MODELING THE MICROBIAL GROWTH OF TWO *Escherichia coli* STRAINS IN A MULTI-SUBSTRATE ENVIRONMENT

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Abstract - The microbial growth in multi-substrate environments may be viewed as an optimal resources allocation problem. The optimization aims at maximizing some biological objective like the biomass growth. The models developed using this hypothesis are called "cybernetic" and they represent the complex cell structure as an optimizing function that regulates the intracellular enzymatic machinery. In this work, a cybernetic model was developed to represent the growth of two *E. coli* strains (JM 109 and BL 21 –DE3–) on a medium containing glucose and glycerol as carbon and energy sources. The model was able to accurately simulate the biomass growth, the substrates consumption and the growth-rate profiles.
Keywords: Cybernetic model; Optimal control; *Escherichia coli*; Multi-substrates; Glycerol.

INTRODUCTION

Modeling the kinetics of a fermentation process in complex media remains a challenge for biochemical engineers and biotechnologists. Although empirical models such as the Monod equation describe the growth kinetics on a single substrate, they are usually not useful for a multi-substrate environment. Different growth phenomena such as sequential utilization of substrates, simultaneous substrates consumption and co-metabolism of substrates may be observed in these environments (Bajpai-Dikshit *et al.*, 2003). There have been numerous attempts to model these phenomena with an unified representation. A successful approach to these phenomena was the cybernetic modeling concept developed by Ramkrishna and co-workers (Kompala *et al.*, 1984; Kompala *et al.*, 1986; Varner and Ramkrishna, 1999; Song and Ramkrishna, 2010).

In biochemical systems, many reactions are catalyzed by enzymes, which may be inhibited or activated by regulatory effects on the genes that code for these enzyme activities. As a result, the changes at the regulatory level may impact the dynamics of the biochemical pathways. Cybernetic models expand the traditional ordinary differential equations (ODE) models by incorporating control laws that manipulate the enzyme synthesis and/or activity in order to drive the pathways toward a stated biological goal. The framework is based on the postulate that biological systems operate according to an optimal resources allocation objective. This objective is developed as a result of evolutionary pressures imposed on the organism. Various criteria have been proposed as the optimality criterion for the allocation of critical resources: maximum biomass productivity (Kompala *et al.*, 1984), maximization of the final biomass concentration (Dhurjati *et al.*, 1985) and matching-law allocation (Kompala *et al.*, 1986). The cybernetic modeling concept hypothesizes that the

cellular objectives of the wild-type and the genetically altered systems are identical (Varner and Ramkrishna, 1998), supporting the use of the same objective function for predicting changes in dynamics due to regulatory effects.

Escherichia coli (*E. coli*) is perhaps the most studied bacterium. It can adapt itself to very different growth conditions. High density cultures of several strains of *E. coli* are currently used to produce recombinant proteins due to its high volumetric productivity; see e.g. Shiloach J. and Fass R. (2005) and Huang *et al*. (2012). This, in practice, has transformed *E. coli* into a "factory" of recombinant proteins and many pharmaceutical products are produced in this way.

E. coli is capable of consuming either glucose or glycerol as carbon and energy (C/E) sources. Also it may consume either organic nitrogen (N) from sources such as peptones or inorganic (N)-salts. It is, therefore, extremely versatile. Although glucose is the classical (C/E)-source, glycerol is an attractive alternative because it has become an inexpensive and abundant (C/E)-source due to its generation as an inevitable by-product of biodiesel (Yazdani and Gonzalez, 2007; Gonzalez *et al*., 2008) and its over-production has become an issue of concern (Albarelli *et al*., 2011). The replacement of glucose by glycerol as (C/E)-source also allows obtaining higher biomass densities, probably because of a higher solubility and a slower catabolism, which avoids acetate production (Kortz *et al*., 1995). This makes glycerol a cheap option as the main substrate for numerous industrial cultures. So, it is desirable to adjust the *E. coli* growth conditions to promote glycerol consumption rather than glucose consumption. Nevertheless, glucose may be useful as a growth initiator. Consequently, in this work a cybernetic model has been used to describe the dynamics of glucose and glycerol consumption by two *E. coli* strains (JM 109 and BL 21 – DE3-) in batch and batch + substrate-injection cultures.

MATERIALS AND METHODS

Microorganisms and Medium

E. coli BL 21 (DE3) and JM 109 strains were grown on MEP medium adjusted according to guidelines proposed by Giordano *et al*. (2010): acid hydrolysate of casein -AHC- (30 g/L); yeast extract (27.5 g/L); NaCl (1 g/L); glycerol (1 g/L) and glucose (1 g/L). Sterilization was done at 121 °C for 15 min. AHC and yeast extract were acquired from Britania, Argentina. NaCl, glycerol and glucose were

acquired from Cicarelli Laboratorios, Argentina. Although the concentrations of AHC, yeast extract and NaCl are relatively high, the increase in the yield with respect to produced biomass overcomes this disadvantage (Giordano *et al*., 2010).

Inoculum Preparations and Culture Conditions

A loopful of each strain (stored in MEP slant agar at 4 °C) was inoculated into a 250 ml Erlenmeyer flask containing 30 ml of sterilized MEP medium and grown overnight on a rotary shaker at 200 RPM and 30 °C. Aliquots of these cultures were pipetted into 250 ml Erlenmeyer flasks containing 30 ml of the same medium to obtain an initial optical density at 600 nm (OD600) of 0.2. The cultures were incubated at 200 rpm at 30 °C. Several samples were withdrawn from each culture.

Samples Analysis

OD600 was measured and used as an estimate of the cell mass (1 OD = 0.20 g dry weight/L). Aliquots were centrifuged (10.000 × g, 5 minutes) and supernatants were stored at -20 °C for further analysis.

Analytical

Glucose and glycerol concentrations were determined in thawed samples. The enzymatic colorimetric assays from Glicemia Enzimática AA kit and TG Color GPO/PAP AA kit were employed for glucose and glycerol quantification, respectively. Both were purchased from Wiener Lab, Argentina. Determination accuracy is 90.7 mg/dL ± 2.20 mg/dL (C.V. = 1.39%) for glucose concentration and 1.14 g/L ± 0.021 g/L (C.V. = 1.82%) for the glycerol concentration.

Computational Tools

ODE and differential-algebraic equations (DAE) were solved by using the MATLAB ODE 45 solver (Mathworks, Natick, MA, USA) and the optimal control problems were solved by the *GPOPS* 4.1 toolbox (Rao *et al*, 2011) running on MATLAB 7.6.

MODEL DEVELOPMENT

The microbial growth is represented here in a way similar to the cybernetic model developed by Kompala *et al*. (1984):

$$X + Y_{X/S_i} S_i \xrightarrow{\ e_i\ } \left(1 + Y_{X/S_i}\right) X \qquad (1)$$
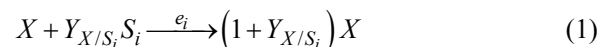
In the assimilation of the ith substrate by the biomass X, it is assumed that there is one lumped key-enzyme which plays the bottleneck role and hence must be synthesized before the substrate is utilized for growth. The synthesis of the ith key enzyme in the presence of the ith substrate can be represented as follows:

$$X \xrightarrow{S_i} X + e_i \qquad (2)$$

The symbols X, S_i and e_i were used to represent the pseudo-reaction "stoichiometry", but subsequently X will represent the biomass concentration, S_i will represent the concentration of the ith substrate and e_i will represent the normalized intracellular level of the enzyme necessary to consume the ith substrate.

The actual specific growth μ will arise from the sequential/simultaneous growth on all substrates. According to the modified Monod's expression (Kompala *et al.*, 1984), μ will be:

$$\mu = \sum_i \mu_i^{max} e_i \frac{S_i}{k_i + S_i} \qquad (3)$$

where i is the substrate indicator, μ_i^{max} is the maximum specific growth rate on the substrate i and k_i is the Monod substrate saturation constant. The ODE for the substrate consumption and the specific enzyme formation rate in a batch environment are given by:

$$\frac{dS_i}{dt} = -\frac{1}{Y_{X/Si}} \mu_i^{max} e_i \frac{S_i}{k_i + S_i} X \qquad (4)$$

$$\frac{de_i}{dt} = a_i u_i - b_i e_i \qquad (5)$$

Some variations in the shape of the above equation were proposed (Kompala *et al.*, 1984 and 1986; Dhurjati *et al.*, 1985; Doshi *et al.*, 1997), but the fundamental structure of the ODE system remains unaltered. To model the *E. coli*-strain growth on a medium with glucose and glycerol, we selected the matching-law allocation model (Kompala *et al.*, 1986) and the whole model-system may be written as:

$$\frac{dX}{dt} = \sum_i \mu_i^{max} \frac{S_i}{k_i + S_i} e_i v_i X \qquad (6)$$

$$\frac{dS_i}{dt} = -\frac{1}{Y_{X/Si}} \mu_i^{max} \frac{S_i}{k_i + S_i} e_i v_i X \qquad (7)$$

$$\frac{de_i}{dt} = \alpha_i \frac{S_i}{k'_i + S_i} u_i - \beta_i e_i \qquad (8)$$

The cybernetic variables u_i and v_i are computed from the following algebraic equations:

$$u_i = \frac{\mu_i^{max} \dfrac{S_i}{k_i + S_i} e_i}{\sum_j \left(\mu_j^{max} \dfrac{S_j}{k_j + S_j} e_j \right)} \qquad (9)$$

$$v_i = \frac{\mu_i^{max} \dfrac{S_i}{k_i + S_i} e_i}{\max_j \left(\mu_j^{max} \dfrac{S_j}{k_j + S_j} e_j \right)} \qquad (10)$$

The parameters α_i and β_i stand respectively for the specific activation rate and degradation rate of the key enzyme e_i. The right-hand-side of Eq. (8) neglects a term of intracellular dilution of the enzyme-level due to the biomass growth that is presented in the model by Kompala *et al.* (1986). Nevertheless, this dilution effect can be lumped into the β_i parameter and the DAE will not lose predictive capability at all. The maximum specific growth rates μ_i^{max} and the saturation constants k_i are usually estimated by fitting data from the exponential phases corresponding to the respective substrates. Also it is usually assumed that $k_i = k'_i$.

RESULTS AND DISCUSSION

Experimental results for *E. coli* strains growing in a medium with glucose and glycerol are summarized in Table A.1 of the appendix. In both cultures we observed three growth stages: a fast growth stage on glucose followed by a slower growth stage on glycerol and a very slow growth phase utilizing the peptides from AHC as (C/E)-source. Hence, the whole culture seems to be triauxic. The model-parameters obtained by minimizing the least square errors between the experimental data and the model predictions are reported in Table 1. To avoid an over-parametrization of the model, the values of parameters α_i and β_i were prefixed. So, growth parameters in the late culture phase, without glucose and glycerol,

were forced to be identical in both least-squares fittings. Figures 1 and 2 compare the experimental data with the model predictions.

Table 1: Model parameters for the least squares fitting of experimental data from both batch runs.

i C/E-source	μ_i^{max}	k_i	$Y_{X/Si}$	α_i^*	β_i^*
E. coli JM 109 strain					
Glucose	0.53	0.01	0.23	1	1
Glycerol	0.46	0.35	0.38	1	1
AHC	0.04	0.01	0.21	1	1
E. coli BL 21 (DE3) strain					
Glucose	0.76	0.01	0.26	1	1
Glycerol	0.54	0.65	0.25	1	1
AHC	0.04	0.01	0.23	1	1

*Prefixed to avoid overparametrization
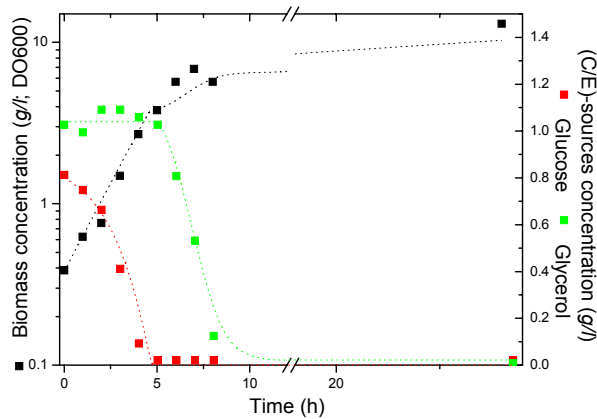
Figure 1: Model predictions (dotted lines) vs. experimental data (square symbols) for the *E. coli* JM 109 strain.
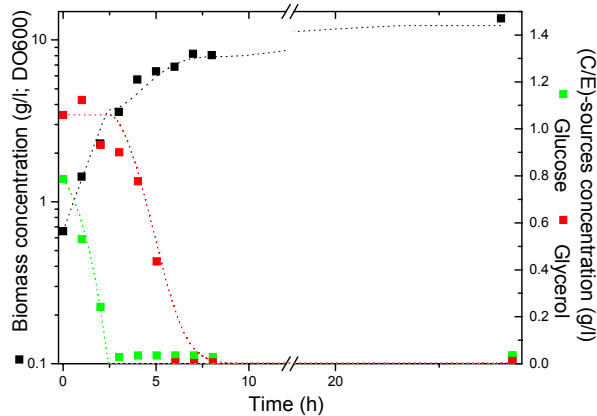
Figure 2: Model predictions (dotted lines) vs. experimental data (square symbols) for the *E. coli* BL 21 (DE3) strain.

Since experimental data for the growth on peptides from AHC is scarce, the parameters for this stage present a significant degree of uncertainty.

Nevertheless, this should not affect the design of a medium with glucose and glycerol as the main (C/E)-sources because the growth on peptides from AHC is much slower than the growth on the former substrates. As our aim is to promote the utilization of glycerol while using glucose just as a growth-initiator, to design a batch + injection culture, we solved an optimal control problem. In that problem, the glycerol concentration may be changed by glycerol injections up to the final culture-time of $t_f = 10$ h. The growth stage on peptides from AHC was neglected in the optimization and the problem can be stated as follows:

$$\text{Maximize } X(t_f)V(t_f) \tag{11}$$

Subject to:

$$\frac{dV}{dt} = F \tag{12}$$

$$\frac{dX}{dt} = \sum_i \mu_i^{max} \frac{S_i}{k_i + S_i} e_i v_i X - F\frac{X}{V} \tag{13}$$

$$\frac{dS_i}{dt} = -\frac{1}{Y_{X/Si}}\mu_i^{max} \frac{S_i}{k_i + S_i} e_i v_i X + \frac{S_i^F}{V}F - F\frac{S_i}{V} \tag{14}$$

$$\frac{de_i}{dt} = \alpha_i \frac{S_i}{k'_i + S_i} u_i - \beta_i e_i \tag{15}$$

$$\frac{dQ_2}{dt} = FS_2^F \tag{16}$$

Here F is the manipulated variable and the states are the culture volume (V), the biomass concentration (X), the glucose concentration (S_1), the glycerol concentration (S_2), the normalized intracellular level of enzymes (e_1 and e_2) and the quantity Q_2 of injected glycerol. Since the injection is glucose-free $S_1^F = 0$ g/L and $Q_1 = 0$ g. The concentration of glycerol in the feed is $S_2^F = 5$ g/L. The objective (Eq. 11) is the maximization, at the final run-time $t_f = 10$ h, of the biomass quantity subject to the constraints (Eqs. (12)-(16)) of a fed-batch bioreactor. The cybernetic variables u_i and v_i are computed from Eqs. (9) and (10). In addition, the constraint (17) imposes an upper bound Q_2^{max} to the quantity of injected glycerol:

$$Q_2 \leq Q_2^{max} \tag{17}$$

The optimal control problem is defined by the objective function (Eq. 11), the differential equations (Eqs. (12)-(16)), the algebraic Equations (9)-(10) and the state constraint (Eq. 17). It was coded on GPOPS 4.1 (Rao *et al*., 2011) and solved in a 2.0 GHz 16 GRAM PC.

We solved several instances of this optimal control problem by varying the initial states and the kinetic parameter values. In all cases, the glycerol addition consisted of an injection just before the start of the glycerol consumption. The explanation of this feed-shape is fairly simple. Since μ_1^{max} for glucose is larger than for other substrates and the glucose-saturation constant k_1 is also very low, the glucose is preferred in the sequential substrate utilization. Because $\mu_1^{max} > \mu_2^{max}$ and $k_1 < k_2$, the optimal profile will always yield a sequential substrates utilization.

So, an early injection of the "slow" substrate will not increase the actual growth rate at all and it might even brake the activation of the enzyme necessary for the degradation of the "fast" substrate (i.e., if μ_1^{max} is slightly larger than μ_2^{max}). Hence, the injection will start just after the glucose depletion and will occur as early as possible. The goal is to minimize the effect of the Monod saturation-term for glycerol, aiming at:

$$\mu_2^{max} \frac{S_2}{k_2 + S_2} e_2 v_2 \approx \mu_2^{max} \qquad (18)$$

An optimal substrate-addition profile is illustrated in Figure 3. The kinetic parameters and initial states of such a profile are reported in Table 2. The kinetic and yield parameters are those that fit the batch culture of the *E. coli* BL 21 (DE3) strain.

After this numerical experiment, we performed a batch + injection culture to grow the *E. coli* BL 21 (DE3) strain under the environmental conditions computed from the rule derived from the above optimal control problem. That rule consists of injecting glycerol just after glucose exhaustion.

Table 2: Model parameters and initial states for the optimal control problem solved to design the glycerol injection profile.

Time horizon					$t_f = 10$ h	
Initial states						
$X(0)$	$S_1(0)$	$S_2(0)$	$e_1(0)$	$e_2(0)$	$V(0)$	$Q(0)$
0.2	2.6	1.2	0.9	0.1	1.0	0
Yield and kinetic parameters						
i C-source	μ_i^{max}	k_i	$Y_{X/Si}$	α_i	β_i	
Glucose	0.76	0.01	0.26	1	1	
Glycerol	0.54	0.65	0.25	1	1	
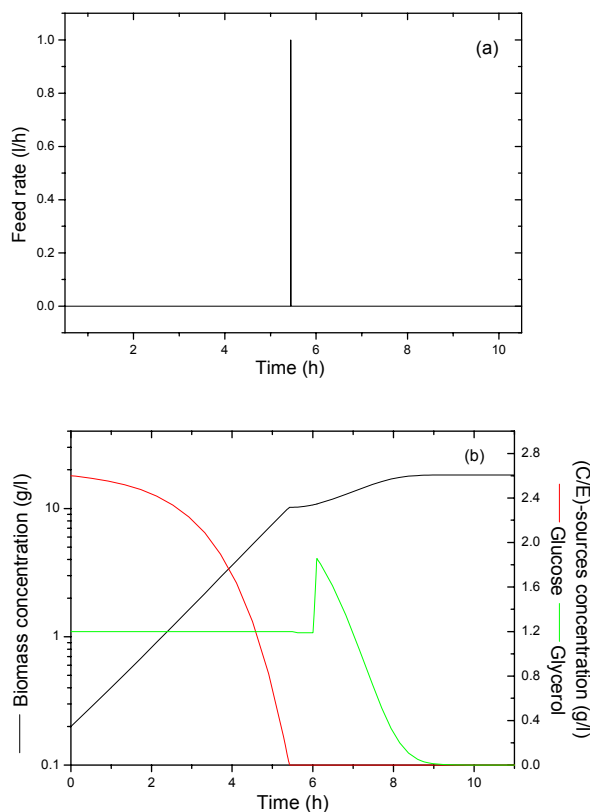
Figure 3: An optimal glycerol injection profile (a) and the evolution of the biomass and substrates concentrations (b).

The experimental results for such a run are summarized in Table A2 of the Appendix. The least squares fit of the model to the data yielded the parameters reported in Table 3 and Figure 4 compares the model predictions with the experimental data.

The experimental results indicate a growth rate on glucose similar to the growth rate observed in the batch culture, but also indicate a simultaneous consumption of glucose and glycerol after $t = 3$ h and up to the time of the glycerol-injection. The simultaneous substrates-consumption was not caused

Table 3: Model parameters for the least squares fitting of the experimental results presented in Table A2.

i C-source	μ_i^{max}	k_i	$Y_{X/Si}$	α_i^*	β_i^*
***E. coli* BL 21 (DE3) strain**					
Glucose	0.75	0.12	0.66	1	1
Glycerol	0.14	0.60	0.25	1	1
Injection time					6 h
Injected volume					0.1 ml/ml culture
S_2^F					5 g/l

*Prefixed to avoid overparametrization
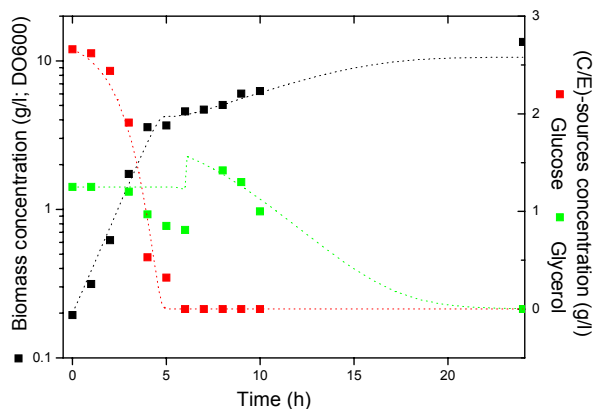
Figure 4: Model predictions (dotted lines) vs. experimental (square symbols) data for the batch + injection culture with the *E. coli* BL 21 (DE3) strain.

by the injection because it occurred before. Before this unexpected behavior and after the glycerol injection, the agreement is quite good. Therefore, the remaining experimental data seems to validate the model also in a batch + injection mode.

CONCLUSIONS

A cybernetic model based on the maximization of the instantaneous specific growth rate hypothesis is able to represent a variety of experimental growth data involving diauxic and triauxic growth. The model utilizes a lumped representation of the cell populations, and the microbial growth on various (C/E)-sources can be treated as a multivariable constrained optimization problem, as proposed by Kompala *et al* (1986). This model was chosen to predict the growth of two *E. coli* strains on a medium with glucose and glycerol. It can accurately represent the growth dynamics of both strains in batch runs.

To design a batch + injection culture, an optimal control problem was subsequently solved. As a consequence, an injection rule was derived and an additional experiment was performed. In that case, the agreement between the model predictions and the experimental data was not as good as for batch cultures, although this cannot be attributed to the glycerol injection. So, the empirical rule remains valid.

ACKNOWLEDGEMENTS

NOMENCLATURE

a_i	Specific activation rate for the ith enzyme	
b_i	Specific inactivation rate for the ith enzyme	
e_i	Normalized intracellular level of the ith enzyme	
$k_i; k_i'$	Monod substrate saturation constant	g/L
F	Feed rate	L/h
Q_i	Mass of the ith substrate injected to the culture	
S_i	Concentration of the ith substrate	g/L
S_i^F	Concentration of the ith substrate in the feed	g/L
u_i	Cybernetic variable for controlling the synthesis of the ith enzyme	
v_i	Cybernetic variable for regulating the activity of the ith enzyme	
V	Culture volume	L
X	Biomass concentration	g/L
$Y_{X/Si}$	Yield of biomass on the ith substrate	g/g

Greek Symbols

α_i	Specific activation rate of the ith enzyme	
β_i	Specific inactivation rate of the ith enzyme e_i	
μ	Actual specific growth rate	h^{-1}
μ_i^{max}	Maximum growth rate on the ith substrate	h^{-1}

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APPENDIX

Table A.1: Experimental growth data for batch culture of *E. coli* strains JM 109 and BL 21 (DE3) on a mixed medium of glucose and glycerol.

Time (h)	Biomass (g/l)	Glucose (g/l)	Glycerol (g/l)
E. coli JM 109 strain			
0	0.39	0.81	1.03
1	0.62	0.75	0.99
2	0.76	0.66	1.09
3	1.49	0.41	1.09
4	2.70	0.09	1.06
5	3.80	0.02	1.03
6	5.70	0.02	0.81
7	6.85	0.02	0.53
8	5.70	0.02	0.12
24	13.06	0.02	0.01
E. coli BL 21 (DE3) strain			
0	0.66	0.79	1.06
1	1.43	0.53	1.12
2	2.29	0.24	0.93
3	3.60	0.03	0.90
4	5.70	0.03	0.78
5	6.39	0.03	0.44
6	6.85	0.03	0.01
7	8.22	0.03	0.01
8	8.05	0.03	0.01
24	13.60	0.03	0.01

Table A.2: Experimental growth data for batch + injection culture of *E. coli* BL 21 (DE3) strain on a mixed medium of glucose and glycerol.

Time (h)	Biomass (g/l)	Glucose (g/l)	Glycerol (g/l)
E. coli BL 21 (DE3)			
0	0.19	2.66	1.25
1	0.31	2.62	1.25
2	0.62	2.44	NA
3	1.73	1.91	1.20
4	3.57	0.53	0.97
5	3.67	0.32	0.85
6	4.56	0.00	0.81
7	4.69	0.00	NA
8	5.04	0.00	1.42
9	6.02	0.00	1.30
10	6.74	0.00	1.00
24	13.4	0.00	0.00