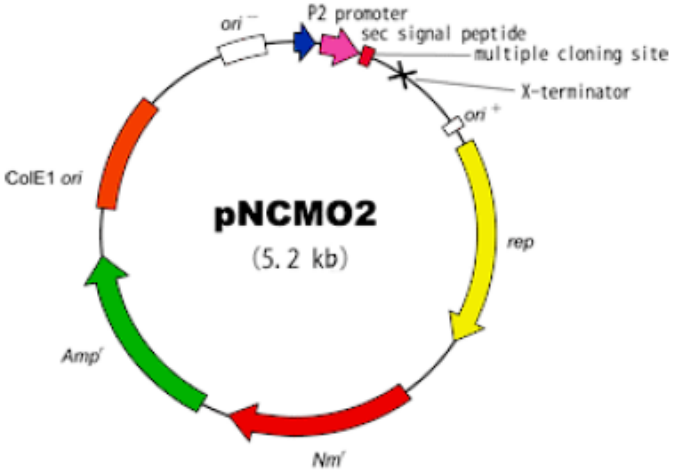

Figure 5 - pNCMO2 DNA vector map [21].

The pNMCO2 vector has single characteristics, namely resistance genes to neomycin as a selection marker in *B. choshinensis*, a secretory signal segment responsible by the protein secretion directly in the culture medium [20]. It contains the P2 promoter which has a strong promoter in *Brevibacillus Choshinensis*, leading to an efficient protein production [20, 21, 23, 24].

1.2.2. Culture Media: Favoring the Growth

The principles of enrichment culture are a technique that has been in use for many years, which consists of incubating a sample in a medium that encourages the growth of an organism of interest, while inhibiting the growth of others [25]. The use of enrichment broths in biotechnology areas, namely for production of recombinant proteins, has been demonstrated during the last decades by great efforts in the media optimization fields [25, 26].

Media composition can dramatically affect protein yields and consequently the overall cost of production [27]. While the media used to enrich for specific microorganisms might differ greatly from each other, the fermentation process requires a balanced medium composition that supplies adequate amounts of nutrients needed for energy, biomass, and cell maintenance [25, 28]. Design of a balanced medium is based on the cell's energy requirements and elemental composition [26]. To satisfy this nutritional requirements, three media types are usually used, the minimal (defined), semi-defined and the complex media [29].

1.2.2.1. Minimal (or Defined) Media

Minimal media supply only minimum nutritional requirements [26]. Some bacteria can grow on defined media because they have the ability to provide his nutritional requirements from a simple carbon source, a nitrogen source, and several salts [26]. Minimal media are composed exclusively by chemical defined substances, including carbon sources (glucose or glycerol), salts, vitamins, and amino acids, avoiding the drawbacks of complex medium components [26]. Therefore, chemical defined media have the advantage that their composition is exactly known [30].

Fermentation processes using minimal media are extremely reproducible [26]. Defined media allow the adjustment and optimization through the addition of components that may increase target proteins yields, resulting in a better process analysis, fermentation monitoring and the specific addition of particular substances which enhance growth or product formation [30]. In comparison to complex media, minimal media enhanced process consistency and improved results during scale-up fermentations, as well as others practical aspects such as better solubility, absence of inhibiting by-products upon sterilization and less foam formation during cultivation even in shake flasks fermentations [26].

It has been reported the growth of Bacillus species in defined medium, namely for polymer production [31], acid lactic [32] and enzymes [33, 34].

1.2.2.2. Semi-defined Media

Semi-defined media receives this denomination because they contain a defined portion (carbon source, salts, trace elements, vitamins) and a complex portion (nitrogen source) [26]. Commonly, the complex components used are yeast extract, casamino acids, and peptones, which supplies growth factors, amino acids, purines and pyrimidines and often supports higher cell densities [26, 35, 36].

1.2.2.3. Complex Media

Culture media consisting exclusively or predominantly of complex components with exact chemical constitution not known, are termed “complex media” [26]. The use of these types of media has clear disadvantages compared to defined and semi-defined media. Namely, the medium components are originate from biological materials and the development of complex strategies for contaminants removals during the downstream steps [30]. The uncertainty about the precise composition due to the presence of undesired substances, impossibility to do stoichiometric yield calculations, formation of undesired

products upon sterilization as well as a higher trend to foaming during scale-up, are another disadvantages of using complex media [30].

1.2.2.4. Media Components

Independently the organism of interest to be produced, media should contain an energy (nitrogen) source, a carbon source, and a source of trace and major (trace) elements [25]. In addition, the pH, temperature, and oxygen tension should be optimize in relationship to the target microorganism [25].

1.2.2.4.1. Nitrogen Source

The bacterial requirement for nitrogen can be satisfied by inorganic or organic sources [29]. Ammonia and ammonium salts, e.g., NH_4Cl or $(NH_4)2SO_4$ are used in minimal media, whereas in semi-defined media the requirement for nitrogen are supplied from complex components, like yeast extracts, peptones, and/or casamino acids [29].

1.2.2.4.2. Source of Carbon

A carbon source provides energy and biomass and is usually the limiting nutrient in cultures [29].

Autotrophic organisms obtain their energy from light making them require more than a source of simple carbon, such as bubbling CO2 gas or adding a carbonate source into the medium [25]. Heterotrophic organisms can obtain their carbon from a wide variety of sources [25]. Carbohydrates are the most common, namely the glucose that is the predominant carbon source, because is inexpensive and metabolized very efficiently providing a higher cellular yield [29]. However, high glucose levels are known to cause undesirable acetate production due to metabolic overflow ("Crabtree effect") [29]. Glycerol is also usually used as carbon source in batch cultures due to the superior capacity face to glucose for reduced acetate and increased recombinant protein formation [28].

Another carbohydrates sources include maltose, lactose, sucrose; acids found in the tricarboxylic acid cycle (i.e., succinic, oxaloacetic, malic, α-ketogluaric); amino acids (i.e., alanine, arginine, glycine, serine); and fatty acids (i.e., lactic, pyruvic, butyric, propionic) [25].

1.2.2.4.3. Salts and Minerals (Trace and Major Elements)

Minerals are necessary for bacterial growth, metabolism, and enzymatic reactions [29]. These can include nitrogen, potassium, sodium, magnesium, and calcium that can be found in a salt form [25]. In general, magnesium, phosphorus, potassium, calcium, and sulfur are normally added as media components [29]. Also, di- and monopotassium phosphates provide potassium and phosphorous and function as buffering agents [29]. Another elements can include iron, zinc, manganese, copper, cobalt, boron, molybdenum, vanadium, strontium, aluminum, rubidium, lithium, iodine, and bromine [25] These elements are usually required in trace amounts and can be supplied by adding a trace-minerals solution [29]. The supplementation of metal ions has been reported to provide good growth and also influence higher enzyme production [37].

1.2.2.4.4. Physical Components

Besides of the focus on medium components that can be added to a fermentation, manipulating the physical environment that the culture is placed, namely the temperature, oxygen tension, and pH, may have an extremely importance in protein yields [25]. These environmental factors have a strong influence in growth rate [28]. Each microorganism has an optimal temperature for his growth [25]. However, the temperature manipulation can be used for increase or decrease specific growth rates [28].

1.3. Statistical Optimization of process parameters: A Perspective

The fermentation process is significantly influenced by various physical and chemical parameters [38]. Therefore, the optimization of these parameters is a topic of central importance in laboratory research and industrial production, with particular attention to biotechnological production processes, in which a small improvement can be critical for commercial success, processes optimization are an undisputed component of any commercial concern [34].

The optimization of cultivation conditions, particularly nutritional and environmental parameters, are laborious and time-consuming when performed by using conventional techniques such as an one-factor-at-a-time method [39]. This kind of conventional design

techniques does not depict the interactive effects among the variables, not guaranteeing the determination of the most favorable conditions [39].

Single variable optimization methods are tedious and can lead to misinterpretation of results because the interaction between the different factors involved are overlooked [40]. These limitations can be eliminated by employing specific design of experiments, a structured and efficient methodology for planning experiments such that statistically valid relationships between factors affecting a fermentation process and its outputs can be established [41]. This kind of statistical based experimental design is a time-saving method and minimizes the error in determining the effect of parameters involved [42]. Statistical experimental designs can be employed at various phases of optimization process, such as for screening experiments or variables and for finding optimum conditions for a desired response [40].

A scheme of a simple strategy to conduct a sequence of experiments involving a reasonable number of factors is shown in figure 6 [43]. Firstly, the focus is the identification of the most dominant factors. Plackett-Burman design, for example, can be used for this first screening [43]. A second stage is conducted after the identification of main factors, in which we can choose to increase the resolution of this first screening, identifying a strong interaction between two factors [43]. After that, or directly after the first stage, a complete evaluation of remaining factors takes place. Complete factorials, including response surface designs, are excellent for these final sequences, in which a statistical modeling of the response as a function of the factors levels lead to a geometric representation of the response, in the case of the factors have quantitative levels [43].

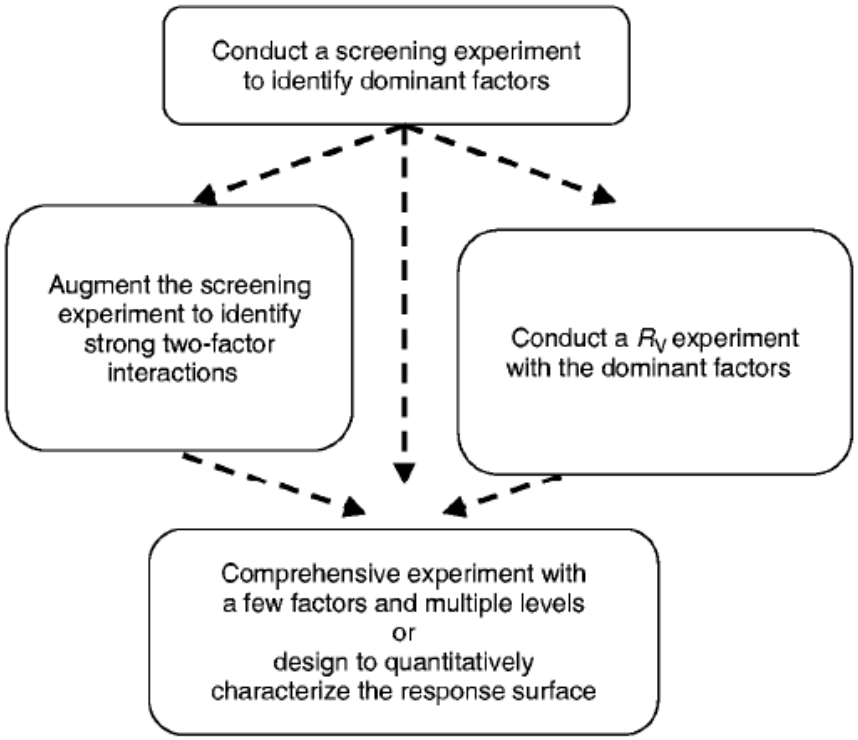

Figure 6 – A simple strategy to optimize bioprocesses [43].

Various statistical methods, predominantly Plackett–Burman design and response surface methodology with various designs, have been successfully employed for optimization in several biological processes, including the optimization of relevant enzyme production [33, 34, 38].

1.3.1. Plackett-Burman Design: A First-Step Optimization

Plackett-Burman design is a well-established and widely used statistical technique for screening of medium components in shake flasks [34]. The Plackett–Burman factorial designs screens the main factors from a large number of process variables, being a design quite useful in preliminary studies in which the main objective is to select variables that can be fixed or eliminated in further optimization processes [34].

Plackett-Burman provides orthogonal designs for factors at two levels for values of *N* (number of trials) multiples of 4 up to *N* = 100, with the exception of the design for *N* = 92 [44]. The Plackett–Burman design is mostly formed by the cyclical shifting of a generator which forms the first row of the design [44]. For example, when *N* = 12 the generator is

$$+ + - + + + - - - + - \quad (1)$$

which specifies the levels of up to 11 factors [44]. The second row of the design is, as shown in Table 3, created by moving (1) one position to the right and the process generates 11 rows where the 12^{th} row contains all factors at their lowest levels [44]. Equivalent designs are found by reversing all + and − signals and by permuting rows and columns [44].

Table 3 - First-order Plackett-Burman design for up to 11 factors in 12 trials [44].

	Factors										
Trial	1	2	3	4	5	6	7	8	9	10	11
1	+	+	−	+	+	+	−	−	−	+	−
2	−	+	+	−	+	+	+	−	−	−	+
3	+	−	+	+	−	+	+	+	−	−	−
4	−	+	−	+	+	−	+	+	+	−	−
5	−	−	+	−	+	+	−	+	+	+	−
6	−	−	−	+	−	+	+	−	+	+	+
7	+	−	−	−	+	−	+	+	−	+	+
8	+	+	−	−	−	+	−	+	+	−	+
9	+	+	+	−	−	−	+	−	+	+	−
10	−	+	+	+	−	−	−	+	−	+	+
11	+	−	+	+	+	−	−	−	+	−	+
12	−	−	−	−	−	−	−	−	−	−	−

The *Plackett-Burman* generators include values of *N* that are powers of 2 and provides an alternative to generate first-order designs from 2^k factorials, *k* is the design order, when *N*

= 2^k [44]. Since the design matrices are orthogonal, the effect of each factor (numeric or categoric) is estimated as if it were the only one in the experiment, resulting in D-optimum designs [44].

1.3.2. Response Surface Methodology: In Search of the Optimum

Response surface methodology (RSM) is a well-known method applied in the optimization of critical cultivation parameters responsible for the production of biomolecules [40].

In general, RSM is a collection of mathematical and statistical techniques used in modeling and analysis of problems in which a response of interest is influenced by several variables with the objective to optimize the response [45]. A general form of this response is represented in the equation (2):

$$y = f(x_1, x_2, \ldots, x_k) \quad (2)$$

where *y* is the response and x_1, x_2,…, x_k are quantitative levels of the factors of interest [43]. This response is called surface response, that is characterized by a geometric representation obtained when a response variable is plotted as a function of the quantitative factors (figure 7a) [43]. The contour plots are a series of lines and curves that identify values of the factors for which the response is constant, corresponding to a particular height of the response surface (figure 7b) [43]. Designs used for fitting response surfaces are called response surface designs [43].

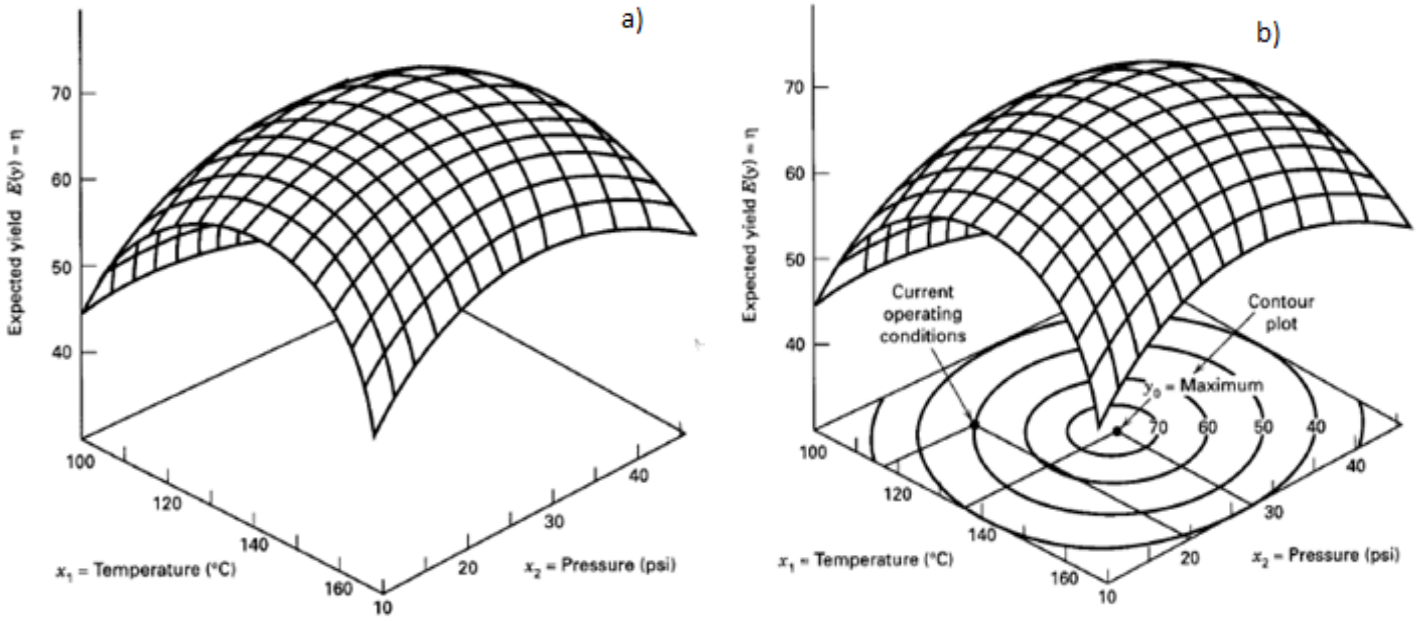

Figure 7 – a) Three-dimensional response surface showing the expect yield in function of two variables and b) Contour plots of a response surface [45].

Usually, one of the RSM problem is that the form of relationship between the response and the independent variables is unknown [45]. Thus, the first step in RSM is to find a suitable approximation for the functional relationship between *y* and the set of independent variables [45]. Polynomial models of first and second order (linear and quadratic equations with interactions) are normally used to model the response surface. [43]. If the response is well modeled by a linear function of the independent variables, then the approximating function is the first-order model

$$\mathbf{y} = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \ldots + \beta_k x_k + \epsilon \quad (3)$$

where, **y** is the response, $\boldsymbol{\beta_0}$ is the model intercept and $\boldsymbol{\beta_{1,\ldots,k}}$ is the linear coefficient, and $\boldsymbol{x_i}$ is the level of the independent variable [45]. If there is curvature in the system, then a polynomial of higher degree must be used, such as the second order-model

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum\sum_{i<j} \beta_{ij} x_i x_j + \epsilon \quad (4)$$

where, $\boldsymbol{y}$ is the predicted response, $\boldsymbol{x_i}$ and $\boldsymbol{x_j}$ the input variables, $\boldsymbol{\beta_0}$ a constant, $\boldsymbol{\beta_i}$ the linear coefficients, $\boldsymbol{\beta_{ii}}$ the squared coefficients and , $\boldsymbol{\beta_{ij}}$ the cross-product coefficients [45].

The RSM is characterized by a sequential procedure and almost all the RSM problems use one or both of these models [45]. Often, when an experience is at a point on the response surface that is remote from the optimum, there is a little curvature in the system and a first-order model will be appropriate [45]. In these cases, the objective is to lead the experimenter rapidly and efficiently along a path of improvement toward the general vicinity of the optimum (figure 8) [45]. Once achieved the optimum region, a more elaborate model (second-order model) may be employed, and an analysis may be performed to locate the optimum profile [45].

Thus, the objective of RSM is to determine the optimum operating conditions for the system or to determine the region of the factor space in which operating requirements are satisfied [45].

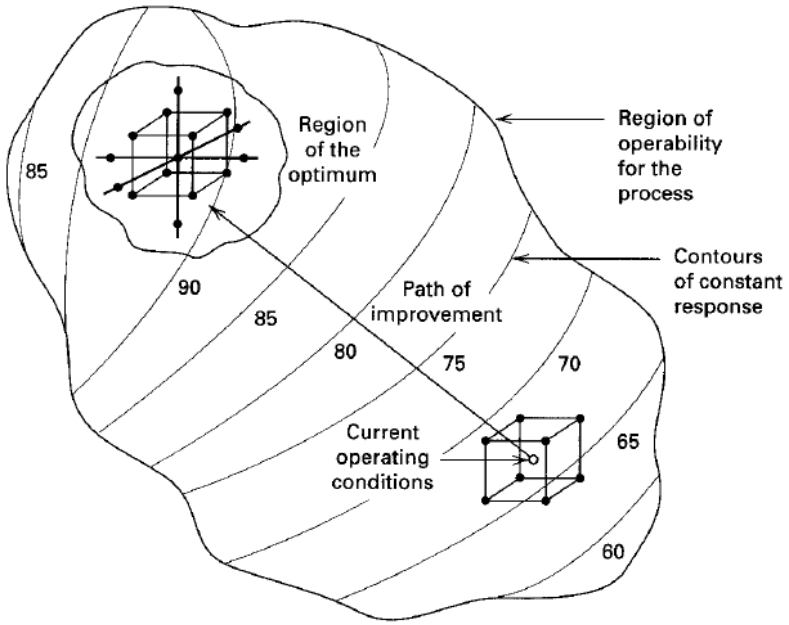

Figure 8 - The sequential nature of RSM [45].

RSM has important features, such as its sequential approach and its ability to the experimental problem into readily understood geometric terms [26]. It can be applied for different number of variables, generating a mathematical equation to predict improved process conditions [46]. RSM reduces the number of possible combinations to a manageable size, because it uses only a fraction of the total number of factor combinations for experimentation [46].

Fitting and analyzing response surfaces is greatly facilitated by the appropiate choice of an experimental design [45]. In this part, we just discuss central composite design (CCD), which is a design for fitting the second-order model due to the purpose of this work, however others designs can be used, such as Box-Behnken design [33, 39].

1.3.2.1. Central Composite Design

The central composite design is the most popular class of design used for fitting the second-order model, which consists in a 2^k factorial with n_F runs, 2k axial or star runs, and n_c center runs, where *k*is the design order, n_c the number of center points and n_F the number of factorial points [45].

The CCD is a very efficient design for fitting the second-order model. In these designs two parameters that must be specified: the distance α of the axial runs from the design center and number of center points n_c [45].

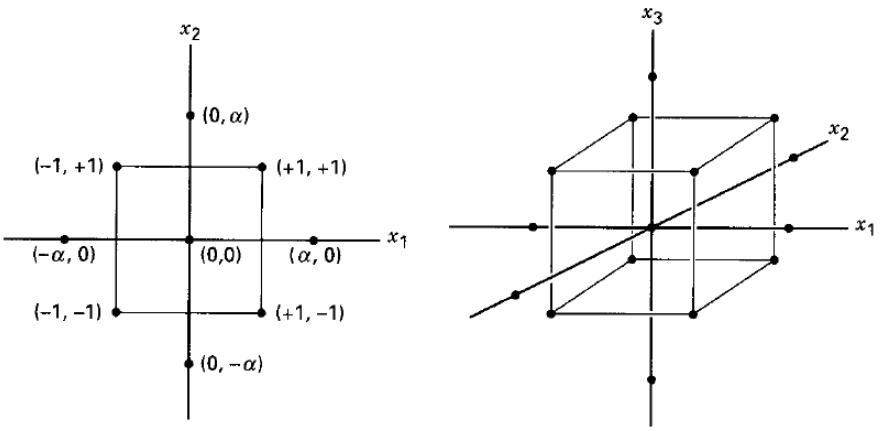

Figure 9 – Central composite designs for k = 2 and k = 3 [45].

It is important in a second-order model to provide good conditions throughout the region of interest [45]. For that it is necessary to require that the model have a reasonably consistent and a stable variance of the predicted response at points of interest. A second order-model response surface design be rotatable, means that the variance is the same at all points **x** and these points are at the same distance from the design center [45]. A design with this property will keep the variance unchanged when the design is rotated with reference to the center, hence, the name rotatable [45].

Rotatability is an adequate basis for the selection of a response surface design [45]. Because the purpose of RSM is the optimization and the location of the optimum, that is unknown prior to running the experiment, it makes sense to use a design that provides equal precision of estimation in all directions [45].

A CCD is made rotatable by the choice of α value, which depends on the number of points in factorial portion of the design; in fact, $\alpha = (n_F)^{1/4}$ yields a rotatable central composite design where n_F is the number of points used in the factorial portion of the design [45].

CCD has been applied in the optimization of a wide range of bioprocesses, becoming a successful statistical method in the present-day for biotechnology [40, 47, 48].

CHAPTER II: Aims

Aims

Due to the importance of human MB-COMT for pharmaceutical industry, the aims of this work focused on optimization of their production, to maximize enzymatic activity levels. This kind of optimization is very important because membrane proteins have a low natural abundance. Furthermore they represent almost all the known drug targets, being necessary for proper functional and structural studies.

The statistical optimization strategy used in this work was applied in two stages. Firstly, the application of Plackett-Burman design to identify the most significant factors of the culture parameters (chemicals and physicals), and after this first screening, the optimization of the selected factors by response surface methodology, by using a central composite design.

CHAPTER III: Materials and Methods

3.1. Materials

Ultrapure reagent-grade water was obtained with a Mili-Q system (Milipore/Waters). Ampicilin (sodium salt), neomycin (trisulfate salt hydrate), glucose, calcium chloride dihydrate, yeast extract, ferrous sulfate heptahydrate, manganese sulfate monohydrate, zinc sulfate heptahydrate, magnesium chloride tetrahydrate, magnesium sulfate heptahydrate lysozyme, cobalt (II) chloride hexahydrate, dithiotreitol (DTT), S-(5'- Adenosyl)-L-methionine chloride, CAPS, DNase, RNase, epinephrine (bitartrate salt), disodium Ethylenediamine tetraacetic acid (EDTA), sodium octil sulfate (OSA), dibutylamine, Bovine serum albumin (BSA), LB-Agar were obtained from Sigma Chemical Co (St Louis, MO, USA). Sodium phosphate dibasic, potassium dihydrogen phosphate monobasic, ammonium sulfate and nickel chloride hexahydrate was purchased from Panreac (Barcelona, Spain). Potassium chloride, sodium chloride and boric acid were supplied by Fluka (Buchs, Switzerland). Bacto Soytone and Polypeptone were purchased from Becton Dickinson (New Jersey, USA). Bis-Acrylamide 30 % was obtained from Bio-RAD, Hercules, CA. The High-Range Rainbow molecular weight markers used for estimation of subunit molecular weight and the anti-rabbit IgG alkaline phosphatase secondary antibody were purchased from GE Healthcare Biosciences (Uppsalla, Sweden). Polyclonal rabbit anti-COMT antibody was produced in Bial using purified recombinant rat COMT [49]. All other chemicals were of analytical grade and used without further purification.

3.2. Microorganism

In this work the plasmid pNCMO2 was used as the expression vector. *Brevibacillus Choshinensis* cells were used in this study for protein recombinant production. Brevibacillus choshinensis cells was grown in the MTNm liquid medium (10.0 g/L glucose, 10.0 g/L Polypeptone, 5.0 g/L Bacto Yeast extract, 10.0 mg/L $FeSO_4.7H_2O$, 10.0 mg/L $MnSO_4.4H_2O$, 1.0 mg/L $ZnSO_4.7H_2O$, 4.1 g/L $MgCl_2$ and 50.0 µg/ml Neomycin) and MTNm plates (MT Liquid medium, 3.75 g/L Agar and 10.0 µg/ml Neomycin), previously described by our research group [5]. Stock cell cultures were maintained at -80ºC until use.

3.3. Media and Biosynthesis

For recombinant human MB-COMT biosynthesis, cells containing the expression construct were grown overnight at 37 ºC in MTNm plates. A single colony was inoculated in

62.5 ml of 2SYNm medium in 250 ml shake flasks. Cells were grown at 30 ºC and 120 rpm until the cell density at 660 nm (OD660) reached 2.6. Subsequently, an aliquot was centrifuged (5000 x g, 10 min, 4 ºC) and the cells resuspended in fresh medium. These cells were grown in 62.5 ml of a mineral salt medium at 30 ºC, 120 rpm and pH 7, on 250.0 ml shake flasks, since the inoculation volume was fixed to achieve an initial OD660 of 0.2 units. The mineral salt medium composition were as follows (l^{-1} distilled water) 4 g Na_2HPO_4, 1g KH_2PO_4, 0.2 g $MgSO_4.7H_2O$, 0.05 g $CaCl_2.2H_2O$, 4 g $(NH_4)_2SO_4$, 0.2 g NaCl, 1 g glucose and 1 ml of trace element solution [31]. The trace elements solution contains (L^{-1} 0.5 M HCl) 5.56 g $FeSO_4.7H_2O$, 3.96 g $MnCl_2.4H_2O$, 5.62 g $CoSO_4.7H_2O$, 0.34 g $CuCl_2.2H_2O$, 0.58 g $ZnSO_4.7H_2O$, 0.6 g H_3BO_3, 0.04 g $NiCl_2.6H_2O$, 0.06 g $Na_2MoO_4.2H_2O$ [31]. After a 50 h growth, cells were harvested by centrifugation (5000 x g, 25 min, 4 ºC) and stored frozen at –20.0 ºC until use.

3.4. Determination of Cell density and Dry cell weight

Cell density (OD_{660}) was measured spectrophotometrically and the assessment of dry cell weight was performed as previously described [50]. For this recombinant strain specifically, one unit of OD_{660} was found to correspond to a dry Brevibacillus choshinensis weight of 0.4415 g/L.

3.5. Cell Lysis

The bacterial cell pellet (62,5 ml) was ressuspended in 10,0 ml of an appropriate buffer (150 mM NaCl, 10 mM DTT, 50 mM Tris, $MgCl_2$ 1 mM, pH 8,0) with protease inhibitors (5,0 g/ml leupeptin and 0,7 g/ml pepstatin) and disrupted by lysozyme treatment (10,0 mg/ml) for 15 minutes at room temperature. After that, cell pellet was subjected to six freeze (- 196 ºC in liquid nitrogen)/thaw (42 ºC) cycles. Finally, desoxyrribonuclease (1 mg/ml) was added to the lysate and the soluble material was removed by centrifugation (16000 x g, 20 min, 4 ºC).

3.6. MB-COMT Solubilization

Unless otherwise stated, solubilization was carried out by incubating the pellet (containing a total protein membrane extract), obtained after freeze thaw/lysis, in an appropriate buffer (150 mM NaCl, 10 mM dithiotreitol, 5 g/ml leupeptin, 50 mM Tris pH 8.0) at 4 ºC until complete solubilization of the pellet, around 8 hours like optimized by our research group [5]

3.7. Total Protein Quantification

Protein amount in fermentation pellets was measured by a Pierce BCA Protein Assay Kit (Thermo Scientific, USA), using BSA as a standard (0.025 – 2.0 mg/ml), according to manufacturer's instructions.

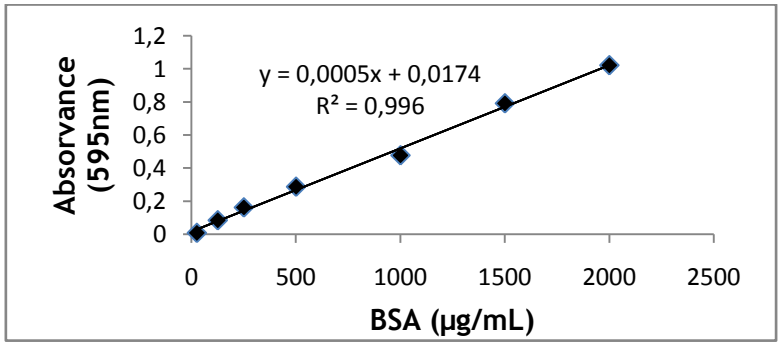

Figure 10 – BSA (25-2000 µg/mL) calibration curve.

3.8. MB-COMT Enzymatic Assay

The activity enzymatic assay was carried out to evaluate the methylation efficiency of recombinant human MB-COMT, by measuring the amount of metanephrine formed using epinephrine as substrate as previously described [51]. In human MB-COMT activity assay, a 150 µg/mL aliquot of the soluble extract was incubated in 5 mM sodium phosphate buffer (pH 7.8) containing 0.2 µM $MgCl_2$, 2 mM EGTA, 250 µM SAMe and 1 mM epinephrine in a total sample volume of 1 mL. Reactions were carried out at 37°C for 15 min in a shaking water bath and were stopped in ice by the addition of 200 µL of 2 M perchloric acid. The supernatants were centrifuged at 6000 rpm for 10 min at 4°C. After that, they were filtered through a 0.22 µm pore size filter to remove precipitated material and then injected into a

high performance liquid chromatography (HPLC) system coupled with a electrochemical detector [51]. MB-COMT activities were calculated as nmol of metanephrine produced/h/mg protein. Protein content of lysates was measured as previously described in 2.7.

3.9. Experimental Design and Optimization

The experimental design was applied in two stages, first to identify the significant nutrients and culture conditions for human MB-COMT production using Plackett-Burman design criteria. Subsequently, the significant variables resulted from Plackett-Burman design were optimized by response surface methodology, by using central composite design.

3.9.1. Selection of Significant Biosynthesis Variables Using Plackett–Burman Design

The Plackett-Burman experimental design was used to evaluate the relative importance of the medium components (described above) and operational conditions with respect to their main effects for human MB-COMT enzymatic activity yields. It is based on the first order model:

$$y = \beta_0 + \sum \beta_i x_i \quad (5)$$

where, y is the response, is β_0 the model intercept, β_i is the linear coefficient, and x_i is the level of the independent variable, which identifies the critical physico-chemical parameters required for high MB-COMT activity levels by screening *n* variables in *n*+1 experiments. Eleven variables representing nine nutritional medium components (Table 1), the initial pH (before sterilization), temperature, were used. These variables were denoted as numerical factors designated as -1 (low level) and +1 (high level). The effects of individual parameters on MB-COMT enzymatic activity was calculated by the following equation:

$$E = (\sum M_+ - \sum M_-)/N \quad (6)$$

where *E* is the effect of parameter under study and M_+ and M_- are responses (MB-COMT enzymatic activity) of trials at which the parameter was at its higher and lower levels respectively and *N* is the total number of trials.

Table 1 represents the different trials and each column represents a different variable. The significance of each variable was determined by applying the student's t-test.

The ranges of each variable of the *Plackett-Burman* design were chosen according to some literature reports and preliminary assays (data not shown).

Table 1 - Plackett–Burman experimental design for screening of significant variables affecting human MB-COMT productivity and activity levels.

Std	A: Na_2HPO_4 (g/L)	B: KH_2PO_4 (g/L)	C: $MgSO_4.7H_2O$ (g/L)	D: $CaCl_2.2H_2O$ (g/L)	E: $(NH_4)2SO_4$ (g/L)	F: NaCl (g/L)	G: Glucose (g/L)	H: Trace Elements Solution (mL/L)	I: Organic nitrogen source	J: pH	K: Temperature (ºC)
1	6	1	0,1	0,1	6	0,5	1	0,1	Yeast extract[a]	8	30
2	2	1	0,5	0,01	6	0,5	20	0,1	Yeast extract[a]	6	37
3	6	0,1	0,5	0,1	2	0,5	20	1	Yeast extract[a]	6	30
4	2	1	0,1	0,1	6	0,05	20	1	Polypeptone[a]	6	30
5	2	0,1	0,5	0,01	6	0,5	1	1	Polypeptone[a]	8	30
6	2	0,1	0,1	0,1	2	0,5	20	0,1	Polypeptone[a]	8	37
7	6	0,1	0,1	0,01	6	0,05	20	1	Yeast extract[a]	8	37
8	6	1	0,1	0,01	2	0,5	1	1	Polypeptone[a]	6	37
9	6	1	0,5	0,01	2	0,05	20	0,1	Polypeptone[a]	8	30
10	2	1	0,5	0,1	2	0,05	1	1	Yeast extract[a]	8	37
11	6	0,1	0,5	0,1	6	0,05	1	0,1	Polypeptone[a]	6	37
12	2	0,1	0,1	0,01	2	0,05	1	0,1	Yeast extract[a]	6	30

[a] Both nitrogen sources (yeast extract and polypeptone) were used with a concentration of 1% (m/v).

3.9.2. Optimization by Central Composite Rotatable Design

Identified the variables having a significant influence on the responses in Plackett-Burman design, it were performed preliminary assays in order to establish the intervals of each of the variables, except the temperature, for the CCD. Twelve assays were performed, testing separately different concentrations for each variable. In these tests, only the concentration of the variable concerned was modified, compared to the medium formulation described in 2.3. The temperature was maintained at 30ºC and the agitation at 120RPM. Thus, four different concentrations were tested for ammonium sulfate - 1(A), 2(B), 4(C) and 6(D) g/L - glucose - 1(E), 15(F), 30(G) and 50(H) g/L - polypeptone - 5(I), 20(J), 40(K) and 60(L) g/L.

The next step of this work was the application of a CCD to determine the optimal concentrations of the significant variables to maximize MB-COMT enzymatic activity. Central composite experimental design allows the establishment of a second degree polynomial with the relationships between the factors and the dependent variable and gives information about interaction between variables (factors) in their relation to the dependent variable. The lowest and the highest levels of variables were given in Table 2.

Table 2 - Levels of variables used in the central composite design.

Factor	Variable	Units	Low Actual	High Actual	Low Coded	High Coded
A	Amonium sulfate	g/L	2	6	-1	1
B	Glucose	%	1	3	-1	1
C	Polypeptone	%	1	3	-1	1
D	Temperature	°C	25	35	-1	1

The experiments were designed by using the Design Expert software version 7.0 (State Ease Inc., Minneapolis, MN, USA). A 2^4 factorial central composite design with eight star/axial points, sixteen factorial points and six replicates at the center points (Table 3) was employed for the optimization of the culture conditions and medium components. The second-order polynomial, equation (7), which includes all interaction terms were used to calculate the predicted response:

$$\hat{Y}_i = \beta_0 + \sum_{i=1}^{4} \beta_i x_i + \sum_{i=1}^{4} \beta_{ii} x_i^2 + \sum_{i,j=1}^{4} \beta_{ij} x_i x_j \qquad (7)$$

like described in 1.3.2. The design expert software was used for regression and graphical analysis of data obtained.

Table 3 – Experimental plan for optimization of MB-COMT enzymatic activity by RSM using CCD.

Std	Sulfato Amónio (g/L)	Glucose (%)	Polypeptone (%)	Temp (°C)
1	2	1	1	25
2	6	1	1	25
3	2	3	1	25
4	6	3	1	25
5	2	1	3	25
6	6	1	3	25
7	2	3	3	25
8	6	3	3	25
9	2	1	1	35
10	6	1	1	35
11	2	3	1	35
12	6	3	1	35
13	2	1	3	35
14	6	1	3	35
15	2	3	3	35
16	6	3	3	35
17	0	2	2	30
18	8	2	2	30
19	4	0	2	30
20	4	4	2	30
21	4	2	0	30
22	4	2	4	30
23	4	2	2	20
24	4	2	2	40
25[a]	4	2	2	30
26[a]	4	2	2	30
27[a]	4	2	2	30
28[a]	4	2	2	30
29[a]	4	2	2	30
30[a]	4	2	2	30

[a] Center points.

CHAPTER IV: Results and Discussion

In general, each microorganism evidences its own idiosyncratic physicochemical and nutritional requirements for growth and enzyme secretion [34]. Genetic manipulation and media engineering are widely used for the economization of the production process, in which enzyme overproduction is essential [34]. Using recombinants systems to increase the production yields may not be stable, making media manipulation the better alternative for the overproduction of enzymes [34, 52].

Nowadays, statistical methods for medium optimization have proved to be a powerful and useful tool for biotechnology and the use of such models to optimize culture medium components and conditions has increase, due to its ready applicability and aptness [34].

In the present study, the significant variables necessary for enhanced MB-COMT enzymatic activity levels at a scale down biosynthesis, were selected using the Plackett–Burman design, with a further optimization by CCD.

4.1. Screening of Significant Variables using a Plackett-Burman design

Plackett–Burman design is a well-established and widely used statistical technique for screening of medium components in shakeflasks in this kind of bioprocesses.

A total of eleven variables in a twelve runs experiment were analyzed with regard to their effects on the response, in this case, in total hman MB-COMT enzymatic activity using a Plackett–Burman design (Table 1). Table 1 represents the corresponding responses (predicted and actual) of the screening experiments using Plackett–Burman design. These responses were obtained at 50 hours of fermentation based on previous studies [5].

Table 1 - Observed and predicted responses for the experiments performed using Plackett-Burman design.

Std	Response (nmol/h)	
	Predicted	Actual
1	15.435	12.730
2	13.802	7.653
3	71.179	65.701
4	70.744	92.770
5	43.049	21.192
6	69.464	77.748
7	13.802	8.142
8	41.769	31.991
9	98.793	85.771
10	14.156	13.113
11	13.721	28.068
12	43.484	64.520

The magnitude of the variable effects on MB-COMT enzymatic activity are provided by statistical analysis, where the effect of each factor is the difference of the response related with the change of the lower level to the higher level. When an effect has a great significance level, the process will present significantly better results for one of these levels. Otherwise, if a factor is irrelevant to the process, no change in the process performance takes place [53].

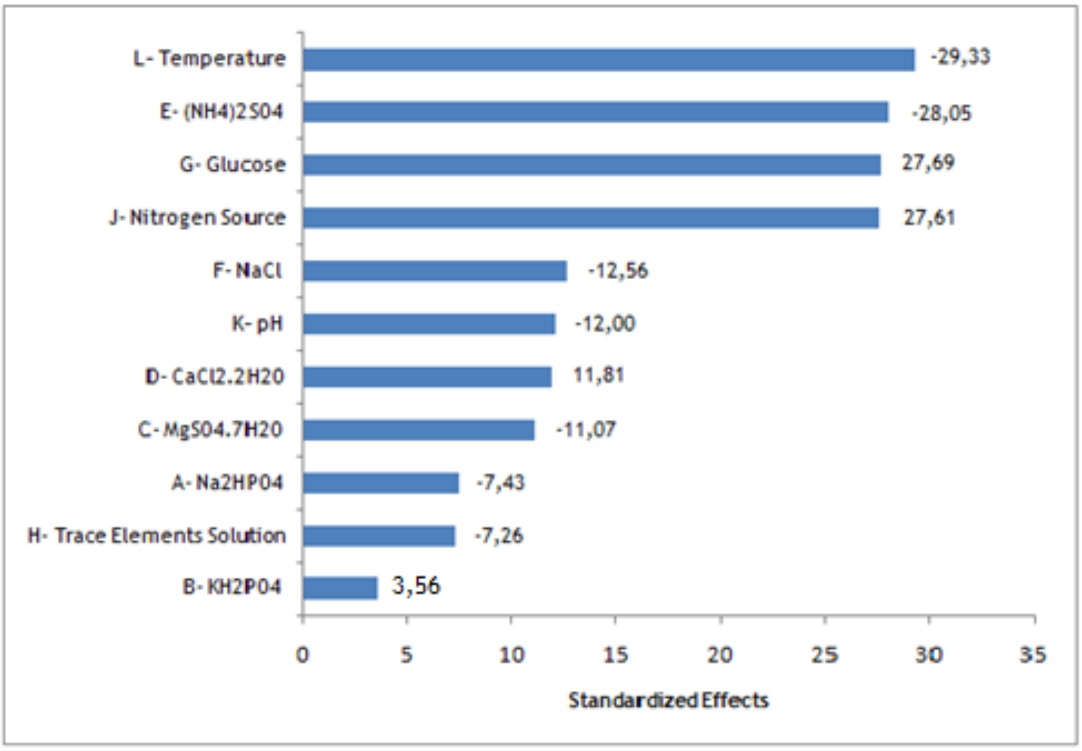

Figure 1 – Pareto chart showing effects of the variables according to his magnitude based on the observations of Plackett-Burman design.

Figure 1 represents the effects of each variable in the levels of MB-COMT enzymatic activity. The negative values indicate that a change of the lower level to the higher level in the corresponding variable produces a decrease in the enzymatic activity, while the positive values reflect an increase in the enzymatic activity due to the change for a higher level [53]. Temperature, ammonium sulfate, glucose and nitrogen source shows a high contribution to MB-COMT enzymatic activity compared to the other variables.

Subsequently, statistical analysis of the model was calculated, and the variables evidencing a statistical significant effect were screened via Student's t-test for ANOVA (Table 2).

Table 2 - Statistical analysis of the model.

Source	Sum of Squares	Degrees of Freedom	Mean Square	F Value	p-value Prob > F
Model	9529.18	4	2382.30	8.12	0.0091
E-Ammonium Sulfate	2360.13	1	2360.13	8.05	0.0252
G-Glucose	2301.04	1	2301.04	7.84	0.0265
J-Nitrogen Source	2287.58	1	2287.58	7.80	0.0268
L-Temperature	2580.44	1	2580.44	8.00	0.0209
Residual	2053.25	7	293.32		
Cor. Total	11582.43	11			

R^2 = 0,8227

The results obtained showed the model *F* value of 8.12 implies that the model is significant and there is only a 0,91% chance that a "Model F-Value" this large could occur due to noise. This model presents a R^2 value closer to 1 denotes an excellent correlation between the observed and predicted values. In our case the value of R^2 (0.8227) indicates good correlation between the experimental and predicted values, which indicated that the model could explain up to 82.27% variation of the data achieved. The multiple correlation coefficient (R^2 value) is always between 0 and 1, and a value >0.75 indicates aptness of the model [53].

Also, factors evidencing *p*-values less than 0.05 were considered to have significant effects on the response, and should be selected for further optimization studies. Therefore, ours experiments E, G, J and L appears as the significant model terms. Specifically, temperature, with a *p*-value of 0.0209, was determined to be the most significant factor, followed by ammonium sulfate (*p*-value of 0.0252), glucose (*p*-value of 0.0265), and nitrogen source (*p*-value of 0.0268). These results corroborates with the lower *p*-values achieved, demonstrating that indicate temperature, ammonium sulfate, glucose and nitrogen source are the most significant factors for MB-COMT activity levels. Based on described above, all other variables were not optimized and maintained at constant level, while pH was maintained at 7. The optimum levels of the four variables selected were further asses by an RSM design.

These results are similar to earlier literature reports about the importance of nitrogen and carbon sources for enzyme production. In microorganisms, nitrogen sources (both organic and inorganic forms) are metabolized, primarily resulting in the production of amino acids, nucleic acids, and cell wall components [34]. Nitrogen and carbon sources exert regulatory effects on enzyme synthesis, as described in [54, 55]. The nitrogen sources also functioned as inducers of enzyme production [56].

Regression analysis was performed and a first order polynomial equation was derived in order to assess MB-COMT total activity as a function of the independent variables (equation 8):

$$Y = +42{,}45 - 14{,}02E + 13{,}85\,G + 13{,}81J - 14{,}66L \quad (8).$$

The resulting equation can be used to predict a response that occurs with a variation of the factors included therein.

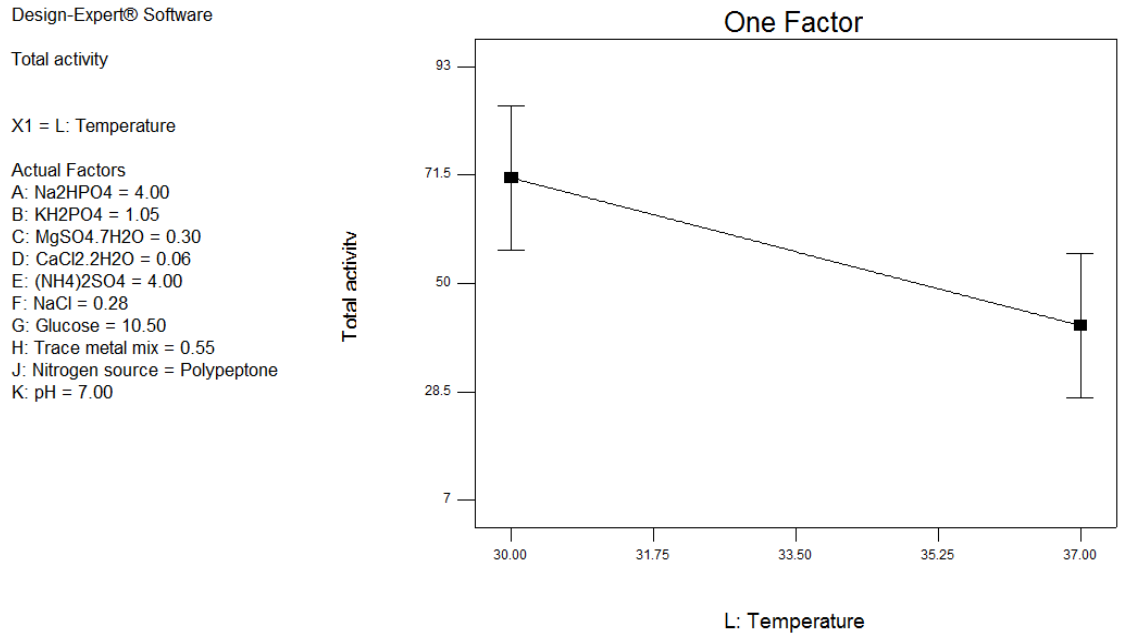

Figure 2 – Temperature effect on MB-COMT enzymatic activity.

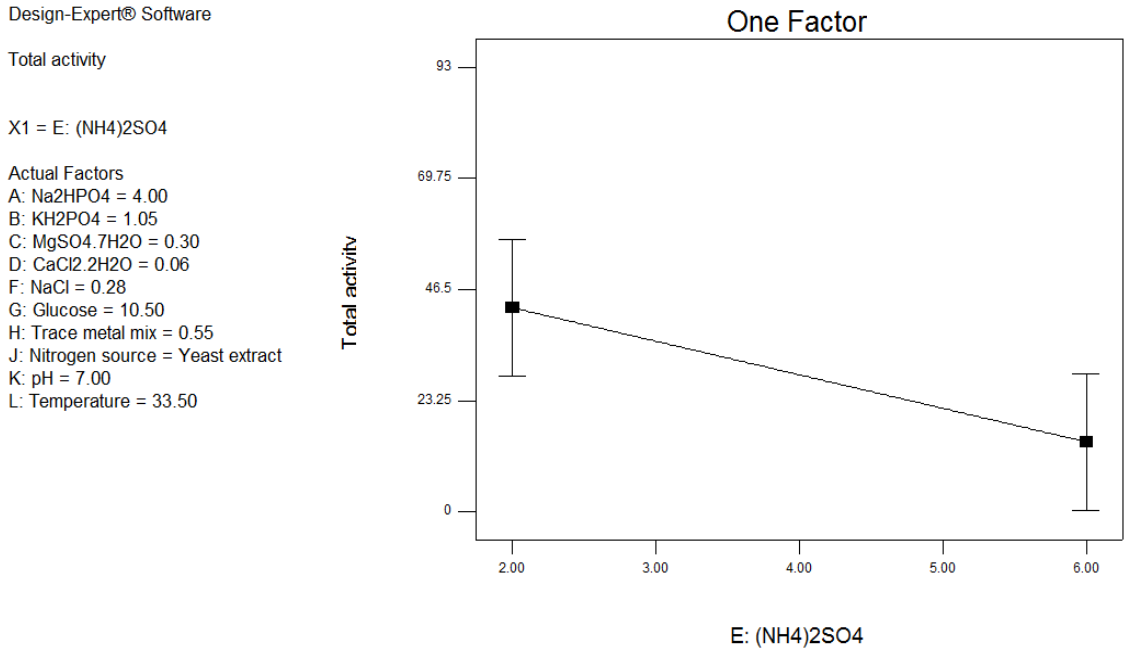

Figure 3 – Ammonium sulfate effect on MB-COMT enzymatic activity.

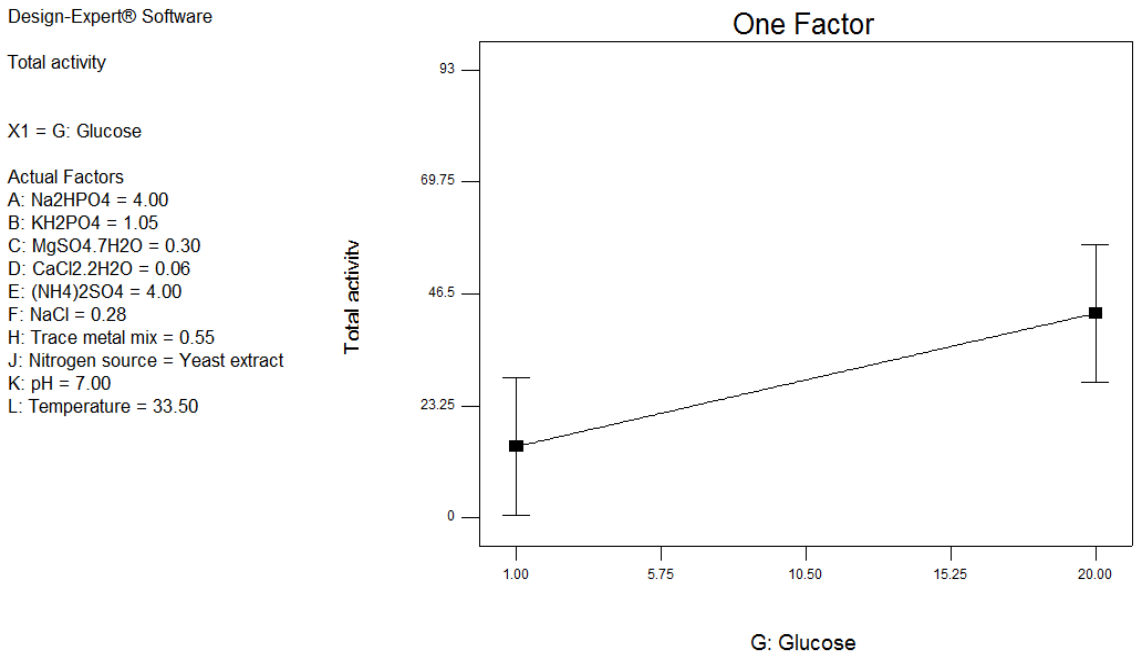

Figure 4 – Glucose effect on the MB-COMT enzymatic activity.

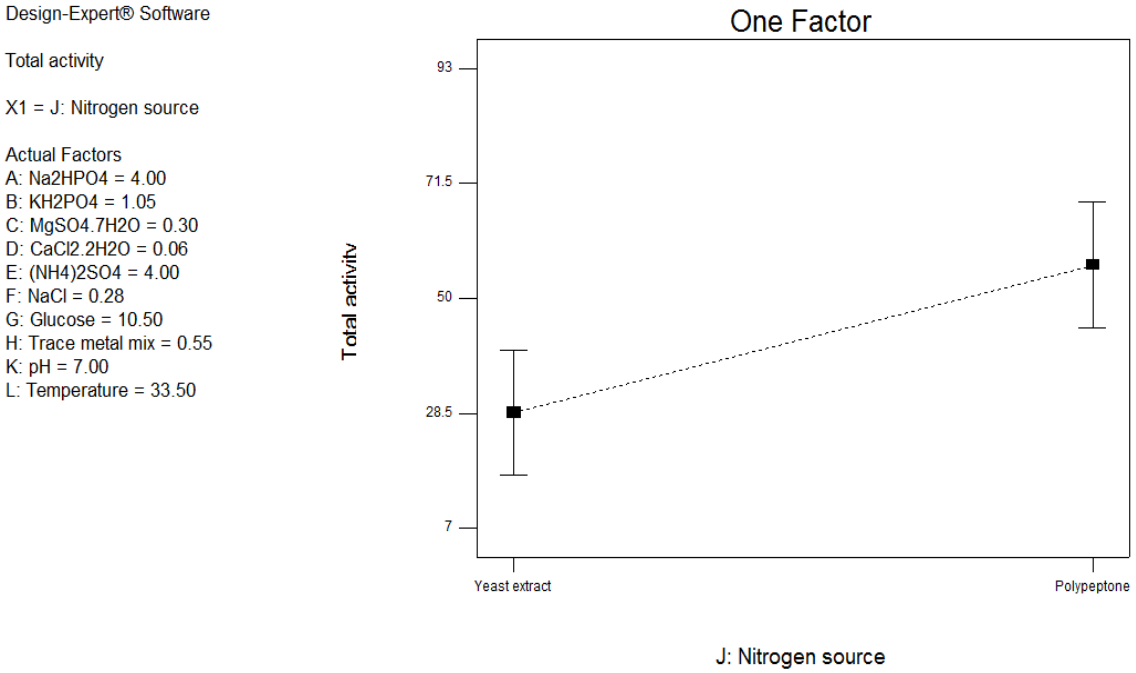

Figure 5 – Effects of two distinct nitrogen sources on MB-COMT enzymatic activity.

The effects on the response (MB-COMT total enzymatic activity) of lower and higher levels for the variables temperature, ammonium sulfate and glucose, are represented in

figures 2, 3, 4, respectively. The effects of each variable show a clear trend in its contribution to the response.

In the case of the temperature and ammonium sulfate a change to the higher level lead to a decrease in the output, while the glucose concentration manipulation caused an increase in the response. Figure 5 represents a categoric variable corresponding at two distinct nitrogen sources (yeast extract and polypeptone). Thus, we can conclude that polypeptone has a better effect in the response, making the yeast extract be neglected. All the others variables showed a low contribution and a weak effect on the response (Figure 1).

The results of the experiments of *Plackett-Burman* design showed an accurate precision to found the most important factors for our purpose, with a clear selection of the four factors to further optimize in CCD. The MB-COMT total enzymatic activity was higher than reported by [5], and the MB-COMT specific enzymatic activity was slightly lower than the results obtained by Pedro and coworkers (2011) (34 face to 44 nmol/h/mg). The discrepancy observed can be related with the solubization step performed in this work. Typically, detergents form spherical micellar structures in which membrane protein are soluble. In our experiments, we omitted the application of any kind of detergents in order to promote environmental conditions near to the enzyme native folding state. So, this fact can lead to a decrease in the concentration of target membrane proteins recuperated in solution and, consequently, reduced the MB-COMT specific and total enzymatic activity.

However, the wide variation (7.653–92.770 nmol/h) observed in MB-COMT total enzymatic activity indicates further optimization necessary.

4.2. Establishment of CCD Variables Ranges

In order to construct the CCD, the ranges for ammonium sulfate, glucose and nitrogen source were established according to results of the assays performed to analyze each variable separately from the others. So, twelve assays (ammonium sulfate – 1g/L(A), 2g/L (B), 4g/L (C) and 6g/L (D) - glucose - 1g/L (E), 15g/L (F), 30g/L (G) and 50 g/L (H) - polypeptone - 5g/L (I), 20g/L (J), 40g/L (K) and 60g/L (L)) were performed. The total enzymatic activity values are shown in Table 3.

Table 3 – Assays performed to establish the central point and the ranges of each variable for the CCD, and their total enzymatic activity values.

Assay	Total Enzymatic activity (nmol/h)
A	2.89501
B	2.44730
C	4.43177
D	3.81016
E	3.97035
F	40.23695
G	2.82641
H	2.76613
I	2.37011
J	18.2963
K	77.7588
L	62.0435

For ammonium sulfate (A-D), the enzymatic activity increased until 4g/L and decreased in the change for 6g/L. Plackett-Burman results shows a decrease of enzymatic activity with the increase of ammonium sulfate concentration. However in these preliminary assays, the media formulation does not contain any nitrogen source and carbon source is present in a very low concentration, leading to lower cell growth kinetics and basal values of enzymatic activity. On the other hand, the similarity of enzymatic activity values for the distinct concentrations does not evidence a major influence of ammonium sulfate on MB-COMT enzymatic activity. Furthermore, the establishment of a center point with a concentration of 2g/L for ammonium sulfate would imply the existence of negative concentrations in the design, due to the axial points. Therefore, the concentration of ammonium sulfate for the center point was established in 4g/L, and the ranges 2 to 6g/L.

For glucose (E-H), a concentration of 15g/L promotes a peak in enzymatic activity obtained, higher than the others concentrations tested. Indeed, these results were in accordance with *Plackett-Burman* design results, while an increase on glucose concentration causes an improvement in the enzymatic activity levels. However, glucose concentration above a certain level, can promote a decrease in the enzymatic activity, probably, due to the acetate formation, compromising cell growth and membrane protein biosynthesis [28, 29]. As the *Plackett-Burman* design results were much higher than the described above, with a glucose concentration of 20g/L, in the next optimization steps the center point was fixed in 20g/L and the ranges established in 10 and 30g/L.

Polypeptone, the main nitrogen source, shows better enzymatic activity at 20g/L, compared to the others concentrations. As described above, about the importance of nitrogen sources in enzyme production, these results show a need of a high nitrogen source

concentration on the medium formulation to increase protein yields and to promote the enzymatic activity.

4.3. Optimization of Screened Variables

Following these assays, the concentration range of the selected variables was established and the CCD was constructed. Thirty experiments (Table 3 – Chapter II) were carried out by RSM using CCD, in order to study the interactions among the significant factors (temperature, ammonium sulfate, glucose and nitrogen source) and also assess their optimal levels. The other variables in the study were maintained at a constant level, like described in 2.3. The predicted and observed responses obtained are depicted in Table 4.

Table 4 - Observed and predicted responses for the experiments performed by RSM using CCD.

Std	Response (nmol/h)	
	Predicted	Actual
1	1.895	1.542
2	2.841	1.748
3	1.668	1.407
4	2.198	1.784
5	2.244	2.167
6	2.879	2.417
7	1.632	2.153
8	1.851	1.162
9	2.228	1.930
10	3.024	2.325
11	1.797	2.080
12	2.178	1.268
13	2.922	3.157
14	3.407	2.680
15	2.107	2.213
16	2.176	2.349
17	1.730	1.068
18	2.745	4.574
19	3.765	4.918
20	2.308	2.321
21	3.389	4.678
22	3.736	3.613
23	0.096	0.927
24	0.754	1.089
25[a]	1.420	1.476
26[a]	1.420	1.488
27[a]	1.420	1.330
28[a]	1.420	1.386
29[a]	1.420	1.501
30[a]	1.420	1.340

[a] Center Points

The results depicted in table 4 are completely unexpected due to the existence of an enormous discrepancy between the CCD results and the previous results of this work. These results show a basal enzymatic activity, while in the *Plackett-Burman* results, were obtained values around 93nmol/h for total MB-COMT enzymatic activity compared to the 5nmol/h of the CCD results.

The calculated regression equation for the optimization of medium constituents and temperature showed that MB-COMT total enzymatic activity (Y) is a function of the

concentration of ammonium sulfate (A), glucose (B), polypeptone (C) and temperature (D). By applying multiple regression analysis on the experimental data, the following second-order polynomial equation was found to represent the MB-COMT enzymatic activity:

$$Y = 1.42 + 0.25A - 0.36B + 0.087C + 0.16D - 0.1\,A*B - 0.078A*C - 0.038\,A*D - 0.096\,B*C - 0.051B*D + 0.086C*D + 0.2A^2 + 0.4B^2 + 0.54C^2 - 0.25D^2 \quad (9)$$

The goodness of fit of the model was examined by determination coefficient R^2. The R^2 obtained was 0.635. This implies that only 63.5% of experimental data was compatible with the data predicted by the model. So, the model does not fit and shows lack of aptness. The *p*-value of the Lack of Fit is <0.0001 implying the Lack of Fit is significant. There is only a 0.01% chance that a "Lack of Fit F-value" this large could occur due to noise. The significant lack of fit is another factor indicating the lack of aptness of the model. The significance of each variable was determined by p-values, listed in Table 5. Also the p-values less than 0.0500 indicate model terms are significant. Therefore a model *p*-value of 0.1219 implies that the model is not significant relative to the noise. There is a 12.19 % chance that a "Model F-value" this large could occur due to noise. In this case B^2, C^2 are significant model terms.

Table 5 - Analysis of variance (ANOVA) for the parameters of CCD fitted to second-order polynomial equation.

Source	Sumo f Squares	Degrees of Freedom	Mean Square	F Value	p-value Prob > F
Model	21.32	14	1.52	1.86	0.1219
A-Ammonium Sulfate	1.55	1	1.55	1.89	0.1891
B-Glucose	3.19	1	3.19	3.90	0.0670
C-Polypeptone	0.18	1	0.18	0.22	0.6449
D-Temperature	0.65	1	0.65	0.79	0.3870
AB	0.17	1	0.17	0.21	0.6519
AC	0.097	1	0.097	0.12	0.7349
AD	0.023	1	0.023	0.028	0.8702
BC	0.15	1	0.15	0.18	0.6767
BD	0.041	1	0.041	0.051	0.8251
CD	0.12	1	0.12	0.15	0.7082
A^2	1.15	1	1.15	1.40	0.2547
B^2	4.48	1	4.48	5.48	0.0334
C^2	7.87	1	7.87	9.63	0.0073
D^2	1.70	1	1.70	2.08	0.1701
Residual	12.26	15	0.82		
Lack of fit	12.23	10	1.22	203.73	<0.0001
Error	0.030	5	6.002E-3		
Total	33.58	29			

$R^2 = 0.635$

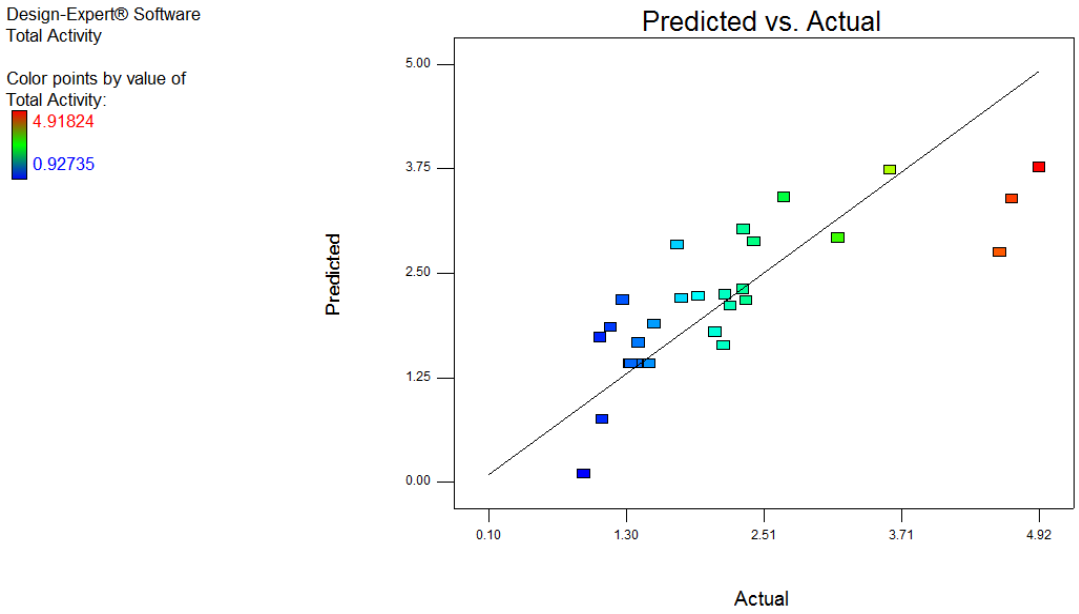

Figure 6 - Parity plot showing the distribution of experimental versus predicted values of MB-COMT enzymatic activity.

As described above the R^2 value was 0.635 what shows an unsatisfactory correlation between the experimental and predicted values of MB-COMT enzymatic activity, wherein, the points cluster around the diagonal does not line, which indicated the lack of fit of the model, since the deviation between the experimental and predicted values was high (Figure 6).

In order to determine the optimal levels of each variable for maximum enzymatic activity, three-dimensional response surface plots were constructed by plotting the response (MB-COMT enzymatic activity) on the Z-axis against any two independent variables, while maintaining other variables at their optimal levels. The response surface plots are represented in Figures 7–12.

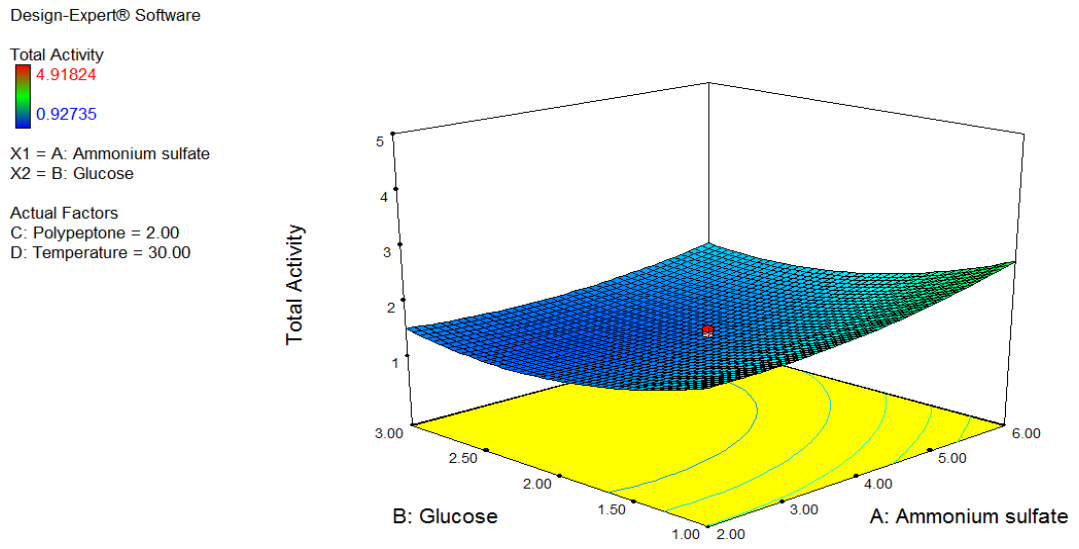

Figure 7 – Response surface graph showing interaction between ammonium sulfate and glucose concentration.

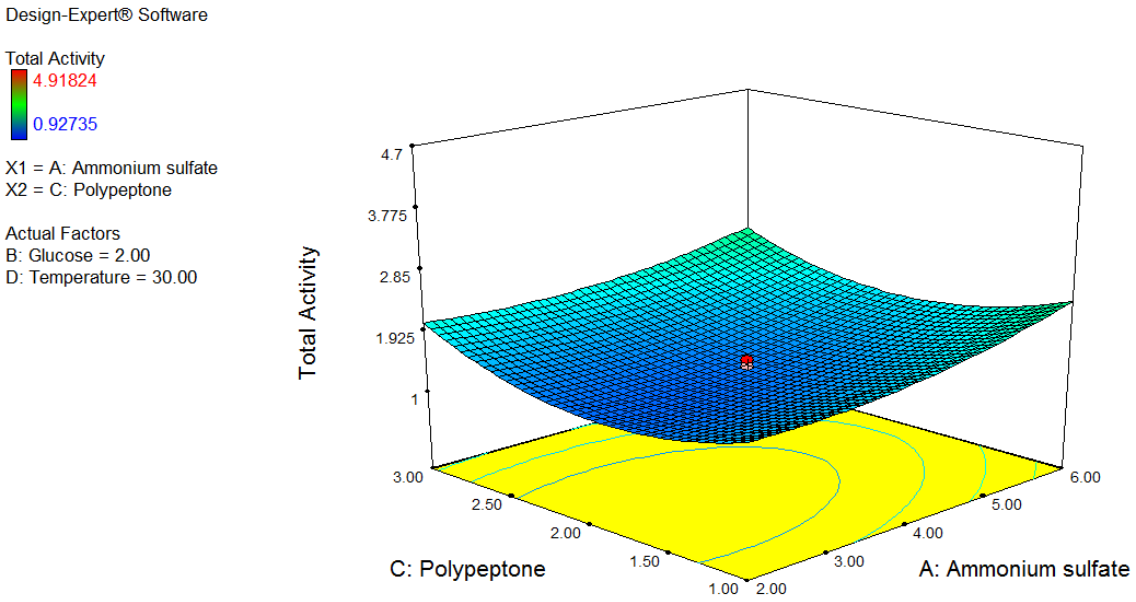

Figure 8 – Response surface graph showing interaction between ammonium sulfate and polypeptone concentration.

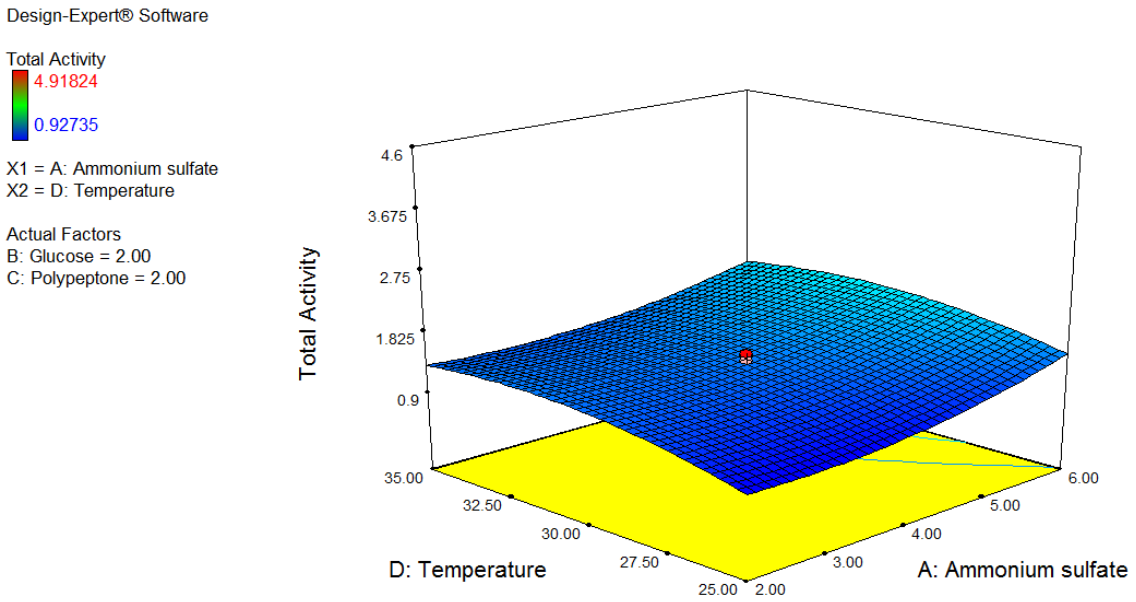

Figure 9 – Response surface graph showing interaction between ammonium sulfate concentration and temperature.

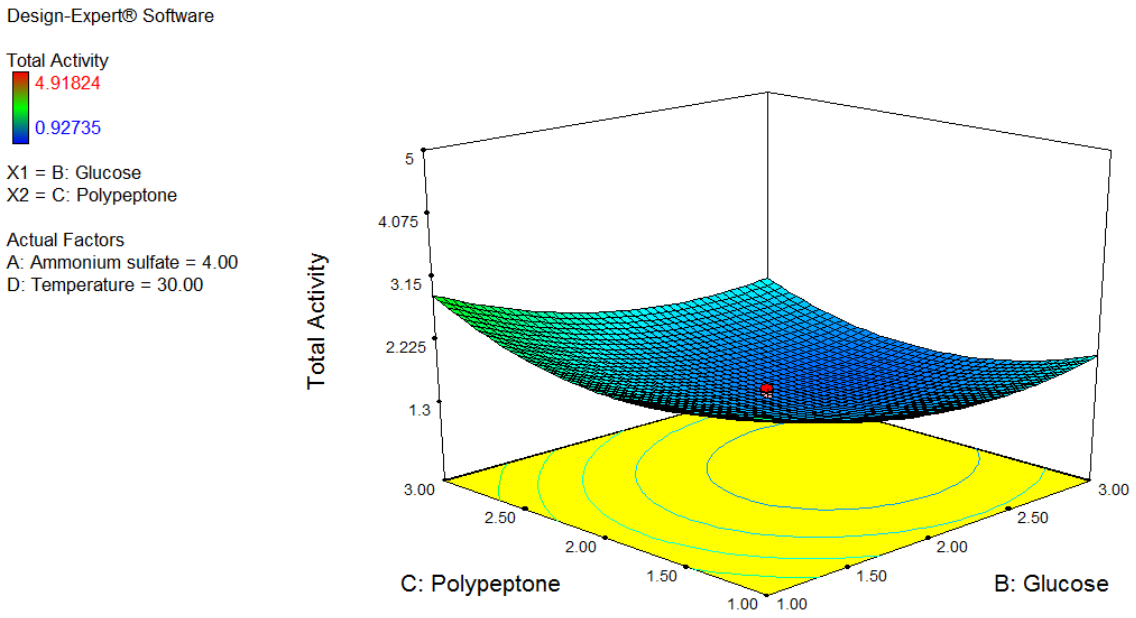

Figure 10 – Response surface graph showing interaction between glucose and polypeptone concentration.

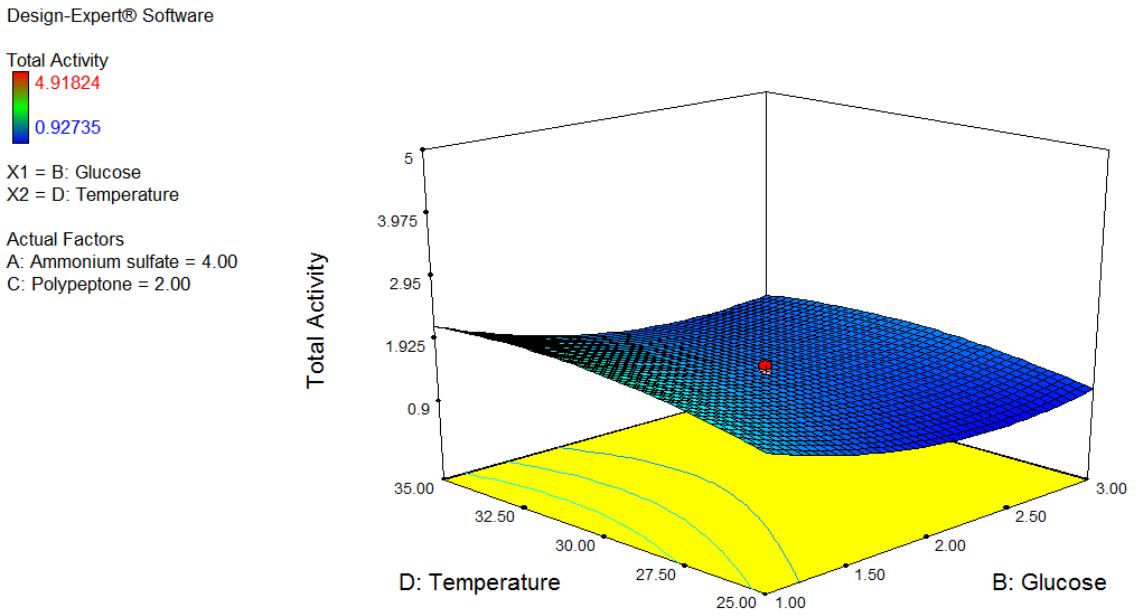

Figure 11 – Response surface graph showing interaction between glucose concentration and temperature.

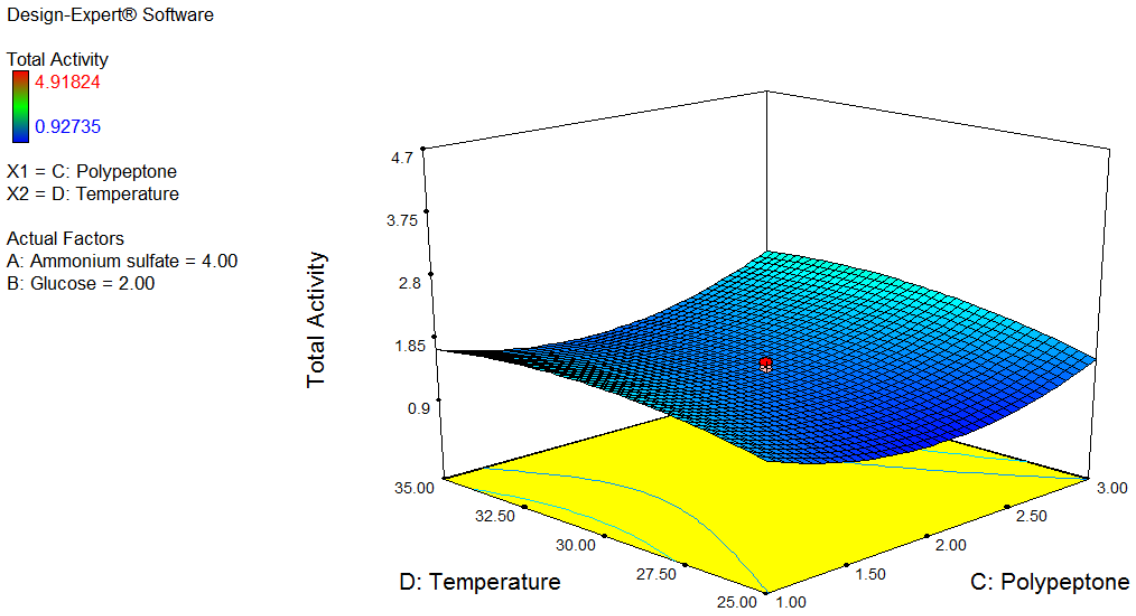

Figure 12 – Response surface graph showing interaction between polypeptone concentration and temperature.

The contour plots are almost horizontal. This indicates that there is no interaction occurring among the independent variables corresponding to the response surfaces. Due to the low levels of MB-COMT enzymatic activity and especially the proximity of the experimented responses observed in the CCD (Table 3), we cannot take conclusions or even describe any weak tendency present in the model.

Comparatively to the Plackett-Burman design responses and the developed assays to establish the center point and the ranges of the CCD, the responses were much lower in the CCD. The maximal response of Plackett-Burman design was around 93 nmol/h and in the CCD was about 5nmol/h for total MB-COMT enzymatic activity. This unexpected and great discrepancy observed between the Plackett-Burman design and the CCD responses may be related to an intrinsic instability of the master *Brevibacillus* stock culture cells during the development of this work, which could lead to a decrease on cell competence to produce the target membrane protein with high yields and maximum massic and volumetric productivities.

CHAPTER V: Conclusions and Future Perspectives

Conclusions and Future Perspectives

The present study involved the use of statistical experimental designs to optimize culture parameters for the biosynthesis of human MB-COMT in order to maximize the enzymatic activity.

In general, at the screening stages it is very important to test as many factors as possible in order to identify the effect of each factor affecting our response (MB-COMT enzymatic activity). In this work, the use of an experimental design allowed the rapid screening of a large experimental domain for optimization. Four variables including: temperature, ammonium sulfate, glucose and nitrogen source were identified by Plackett– Burman design as most important factors for MB-COMT enzymatic activity, that were further optimized using CCD involving RSM.

As far as we knew, this was the first trial to optimize the MB-COMT enzymatic yields using statistical methods.

Plackett-Burman design has been proved to be effective in optimizing MB-COMT enzymatic yields, showing significant results for enzymatic activity. The experimental results show, as reported in the literature, the importance of the carbon and nitrogen sources and its combination with physical parameters of extreme importance in bioprocesses, namely temperature.

CCD converts the bioprocess factor correlations into mathematical models that predict where the optimum is likely to be located. The CCD approach of this work failed and it was not possible locate our maximum response. The high discrepancy of the CCD and Plackett-Burman design responses, leads us to believe in the possibility of the existence of a problem with the cells. Nevertheless, this kind of methodology as a whole is widely applied and described in the literature and has proven to be an adequate and promising strategy for the design and optimization of bioprocesses in order to produce several therapeutic soluble proteins.

This study also encourages putting more emphasis on the development of chemically defined media because of the advantages they demonstrate over complex media including the inherently higher process consistency. From a regulatory point of view, chemically defined media are especially attractive for the production of bio-pharmaceuticals where the production process is considered as an integral part of the final product definition.

Future plans include the need to perform some readjustments to the process and then try to validate this model with this approach, where better results than *Plackett-Burman* are expected. A scale-up step, with the optimum culture parameters obtained from the CCD at the scale-down experiments, is another stage for future work.

CHAPTER XI: References

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