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Optimization of Culture Medium for Large-Scale Production of Heterologous Proteins in *Pichia pastoris* to be used in Nanoscience and other Biotechnological Fields

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Abstract

The methylotrophic yeast *Pichia pastoris* became an excellent and successfully expression system for therapeutics and other useful heterologous proteins at large-scale. Its capacity to reach a high-cell-density-concentration during its fed-batch fermentations in a chemically-defined culture medium is one of the main features of this system. The standard culture medium BSM developed by Invitrogen Co. has been the most widespread used. However, it contains certain components in improper ratios that lead to precipitation of some of its components during preparation, mainly after sterilization and/or during pH adjustment. In this study, we propose a modified version of the BSM culture medium, named MBSM. These simple modifications by employing a linear optimization technique solves the precipitation problems observed on BSM and other culture media. Beside it, is rather simple, easy to prepare, and inexpensive, and it has impact positively over the environment by reducing the pollution load of the fermentation waste and also diminish the cost of the industrial scale fermentation process. By using this modified medium, were reached cell densities of 100 gDCW·L^{-1} and 60 gDCW·L^{-1} for mini-proinsulin and exo-levanase under the inducible AOX1 and the constitutive GAPDH promoters, respectively. These results allow to get vast amounts of proteins able to be used in nanoscience and other biotechnological applications.

Keywords: Chemical defined medium; *Pichia pastoris*; Heterologous protein expression system

Introduction

The methylotrophic yeast *Pichia pastoris* has become over the years in one of the most used host organisms to produce therapeutic and industrial recombinant proteins. This success story is due to its GRAS (General Regarded as Safe) status and the ability to grow at high cell density in inexpensive chemical-defined media. Also, this yeast allow efficient expression of foreign genes using inducible and constitutive promotors in both ways, secreted to the culture media or intracellularly, producing the corresponding well folded proteins at high yield even with the human-like glycosylated pattern [1,2].

The culture medium is a key player when high cell density of any microorganism is desired. Additionally, its properties and design result of great influence in the efficiency of the production process at industrial scale so, becomes in an important component in terms of economic efficiency of the whole process. Although the capacity of *P. pastoris* to reach high cell concentrations with chemical-defined media, literature postulates that together with the optimum composition of the culture medium also, the chosen strain and the particularities of the foreign protein to be expressed must be considered to achieve the best results [3-5].

It is strongly recommended the use of the basal salt medium BSM developed by Invitrogen Co. (Carlsbad, CA, USA) to start protein expression studies using *P. pastoris* as host [6], which has been extensively and successfully tested for the expression of hundreds of different heterologous proteins [7].

Despite the success accumulated by BSM use, this media presents some problems easily observed during its preparation and sterilizing procedures by the presence of a white-turbidity formation before inoculation, especially where pH ≥ 5 [8]. This turbidity is ascribed to the low solubility of the polyvalent cations calcium (Ca^{2+}) and magnesium (Mg^{2+}) present in the supplement PTM1, in the presence of ortho-phosphate anions (HPO_4^{2-}) [9]. Despite that this precipitation matter tends to disappear during the cultivation time, clearly leads to certain nutrient imbalance problems, especially if the medium pH increases above pH 5 [8].

The aim of this work was to find a chemically defined culture medium, simple, easy to prepare and inexpensive, to serve for the production of heterologous proteins in *P. pastoris* at industrial scale that avoids the formation of precipitates during the preparation and sterilization of culture medium so, reaching the exact nutrient availability during the first fermentations stages. This media will guarantee both, high cell densities an adequate expression levels of the studied proteins for further nano-applications in therapeutics and industrial approaches.

Materials and Methods

Originally, the Invitrogen's BSM was developed from Wegner research [10] using continuous culture and analyzing the ranges for each essential chemical element required to grow *P. pastoris* to high

cell densities. Atomic composition for main BSM nutrient elements was determined as shown below on Table 1. Other chemical-defined alternative media to achieve high cell densities in cultures of *P. pastoris* are showed in the same Table 1 [5,11-13].

For this culture optimization studies, the culture medium cost was used as the objective function and as the constraint equations regarding the minimum and maximum ranges reported by Wegner [10] for each culture medium components. Also, the solubility of each component was used to find a model that could be optimized to find out the minimal composition of the culture medium that minimized both, its elaboration cost and also, eliminate the turbidity caused by nutrient imbalances and precipitations of certain media components after sterilization. For this purpose, the Solver tool of Excel (Microsoft Co., Redmond, Washington, USA), which uses an optimization algorithm Simplex LP was used.

Results

When comparing suggested Wagner's ranges in the BSM media and those reported by D'Anjou and Daugulis [12], and FM22 described by Stratton and co-workers [13], it appears certain deficits or surpluses for some atoms-elements of the BSM and FM22 media respect to the Wagner`s recommended range, specially respect to phosphorous and potassium concentrations. Only the medium suggested by D'Anjou and Daugulis [12] fulfill all of requirements suggested by Wagner [10].

In the present work the function corresponding to the culture medium cost $\left(\sum_{i=1}^{n} c_i \times K_i\right)$ was minimized so, two additional restriction functions appears. The first, deals with the minimum concentration of each culture media components within the range studied by Werner [10]; and the second involve the concentration below the limit of aqueous solubility of the component in the aqueous medium (approximately below 85% of this value). The mathematical expression then is (Equations 1-3):

$$\min_{c_i} \subset s \sum_{i=1}^{n} c_i \times K_i \; S := \{S_1, S_2\} \qquad (1)$$

$$S_1 : c_{i_{\min}} \leq c_i \leq c_{i_{\max}} \qquad (2)$$

$$S_2 : c_i < 0.85 \times Sol_i \qquad (3)$$

where:

c_i: concentration of component i in the culture medium, g•L-1

$c_{i_{\min}}$, $c_{i_{\max}}$: Minimal and maximum concentration of the component i, according to the values reported by Wegner [10], g•L-1

K_i: unit price of component i in the culture medium, US$•g-1

Sol_i: solubility of the component i in the water, g•L-1

The solution of this optimization problem of course depends on the updated prize of the component i in the culture medium and its solubility. The unit price of the needed components in the culture medium depend on the supplier, the component quality and quantity. As noted elsewhere [5] in general, the BSM medium provides high concentrations of basic elements, whilst the D'Anjou and Daugulis [12] has a low concentration of chemical elements with respect of the Wegner`s reported range [10].

Respect to nitrogen source, in BSM, FM22, and MBSM, this element is added as ammonium hydroxide when controlling pH. Contrary, in the D'Anjou and Daugulis [12] culture medium all nitrogen source is supplied at the initial formulation and is not added during the culture.

The composition of each BSM atom-element [6], FM22 [13], of the D'Anjou and Daugulis [12], and MBSM proposed here is shown in Table 1.

Atom	Wegner [10]	BSM [6]	FM22 [13]	D'Anjou and Daugulis [12]	MBSM (This work)
Macro Elements [g·L^{-1}]					
N	-	NH_4OH (as pH control)	1.06 + NH_4OH (as pH control)	4.24	NH_4OH (as pH control)
P	2.2 – 10.0	7.17	0.98	2.73	2.27
K	1.5 – 10.0	11.04	1.23	3.44	2.87
Mg	0.3 – 1.2	1.47	0.31	0.46	0.31
Ca	0.08 – 0.8	0.27	0.07	0.1	0.1
S	0.2 – 5.0	5.56	0.46	0.66	0.46
Trace Elements [mg·L^{-1}]					
Fe	9.0 – 80.0	56.83	65.05	65.05	56.14
Zn	3.0 – 40.0	41.73	22.66	22.66	19.55
Cu	1.0 – 10.0	6.64	7.61	7.61	6.57
Mn	0.9 – 8.0	4.24	4.87	4.87	4.19
Na	-	0.22	0.95	0.95	0.82
I	-	0.29	1.61	1.61	1.38
Mo	-	0.35	1.98	1.98	1.71
B	-	0.015	0.09	0.09	0.08
Co	-	0.99	-	-	-
Cl	-	46.45	173.29	173.29	168.79
pH Control	-	NH_4OH & H_3PO_4	NH_4OH & H_3PO_4	KOH & H_3PO_4	NH_4OH & H_3PO_4

Table 1: Comparative atomic composition for main elements of different culture media for *Pichia pastoris*.

Starting from the simplest culture medium reported by D'Anjou and Daugulis [12], the modified basal saline medium (MBSB) proposed here was designed. For this purpose, it was established two conditions:

1- that all the elements of the designed medium, always fix the proposed by Wegner`s range [10] and 2- the use of the medium cost as objective function. Then, through the optimizing on Excel sheet by using a Simplex LP algorithm in the Solve tool of Excel, the composition of the new cheaper and simplest MBSM medium, has been obtained (Table 2).

Compound (for 1 L)	D'Anjou and Daugulis [12]	MBSM (This work)
C_3H_8O (glycerol)	50 g	50 g
$(NH_4)2SO_4$	20 g	-
KH_2PO_4	12 g	10 g
$MgSO_4{\cdot}7H_2O$	4.7 g	3.2 g
$CaCl_2{\cdot}2H_2O$	0.36 g	0.35 g
Trace Solution	-	4.3 mL
Vitamins Solution	2.5 mL	2.5 mL
Trace Elements		Trace Soln. (for 1 L)
$FeSO_4{\cdot}7H_2O$	-	65 g
$FeCl_3{\cdot}6H_2O$	44.5 μM	-
$CuSO_4{\cdot}5H_2O$	-	6 g
$CaSO_4{\cdot}5H_2O$	0.2 μM	-
$ZnSO_4{\cdot}7H_2O$	17.5 μM	20 g
$MnSO_4{\cdot}H_2O$	4.5 μM	3 g
KI	1.25 μM	0.42 g
H_3BO_3	0.75 μM	0.1 g
$Na_2MoO_4{\cdot}\ 2H_2O$	2 μM	1 g
H_2SO_4 98% (v/v)	-	10 mL
Vitamins Soln. (for 1 L)		
Calcium panthotenate	0.8 g	
myo-inositol	8.0 g	
Thiamine dichloryde	0.8 g	
Pyridoxine hydrochloride	0.8 g	
Nicotinic acid	0.2 g	
D(+)- biotine	0.8 g	
K_2HPO_4	4.0 g	

Table 2: Comparative composition of the D'Anjou and Daugulis [12] culture medium, and proposed MBSM.

The MBSM has been successfully employed in the expression of several recombinant proteins in *P. pastoris* at laboratory and pilot-scale, using both, inducible AOX1, as well as, the constitutive pGAP expression promoters. A summary of these practical applications of MBSM medium is shown in Table 3.

The results of growth of *P. pastoris* and expression of several heterologous proteins by using the MBSM proposed here (Table 3) are similar or higher to those reported by other authors including the popular BSM culture media [7].

Recombinant Protein	Promoter	Expression level / Biomass concentration	Reference
Mini-proinsulin, MPI	AOX1	0.3 $g{\cdot}L^{-1}$ / 100 g $DCW{\cdot}L^{-1}$ at 160 h	[14,15]
Levansucrase (LsdA)	GAPDH	0.2 $g{\cdot}L^{-1}$ (4000 $EAU{\cdot}L^{-1}$) / 90 g $DCW{\cdot}L^{-1}$ at 39 h	[16,17]
Exolevanase (LsdB)	AOX1	21.1 $EAU{\cdot}L^{-1}$ / 115 g $DCW{\cdot}L^{-1}$ at 96 h	[18,19]
	GAPDH	26.6 $EAU{\cdot}L^{-1}$ / 60 g $DCW{\cdot}L^{-1}$ at 39 h	

Table 3: Some proteins expressed under inducible and constitutive promoters in *P. pastoris* by using MBSM.

Discussion

By far, the most common medium for high cell density fermentation of methylotrophic yeast *Pichia pastoris* is the basalt salt medium BSM proposed by Invitrogen Co. However, as reported by other researches [5,9], some problems during its preparation and sterilization arise due to component imbalances.

The BSM is commonly used at the initial studies of expression of heterologous proteins in *P. pastors*, for further high scale fermentation it should be adjusted and improved, according to the requirements of the production process.

The proposed MBSM offers some advantages over its BSM counterpart because not only solves the imbalance problems of some atom-elements of the culture media previously reported [5,9], but also it is less expensive (accounting near $2 per liter, depending, of course, on supplier) and it is easy to prepare (it is not necessary a defined order to add the components nor adjust pH before to finish the addition of components of the culture medium) compared to BSM and other existing culture media used for *P. pastoris*, at large-scale protein production purposes. Besides, MBSM as shown in Table 3, can be useful for both *P. pastoris* developed promoters and scales, lab research studies and also suitable for large-scale production of recombinant proteins in biopharmaceutical, food, nanoscience, and chemical industries.

Additionally, due to its simplest chemical composition (the proposed culture medium required in summary 27% less mass of chemical components than the previously simplest culture medium), the further pollution load of the fermentation wastes on ecosystems could be reduced by using the MBSM at large-scale production of heterologous proteins in *Pichia pastoris*.

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