Probiotics Production: An Interesting Example for the Undergraduate Analytical Chemistry Laboratory

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Abstract: An analytical chemistry laboratory experiment for undergraduates that combines traditional analytical chemistry techniques with the interdisciplinary field of biotechnology is presented. It involves a process in which the combination of a yeast (*Schizosacaromyces pombe*) and a bacteria (*Acetobacter xylimun*), usually named *Kombucha*, in a very simple culture of commercial black tea and glucose produces metabolites that are sampled and quantified by students. Students determine the biomass and quantify the production of several organic acids, and the consumption of glucose. This is an interdisciplinary project where students apply many tools of the general analytical process and combine results obtained by all the participating students to learn the characteristics of a biotechnological process. Overall, the system discussed is easy to implement because the selected micro-organisms are not pathogenic, are safe and easy to manipulate, and are resistant to contamination.

Introduction

The word probiosis, taken from the Greek (*pro*, for; *biosis*, life), has opposite meaning to the term antibiosis. Instead of killing microbial cells, as does an antibiotic, a probiotic product is designed to promote the proliferation of beneficial species. It is a substance, generally produced by micro-organisms, that performs a useful action on other living beings, including humans and superior animals [1]. An important branch of biotechnology is presently devoted to the development of processes for probiotics production on different scales [2], and, on this basis and due to the multidisciplinary nature embracing this type of process, an experiment for basic analytical chemistry undergraduate students was developed. Another valuable aspect of the experiment is the interaction among the different laboratory groups needed to obtain the final results. At our university it is used by licentiate students in biotechnology and biochemistry.

Students study the production of several organic acids, based on the glucose fermentative degradation produced by a combination of a yeast (*Schizosacaromyces pombe*) and a bacteria (*Acetobacter xylimun*), usually named *Kombucha*, which constitutes a generous probiotic producer [3]. The exercise is based on research of the kinetics of product formation and reagent consumption in a very simple batch system formed by an infusion of tea, glucose, and the *Kombucha* bacteria.

The experiment is suitable for undergraduate students for the following reasons: (1) The microorganisms used are not pathogenic; they are safe and easy to manipulate and resistant to contamination. (2) Many steps of the general analytical process are applied, namely, definition of the problem, measurement of needed analytical variables, and calculation of the results. (3) It offers the possibility of direct sampling and analysis during the course of the process (eight days). These analyses are relatively rapid and can be completed in the course of a laboratory period (approximately four hours). Indeed, one acid–base titration and a visible photometry are carried out [4–11]. This is an interdisciplinary experiment in which students obtain a product biotechnologically and quantify some of the substances produced and consumed in the process. This allows students to gain insight into the characteristics of the process. Our main objective is to teach students real-world chemical applications in analytical chemistry laboratories without the need to go too deeply into the kinetics of the fermentation process.

Experimental Procedure

Batch Reactor Setup. An infusion of commercial black tea and glucose was used as the medium and a 3-L cylindrical vessel of thermal glass was used as the reactor (Figure 1). After sterilisation at 105 ºC, the reactor was charged with 2 L of medium to which 20.0 g of *Kombucha* wet mass was added. The system was stored at ambient temperature.

Details regarding the purchase of reagents, the experimental set-up, and the experimental procedure are located in the supporting material (520067hgs1.pdf).

Sampling. During the 8-day process, students obtained samples every 24 hours at the beginning of each laboratory class. The students were divided into five groups (one group for each day of the week). Each group took 20-mL culture samples and analysed them for glucose consumption and organic acid formation. The instructors performed the sampling and analysis during the following three days with the total process lasting for eight days.

Biomass. Before and after the experiment, a portion of the microorganisms (ca. 2 g) was cut and used to obtain humidity; the remainder was drained and weighed by the instructors. The weight of dry biomass yield was calculated by difference. For the drying procedure the microorganisms were put into an empty capsule of known weight and weighed, placed in a 110 ºC oven, and, then weighed to constant weight. From the weight difference between the

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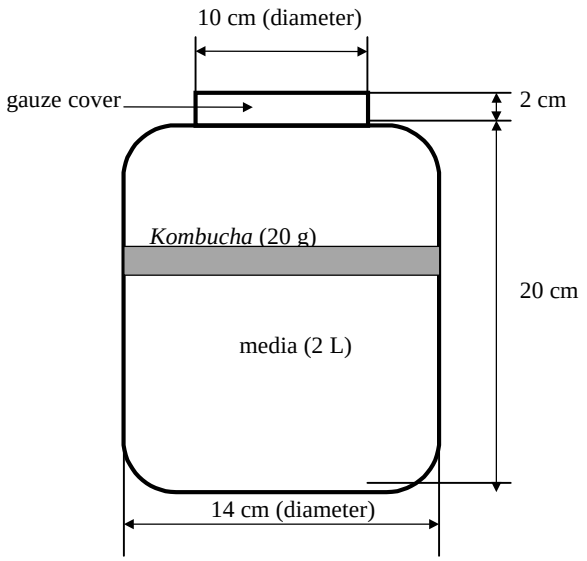

Figure 1:. Schematic of the batch reactor set-up used in the glucose fermentation process in an infusion where *Kombucha* was added.

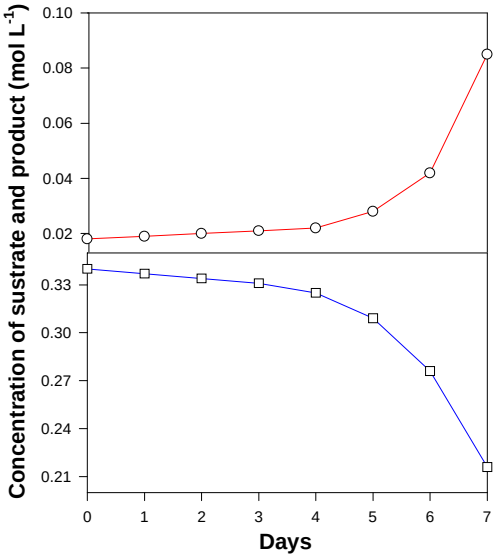

Figure 2. Concentration of substrate and product as a function of the fermentation time: acids production in mol L^{-1} (red) and glucose consumption in mol L^{-1} (blue). In all cases the time is measured from the beginning to the end of the process.

empty capsule and the capsule plus dried microorganisms, the weight of the microorganisms was determined.

Acid determination. Solutions of standardised NaOH (0.02 mol L^{-1}) and thymolphthalein (1 g L^{-1}) in ethanol were used. Four replicates of 2.00 mL were titrated to blue.

Glucose determination. The Trinder enzymatic method, using a diagnostic kit, was used to determine glucose [9–11]. Four replicates of 20 µL of diluted sample were analysed. The samples were diluted in order to lie in the dynamic range of the calibration curve.

Analysis of Results. To obtain the average and standard deviation, four replicate measurements were made. The students calculated the confidence limits ($\pm t_{(n-1)} \times s/n^{1/2}$, where n = 4, and $t_{(n-1)}$ is the appropriate coefficient having (n-1) degrees of freedom at the 95% confidence level).

At the end of the experiment, students presented a report indicating the measured variable values within the confidence limits corresponding to that day of work. During class, a week after the culture development, each group of students presented the results that they obtained. Using all the data collected throughout the process, students constructed a graph showing the process evolution and from it drew conclusions about the culture and its development.

Results and Discussion

For every mole of glucose metabolised by the microorganisms, one mole is converted to CO_2 and organic acids and the other mole is assimilated to biomass. The relation can be expressed in terms of the following equation:

$$C_6H_{12}O_6 + a\ (\text{nitrogen}) + b\ O_2 \rightarrow$$

$$c\ CO_2 + d\ C_2H_4O_2 + e\ (\text{biomass}) \qquad (1)$$

where the nitrogen source comes from the tea and the coefficient values depend on the fermentative conditions.

The system is influenced by two kinds of variables: operatives like temperature (kept constant throughout this experiment) and variables such as the initial concentration of glucose, tea, and biomass (they are defined by the initial conditions). Therefore, the consumption of glucose and the production of organic acids as function of time can be used to reveal the characteristics of the process. In order to simplify the experiment, we did not analyse for nitrogen or CO_2.

To completely understand the entire process, 24-hour fermentation values are needed. These were measured and integrated with the results obtained by the student groups working in the laboratories. As can be seen in Figure 2, the changes in substrate and the product (acids) as a function of time show a nonlinear variation. For this reason, it is very important to know the concentrations during fermentation to obtain data on the kinetics of fermentation.

Kinetic studies are necessary to gain an understanding of any fermentation. As the word implies, fermentation kinetics in concerned with the rates of cell synthesis and fermentation product formation and the effect of environment on these rates [12]. Measuring these kinetic parameters may allow us to know the characteristics of the fermentative process. They are indispensable elements in the design of biochemical reactors [12]. One of the most important kinetic parameters is the rate of reaction, which is simply defined as the rate of change of concentration for reactants or products [7, 13, 14]. For example, the rate, R, of the above reaction may be expressed as:

$$R = -\frac{d[\mathrm{C}]}{dt} \qquad (2)$$

where the term in square brackets is the concentration of glucose. The negative sign indicates that during the reaction the concentration of glucose decreases. The rate equation for a chemical reaction contains a proportionality constant, k, known as the rate constant (or specific rate):

$$R = k[C]^x \qquad (3)$$

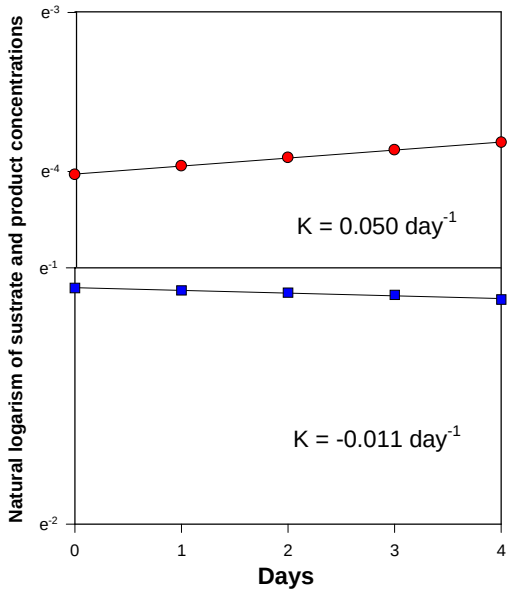

Figure 3. Plot of the natural logarithm of the concentration of acid (red) and glucose (blue) as a function of the fermentation time for the first four fermentation days. The black line is the least-squares best-fit line.

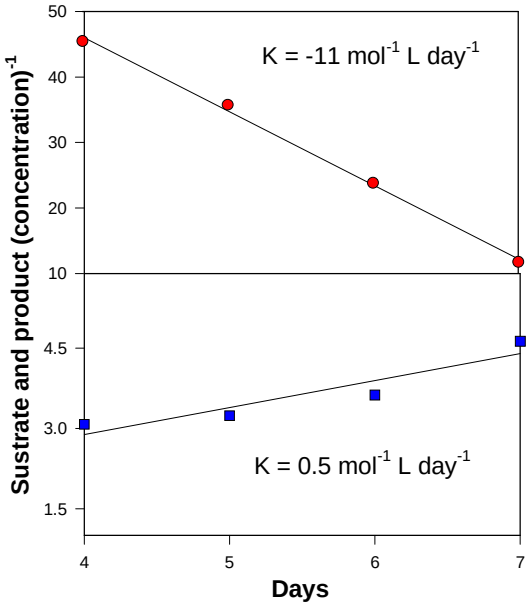

Figure 4. Plot of 1/[C] versus *t* for the concentration of acid (red) and glucose (blue) for fermentation days 4–7. The black line is the least-squares best-fit line.

where the experimentally determined exponent, *x*, is called the order of the reaction for the component, C. For the great majority of reactions this number is between 0 and 3. The dimensions of *k* are (concentration)$^{1-\text{order}}$ (time, *t*)$^{-1}$.

In the present work, the nonlinearity, seen in Figure 2, indicates the presence of first or greater reaction order. First-order reactions are those in which the integration of the differential equation ($x = 1$) gives:

$$\ln [C] = \ln [C_o] - kt \quad (4)$$

and a plot of ln [C] against *t* will be linear with slope of *k* and intercept of ln [C_o].

Second-order reactions are those having the differential rate equation:

$$-\frac{d[C]}{dt} = k[C]^2 \quad (5)$$

which integrates to:

$$\frac{1}{[C]} = \frac{1}{[C_O]} + kt \quad (6)$$

where a plot of 1/[C] versus *t* will be linear with slope of *k*.

Using these equations, the following methodology is applied in order to understand the kinetic process. First, make plots of ln [C] or 1/[C] versus time. Second, calculate the slope of the curves generated. Figure 3 shows the plot of ln [C] versus *t* for acid production and glucose consumption for the first four days. The curve was found to be linear over this range for this system. The best fit to the curves for the production of organic acids and the disappearance of glucose is first-order. The specific rates were determined graphically and were found to be $k_{acids} = 0.050 \pm 0.002$ day^{-1} ($r = 0.999$) and $k_{glucose} = -0.011 \pm 0.002$ day^{-1} ($r = 0.999$). On the other hand, Figure 4 shows the plot of 1/[C] versus *t* for acids and glucose for days 4–7. In this case the best fit for the kinetics of the production of organic acids and consumption of glucose is second-order. The specific rates were determined graphically and were found to be $k_{acids} = -11 \pm 1$ mol^{-1} L day^{-1} ($r = 0.999$) and $k_{glucose} = -0.5 \pm 0.3$ mol^{-1} L day^{-1} ($r = 0.934$). Surprisingly, the reaction kinetics changes on day four. This is probably due to the high complexity of the matrix sample studied.

At the end of the process, the student results showed that the consumption of one mole of glucose yielded 14.5 g of biomass and (0.54 ± 0.02) moles of organic acids. Equation 1 gave the stoichiometric coefficient corresponding to organic acids for this experimental data as 0.54 ± 0.02.

Conclusion

We have presented a laboratory experiment that presents real-world chemical problems to analytical chemistry students. This experiment is not only interdisciplinary, combining biotechnology with analytical chemistry, but also cooperative; students must share data between groups to put together a picture of the entire process. This experiment is easily implemented because simple instruments and nontoxic reagents are used throughout. It is both safe and easy to use in a basic analytical chemistry laboratory course.

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