Short communication

Screening of *Bacillus coagulans* strains in lignin supplemented minimal medium with high throughput turbidity measurements

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**ABSTRACT**

The aim of this study was to extend the options for screening and characterization of microorganism through kinetic growth parameters. In order to obtain data, automated turbidimetric measurements were accomplished to observe the response of strains of *Bacillus coagulans*. For the characterization, it was decided to examine the influence of varying concentrations of lignin with respect to bacterial growth. Different mathematical models are used for comparison: logistic, Gompertz, Baranyi and Richards and Stannard. The growth response was characterized by parameters like maximum growth rate, maximum population, and the lag time. In this short analysis we present a mathematical approach towards a comparison of different microorganisms. Furthermore, it can be demonstrated that lignin in low concentrations can have a positive influence on the growth of *B. coagulans*.

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1. Introduction

Lactic acid is widely used in the food processing, cosmetics, pharmaceutical and chemical industry. Increasing prices of fossil fuels lead to increasing interests in lactic acid as a component for the production of biodegradable polymer poly(lactic acid) [24]. There have been various attempts to produce lactic acid efficiently in bio-refineries from inexpensive feedstock such as lignocellulosic raw materials, e.g. wheat straw or hard- and soft-wood [4,16]. Lignocellulose as part of the secondary cell wall of rooted plants is one of the most abundant natural materials. It contains cellulose, hemicellulose and lignin [8]. Cellulose and hemicellulose represent polymeric carbohydrates formed from glucose, xylose, and arabinose amongst other sugars [22,16]. Therefore, lignocellulose is also the most abundant carbon storage. After a hydrolysis process, lignocellulose can serve as a potential substrate in a biotechnological microbial fermentation for the formation of valuable products such as lactic acid [11,12,23]. Unfortunately, a non-specific chemical hydrolysis treatment, e.g. high temperature acid or alkali pre-treatment, leads to solvation of lignin and to the formation of complex sugars and inhibitory compounds such as furfural [18–21]. One way of reducing the inhibitory effect of lignin for process optimization is the reduction of the lignin concentration in the fermentation medium [7]. Another option is the use of microorganisms inhibited by lignin only to a low level, or those that can transform lignin into another compound like vanillate [10,13].

In order to improve the screening of microorganisms usable in complex and inhibitory media like lignocellulosic hydrolysates, it is necessary to characterize their growth behaviour. High throughput methods for kinetic analysis of the lignin inhibition are useful to achieve information about the lag time (\(\lambda\)) and the maximum growth rate (\(m\)). These screening methods provide the chance to investigate the growth behaviour under different working conditions. In order to get access to lignin stable natural microorganisms (MOs) it is crucial to screen interesting bacteria in an inhibitory environment.

In this study, rapidly automated optical density (OD) measuring was applied to determine the growth response of *Bacillus coagulans* strains. The used strains are thermophilic bacteria, frequently utilized in our processes at technical scale. In studies taking place under non-sterile conditions, *B. coagulans* was shown to be the most predominant species [1]. Furthermore, the *B. coagulans* strains are known for their inhibitor tolerance [17] and their capability of utilizing pentose sugars from the hemicellulose fraction of lignocellulose [24]. These facts provide for the possibility to ferment difficult media under semi-sterile condition. Prior the fermentation in technical and pilot scale, kinetic data is
needed to gain a basic understanding of the characteristics of the MOs for later fermentation processes and their design. Growth models are used to obtain the basic growth parameters, such as specific growth rate and duration of lag phase, in order to classify and differentiate microorganisms in respect to their behaviour towards diverse lignin concentrations. Numerous models were developed for the representation of growth curves. Widely known models are the logistic [28], Gompertz [14,25,26,28], Champbell-Richards and Stannard [28], and the model offered by József Baranyi [3]. These models have been established to fit the equations to the sigmoidal shape of a typical growth curve.

2. Materials and methods

2.1. Microbes and media

_Bacillus coagulans_ strains were isolated from different environmental areas. They were stored in cryogenic vials (VWR, 822074ZA) at −70 °C and reactivated on MRS broth (Merck, 1.10661.0500) at 52 °C for 24h). After reactivation the microorganisms were cultivated on slant culture tubes with MRS agar (Merck, 1.10660.0500) and stored at 4 °C for further use in inocula. The used strains were officially microbiologically characterised through the Leibniz Institute’s German Collection of Microorganisms and Cell Cultures (DSMZ). Strain-1 (DSM No. 2314) was isolated from potato washing water, strain-2 (DSM ID 14-301) was isolated from chicken feed, and strain-3 (DSM ID: 14-298) was isolated from rotten foliage.

2.2. Inoculum, culture conditions

Inocula were cultivated on 60 ml MRS (Merck, 1.10661.0500) broth in shaking flasks (52 °C, 100 rpm, 15 h). These were transferred into 5 ml tubes for centrifugation (5000 rpm, 15 min, 4 °C). Centrifuged bacteria were resuspended in minimal medium for the lignin test (60 g/l D-(+)-glucose, 5 g/l yeast extract, 0.025 mol/l sodium-acetate-buffer at pH 6.0). A set of five different lignin concentrations (Sigma, 471003), (0.0, 0.2, 0.4, 0.6, and 0.8 g/l) was applied.

2.3. Optical density measurement

A Bioscreen C from Oy Growth Curves Ab Ltd., was used for the optical density experiments. Measurements were taken with a wide band filter (420–580 nm).

2.3.1. Calibration curve

For the calibration curve, Bioscreen C microarray honeycomb plates were prepared as follows: all wells, except the wells of the 10th row, were filled with 250 μl of the minimal medium. The wells of the 10th row were filled with 500 μl inocula. 250 μl were removed from these wells and transferred into the next upper row. Appropriate serial 2-fold dilutions were made up to the 2nd row, mixed by repeated syringing. The 1st row was used as the medium blank. The filled plates were placed in the Bioscreen C followed by a short measurement. The OD from the non-inoculated wells was subtracted from the growth data to minimize the effect of the signal draft. The concentrations of the colony forming units (cfu) were determined by an Abbe counting chamber. On demand, additional 10-fold dilutions were prepared for counting.

2.3.2. Optical density measuring of bacterial growth

The honeycomb plates were prepared as described in Section 2.3.1. The incubation temperature was set to 52 °C with interval shaking, changing to medium and slow intensity for 30 s prior and after OD reading. Measurements were taken every 5 min for 32 h. At least two replicate wells were used in one experiment for the determination of maximum growth rate for each lignin concentration.

2.4. Models and parameter estimation

Presupposing that the cell concentration increases in sigmoidal shape, different models were used to simulate the bacterial growth curve [3,15,27]. Although these models had the same key parameters, they differed in shape and number of parameters. A logistic, the Gompertz and the Richards and Stannard model were used in a modified and reparameterised shape as it had been offered by Zwietenring et al. [28]. The Baranyi equation [2] was used as a two (μm, λ) and three (μm, λ, ν) parametrical model [5,9].

- natural logarithm of the quotient of the cell concentration (N) and minimal cell concentration (Nmin)

\[ y = \ln \left( \frac{N}{N_{\text{min}}} \right) \quad \text{with} \quad |y| = 1 \]

- natural logarithm of the quotient of the initial cell concentration (N0) and minimal cell concentration (Nmin)

\[ y_0 = \ln \left( \frac{N_0}{N_{\text{min}}} \right) \quad \text{with} \quad |y_0| = 1 \]

- natural logarithm of the quotient of the asymptotic cell concentration (Nmax) and the initial cell concentration (Nmin)

\[ y_{\text{max}} = \ln \left( \frac{N_{\text{max}}}{N_{\text{min}}} \right) \quad \text{with} \quad |y_{\text{max}}| = 1 \]

- difference of logarithmic cell concentrations

\[ \Delta y = y_{\text{max}} - y_0 \quad \text{with} \quad |\Delta y| = 1 \]

![Fig. 1. Third order calibration curve between cell concentration and optical density of the different B. coagulans strains.](image)
The models were implemented in MATLAB®. A simulated annealing algorithm was used to obtain the statistical global solution with standard properties. The Euclidean distance was used as optimization criteria.

3. Results and discussion

3.1. Calibration curve

The relationship between a certain concentration of colony forming units per millilitre medium (cfu/ml) and the resulting measurable OD can be used to construct a calibration curve. The calibration curve is used to equate the concentration of the cells at a given time of the experiment. The calibration curve is shown in Fig. 1 and described with a regression of a third order binomial equation in Eq. (1). Using the calibration curve, the values of the measured OD can be directly converted into the microbial concentration.

\[
\text{cfu/ml} = 4.3555 \times 10^{12} \times \text{OD}^3 + 6.9824 \times 10^{-2} \times \text{OD}^2 \\
\times 4.8828 \times 10^{-4} \times \text{OD} 
\]

\( R^2 = 0.92601 \)

3.2. Data analysis

The general shape of the bacterial growth curve is known and characterized by the lag phase, the exponential growth phase, and the stationary phase. In this study, the simulated annealing algorithm is used and the models are matched to the growth data already published by [15,13]. This step is important to check the discrepancy of the optimization results between the key parameters \( \mu_m \) and \( \lambda \) compared to the mentioned published results and to each other. Table 1 constitutes a summary about the results of this test. Based on the simulation results it is decided to use the average value of \( \mu_m \) and \( \lambda \) of the different models.

3.3. Experimental data

Growth curves for different initial inocula of strain-1 are shown in Fig. 2. Each individual curve shows the same growth characteristics. Independent from the inoculum dilutions they reached nearly the same maximum cell concentration. Obviously, the lag time and the maximum growth rate differ from dilutions (DL) in a

![Fig. 2. Incubation time plot of multiple initial inocula dilutions of strain-1. Each curve represents the growth of a single initial inoculum dilution. A: Growth without lignin; B: growth with 0.4 g/l supplement of lignin.](#)
dependent way. This effect was also described by Baranyi et al. [4,6] with a mathematical background. Furthermore, the data lead to the assumption that there exists a minimum lag time with an optimal cell concentration. That means that the lag time cannot be reduced by a further increase of the cell concentration (Fig. 2A).

A slight decrease of the cell density within the first hours of the experiments can be noticed (Fig. 2B). This is possible due to a lysis process during the adaptation period of the MOs to the new environment. Also a reduction of the cell density can be detected at the end of the final cell concentration. If the inocula concentration is about ln($N_0$) = 25 ln(cfu/ml), no increase of OD is detected (1:2 DL in Fig. 2A and 1:2, 1:4, and 1:8 DL in Fig. 2B). The other DL leads to the same final concentration of strain-1 of about ln($N_{max}$) = 28.913 ± 0.049 ln(cfu/ml) without lignin and ln($N_{max}$) = 26.103 ± 0.396 ln(cfu/ml) with 0.4 g/l of lignin. Consequently, a threshold exists for the highest achievable concentration depending on the lignin concentration.

3.4. Parameter estimation

The parameters of growth characteristics, $\mu_m$ and $\lambda$, are estimated and the average values are taken. In Fig. 3 an exemplary survey of the parameters for the different inocula dilutions of strain-2 and strain-3 is shown. All parameters show direct dependence on the initial inoculum. With increasing inoculum concentration $\mu_m$, $\lambda$, and the differences in the maximum of the achieved cell concentration, $\Delta y$ shows a decreasing behaviour, as can be expected. In Fig. 3A, a general lower $\mu_m$ of strain-2 compared to strain-3 (Fig. 3B) is visible. Likewise, strain-2 does not vary much in the value of $\mu_m$, and $\lambda$ about 0.6 g/l of lignin. Also $\Delta y$ (Fig. 3C) is very low and does not indicate any growth. The high cell density only leads to little growth of the microorganisms and might be the reason for the growth impulse at the point of higher inocula. Unexpectedly, strain-2 shows a slightly higher value of $\mu_m$ and also a decrease in lag time concerning 0.2 and 0.4 g/l of lignin. The growth is detected only with higher inoculum concentrations.

Strain-3 shows growth on all indicated lignin concentrations, with a steady decrease of $\mu_m$ (Fig. 3D). The parameter $\lambda$ of strain-3 (Fig. 3E) also shows a little variance, just like $\Delta y$ (Fig. 3F). As a result of the aspect, it gets clear that the estimated parameter cannot be used directly to distinguish between the capabilities of the MOs to withstand higher concentrations of lignin.

A dimensionless parameter $\alpha = \exp(-\mu_m\lambda)$ is described by by Baranyi [4,6] to quantify the physiological state of an initial population. However, this dimensionless parameter $\alpha$ does not give more information than $\mu_m$ and $\lambda$ itself due to the equation to a mathematical product. A redefinition of $\alpha$ as quotient provides more information (Eq. (2)).

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Fig. 3. Means of estimated parameters as result of different lignin supplementations dependent on different inoculum concentrations of strain-2 and strain-3. Strain-2: A: maximum growth rates $\mu_m$; B: lag time $\lambda$; C: difference $\Delta y$; strain-3: D: maximum growth rates $\mu_m$; E: lag time $\lambda$; F: difference $\Delta y$. 

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\( \beta = \frac{\mu_m}{\lambda} \) with \( \left| \beta \right| = 1/\text{h}^2 \) (2)

\( \beta \) can be interpreted as the efficiency rate of an increased maximum growth rate in respect to the limitation of a higher lag time. A higher \( \beta \) indicates a higher efficiency of the MOs to endure lignin in fermentation. Fig. 3 shows the dependence of growth parameters on the inoculum concentration. Due to this behaviour it seems of interest to interpret \( \beta \) in context of the cell concentration as shown in Eq. (3). This procedure allows looking at the behaviour of \( \beta \) with increasing lignin concentration.

\[
\gamma = \frac{\mu_m}{\lambda \Delta y y_0} \text{ with } \left| \gamma \right| = 1/\text{h}^2
\] (3)

In Fig. 4, there are shown \( \beta \) and \( \gamma \) of the three strains. In Fig. 4A it becomes apparent that strain-1 and strain-2 show a raising curve of \( \beta \) until 0.2 g/l of lignin. After that small increase the decrease of the parameter occurs. Strain-1 and strain-2 in Fig. 4B display the increase of the efficiency parameter \( \gamma \) until 0.2 g/l of lignin as well, but strain-1 shows the higher value. Strain-3 displays a steady falling in \( \beta \) and \( \gamma \), thus, descent is not as rapid as the descent of strain-1 and strain-2. Continuing, the efficiency of strain-1 and strain-2 is lower than the efficiency of strain-3 at an inhibitor concentration that is higher than 0.6 g/l. Furthermore, in Fig. 4B there is an indication of an interception point of \( \gamma \) for the three strains about 0.5 g/l of lignin.

For the further comparison of the MOs, the interception point with the x-axis of a linear interpolation of the descending part of \( \beta \) or \( \gamma \) is used (Fig. 4A and B). A higher interception point of the x-axis represents a more effective tolerance of lignin of the MOs. The interception indicates the highest possible lignin concentration in which growth is possible under the current unregulated bioscreen conditions.

Regarding to the dependence of the estimated parameters of the cell concentration, Fig. 4C and D shows the values of \( \beta \) and \( \gamma \) of strain-3 in respect to the inoculum concentrations. While \( \beta \) shows a decreasing behaviour, \( \gamma \) is nearly constant during the increase of the inoculum concentration. This circumstance indicates that \( \gamma \) might be more independent from the inoculum concentration and seems to be a more efficient parameter than \( \beta \). For example, it can be usable as characterization parameter prior to a process scale-up. Based on the interpolation results it is assumable that the MO with higher interception is a better MO for a scale-up process. Strain-1 and strain-2 have nearly the same effectiveness to the phenolic compound. Theoretically \( \beta \) indicates a growth of strain-1 and strain-2 to lignin tolerance below 1 g/l (Eqs. (4) and (5)). \( \gamma \) indicates a growth of strain-1 until 0.9 g/l (Eq. (7)) and a possible growth of strain-2 up to 1.3 g/l (Eq. (8)). The interpolation of strain-3 shows the strongest effectivity in \( \beta \) and \( \gamma \). The interception indicates a tolerable lignin concentration of strain-3 at a maximum of 1.7 g/l in \( \beta \) (Eq. (6)) and of 1.84 g/l in \( \gamma \) (Eq. (9)) in current bioscreen cultivation conditions.

Strain – 1: \( \beta = -2.473x + 1.983 \quad x (\beta = 0) = 0.80 \text{ g/l} \) (4)

Strain – 2: \( \beta = -2.215x + 2.070 \quad x (\beta = 0) = 0.97 \text{ g/l} \) (5)
Strain – 3: \[ \beta = -0.830x + 1.418 \quad x(\beta = 0) = 1.70 \text{ g/l} \] (6)

Strain – 1: \[ \gamma = -0.0117x + 0.0105 \quad x(\gamma = 0) = 0.90 \text{ g/l} \] (7)

Strain – 2: \[ \gamma = -0.0072x + 0.0089 \quad x(\gamma = 0) = 1.13 \text{ g/l} \] (8)

Strain – 3: \[ \gamma = -0.0036x + 0.0067 \quad x(\gamma = 0) = 1.84 \text{ g/l} \] (9)

In summary, the strains show a different behaviour in the reduced controllable environment of the well plates of the Bioscreen C screening. It is also noticeable that the lignin concentration has different effects on diverse bacterial strains. The raising of the lignin concentration in concentration should be done with strain-3. A procedure as described above facilitates the identification of more interesting bacteria e.g. for the benefit of use in other complex inhibitory environments. With the help of a mathematical approach it was possible to characterize lactic acid producing bacteria for a lignocellulose biorefinery. It was shown that a strain, isolated from a natural lignin containing environment, had the best growth results and it could show as well that low concentrations of lignin can stabilize the growth of two other strains. Our described mathematical approach can help to identify the amount of a substance, e.g. lignin, which might stabilize bacterial growth.

4. Conclusion

The procedure as described above facilitates the identification of more interesting bacteria e.g. for the benefit of use in other complex inhibitory environments. With the help of a mathematical approach it was possible to characterize lactic acid producing bacteria for a lignocellulose biorefinery. It was shown that a strain, isolated from a natural lignin containing environment, had the best growth results and it could show as well that low concentrations of lignin can stabilize the growth of two other strains. Our described mathematical approach can help to identify the amount of a substance, e.g. lignin, which might stabilize bacterial growth.

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