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USE OF RESIDUAL YEAST FOR THE DEVELOPMENT OF ALTERNATIVE CULTIVATION MEDIUM FOR *Bacillus* spp.

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ABSTRACT

The development of alternative culture media from waste has shown promising results in the cultivation of microorganisms. The residual yeast contains nutrients that favor the growth of *Bacillus* spp. Through a mixture design, the optimized culture medium, using 20% of this residue for the cultivation of *Bacillus subtilis, Bacillus megaterium*, and *Bacillus thuringiensis israelensis*, reached 9.25, 8.04, and 8.99 log UFC.mL^{-1}, respectively, in 30 hours of microbial growth. The results demonstrate that it is possible to use residual yeast from the brewing industry, contributing to waste recovery, the circular economy, and the reduction of production costs in both the brewing industry and the multiplication of microorganisms.

Keywords: Waste valorization. *Bacillus subtilis*. *Bacillus megaterium. Bacillus thuringienses israelensis*. Mixture Simplex-Centroid design.

1 INTRODUCTION

Beer is a drink of great popularity worldwide. The high consumption of this product results in a significant generation of waste, as 30% of the raw material, such as malt and yeast, invested in its production becomes a by-product [1]. In general, yeast residue (YR) from the brewing industry has the yeast *Saccharomyces cerevisiae* as the main component [2]. YR has significant concentrations of nitrogen and other nutrients, such as carbon, phosphorus, sulfur, and calcium, which can act positively on the growth of microorganisms. These nutrients are used in the cellular structure as a source of energy for their growth and thus represent an opportunity to use such residue as raw material in alternative cultivation media (ACM) [3]. ACM is an option to promote the use and valorization of waste and by-products from the most diverse categories, resulting in several economic, social, and environmental benefits [4,5].

The valorization of wastes in the biotechnological context is a current and growing topic, as it encompasses aspects of the circular economy and bioeconomy. This can have positive impacts on several Sustainable Development Goals (SDGs) of the United Nations (UN), especially the SDG 12 (Sustainable consumption and production). Several *Bacillus* strains are capable of using ACM for their growth. After the fermentation process, these microorganisms have applicability in several areas, especially for environmental, pharmaceutical, and agricultural uses [6]. Therefore, understanding the notoriety of the application of this genus, it is important to explore its fermentative process, especially in relation to substrates, as a way to optimize cell growth and the generation of metabolites, as well as to reduce the cost of bioprocesses [7]. Given this context, the present work aimed to evaluate the applicability of using YR as a component in the *Bacillus* spp. cultivation medium.

2 MATERIAL & METHODS

The YR used was provided by a brewery located in the municipality of Passo Fundo, Rio Grande do Sul. Before use, the YR was autoclaved at 121 °C for 30 minutes to sterilize and inactivate the residual yeast. Soy extract (SE) was used as a second organic source of nitrogen. Urea and glucose were used as an inorganic nitrogen and carbon source, respectively. The microorganisms tested were: *Bacillus subtilis, Bacillus megaterium*, and *Bacillus thuringiensis israelensis*, obtained from the strain collection of the company Beifiur LTDA, located in the municipality of Garibaldi, Rio Grande do Sul. The YR and SE were characterized, resulting in 18.10±0.62% and 0.95±0.14% humidity; 0.81±0.02% and 5.74±<0.01% ash; 36.46±0.06% and 34.19±1.39% proteins; 2.02±0.45% and 1.43±0.18% fiber; 9.01±2.19% and 3.43±0.11% lipids; 33.60% and 54.26% carbohydrates, respectively. Urea and glucose were not characterized as they were of analytical standard.

The optimization of the ACM was carried out through an experimental design in the Statistica software (version 28.0.1), using the Mixture Simplex-Centroid design type, in which the sources varied from 1-5 g.L^{-1} of YR, 1-5 g.L^{-1} of SE, 0-4 g.L^{-1} of urea, and 4-8 g.L^{-1} of glucose. 15 formulations were tested, in a volume of 50 mL each, in duplicate. Based on the results obtained from the experimental design, the statistical analysis proposed an optimal cultivation medium formulation, and concentrations of 5, 10, and 20 g.L^{-1} of ACM were tested, in a volume of 50 mL each, in duplicate. The scale-up (100X) of the optimal ACM occurred with the use of a benchtop bioreactor with a capacity of 5 L (Tecnal, Tec-bio-flex model). The experimental conditions were: 30ºC temperature, 250 rpm, 1 vlm O_2. pH and dissolved oxygen analyses were carried out, with results provided by the reactor module itself. The determination of viable cells, from aliquots obtained every 3 h, was carried out using the colony-forming unit (CFU) counting method using serial decimal dilutions and subsequent Spreader Plate plating in standard PCA medium [8].

3 RESULTS & DISCUSSION

Table 1 presents the concentrations of the components used in each formulation and the microbial growth results obtained for each microorganism tested.

Table 1 – Mixture design and microbial growth results in 24 hours of process.

	Growing Medium Ingredients (g.L^{-1})					log 10 UFC.mL^{-1} in 24h		
	Glucose	Yeast residue	Soy extract	Urea	C:N	*Bacillus subtilis*	*Bacillus megaterium*	*Bacillus thuringiensis israelenses*
1	8	1	1	0	26.7	5.14±0.06 ^{f}	4.07±0.03 ^{d}	7.78±0.29 ^{bc}
2	4	5	1	0	11.6	5.85±0.78 ^{def}	3.68±0.03 ^{d}	4.90±0.51 ^{e}
3	4	1	5	0	12.7	8.27±0.23 ^{a}	8.24±0.09 ^{a}	8.37±0.14 ^{abc}
4	4	1	1	4	41.2	7.15±0.02 ^{abcd}	7.31±0.28 ^{a}	6.18±0.00 ^{d}
5	6	3	1	0	16.7	5.68±0.47 ^{ef}	3.98±0.31 ^{d}	5.54±<0.01 ^{de}
6	6	1	3	0	17.4	5.79±0.01 ^{def}	8.07±0.20 ^{a}	8.08±0.20 ^{abc}
7	6	1	1	2	39.4	7.87±0.18 ^{ab}	7.68±0.08 ^{a}	8.51±0.00 ^{ab}
8	4	3	3	0	12.2	6.72±0.17 ^{bcde}	5.74±0.10 ^{bc}	8.19±0.01 ^{abc}
9	4	3	1	2	34.8	7.96±0.08 ^{ab}	7.25±<0.01 ^{ab}	7.45±0.14 ^{c}
10	4	1	3	2	35.0	8.11±0.36 ^{a}	7.72±0.20 ^{a}	8.49±0.07 ^{abc}
11	5.33	2.33	2.33	0	15.1	6.29±0.05 ^{cdef}	4.35±0.49 ^{cd}	8.19±0.16 ^{abc}
12	5.33	2.33	1	1.33	34.0	7.72±0.60 ^{ab}	7.05±1.24 ^{ab}	8.09±0.36 ^{abc}
13	5.33	1	2.33	1.33	34.2	7.99±<0.01 ^{ab}	7.65±0.15 ^{a}	8.47±0.16 ^{abc}
14	4	2.33	2.33	1.33	30.9	7.72±0.32 ^{ab}	7.48±0.21 ^{a}	8.96±0.40 ^{a}
15	5	2	2	1	30.5	7.45±0.53 ^{abc}	7.43±0.28 ^{a}	8.78±0.53 ^{ab}

Equal letters indicate that there was no statistical difference ($p < 0.05$) by the Tukey test.

Based on the results obtained in the mixture design, the optimal medium (desirability) proposed by the software is composed of 4.51 g.L^{-1} glucose, 1.32 g.L^{-1} YR, 1.89 g.L^{-1} SE, and 2.27 g.L^{-1} urea. A C:N ratio of 40.0 obtained a better response than the other formulations pre-established by the design, indicating a preference for a greater amount of C (less complex source) in relation to the amount of N. The results indicated a greater tendency for the bacteria to prefer an inorganic source of nitrogen (urea), rather than organic (YR and SE), at the beginning of cultivation. This is considering that nitrogen from an inorganic source is more available, enabling rapid consumption [9]. Over time, there is a predisposition to increase the percentage of organic N source, given the fact that its consumption is slower.

For microbial kinetics in a 5 L benchtop bioreactor, the pre-inoculum time was set at 24 h, with OD_{615} 0.9 – 1.0 (approximately 8.386 CFU.mL^{-1}). The optimized culture medium was composed of 10 g.L^{-1} of glucose, 4 g.L^{-1} of YR, 4 g.L^{-1} of SE, and 2 g.L^{-1} of urea, totaling a medium with a concentration of 20 g.L^{-1}. This concentration showed the greatest growth between 5, 10, and 20 g.L^{-1}. Microbial growth, pH, and dissolved oxygen were monitored for 30 hours and generated the graphs shown in Figure 1.

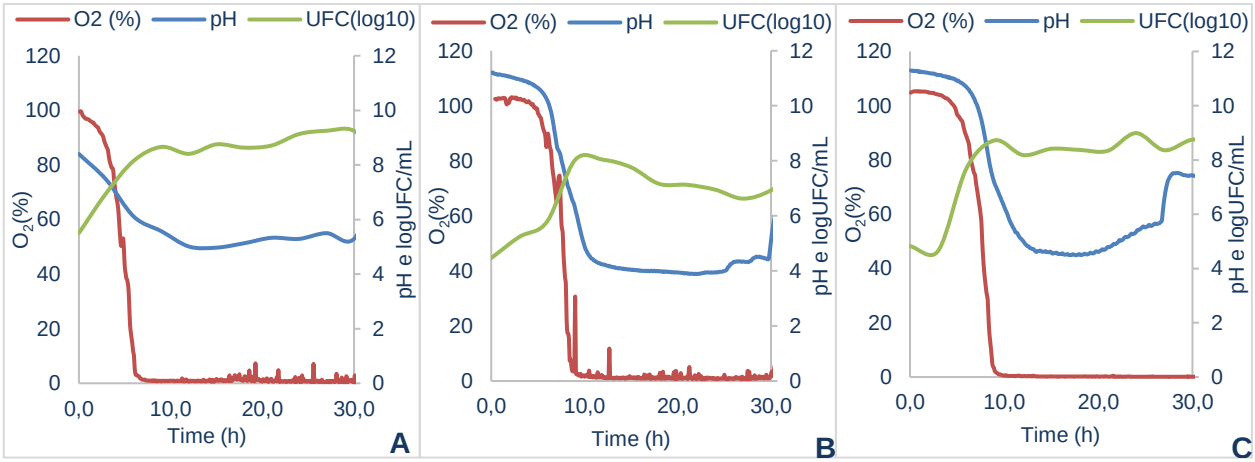

Figure 1. Behavior of the fermentative process of *Bacillus subtilis* (A). *Bacillus megaterium* (B) and *Bacillus thuringienses israelensis* (C) in alternative culture medium for 30 hours.

Figure 1 shows a reduction of O_2 as the microbial growth curve rises, around 5 hours after the start of the process. This may be associated with microorganisms being aerobic and using oxygen as a kind of substrate for their multiplication, especially in the exponential phase [10]. When high cell growth occurs in the fermentation medium, oxygen can become a limiting reactant, since O_2 consumption is greater than the supply of the component to the medium. Thus, cell growth is influenced by low oxygen availability and can be reduced. Likewise, a tendency to decrease pH is observed. This is due to *Bacillus* being capable of producing organic

and inorganic acids, acidifying the medium for the release of some minerals [11], indicating microbial multiplication in the proposed alternative means.

As for microbial growth expressed in log10 CFU, the three *Bacillus* showed good growth results in the proposed alternative medium. All exponential phases occurred before completing 10 hours of the fermentation process. *Bacillus subtilis* reached 9.25 log CFU.mL^{-1} in 27 hours of the process and pH 5.5; while *Bacillus megaterium* reached 8.04 log CFU.mL^{-1} in 12 hours of the process and pH 4.23; and *Bacillus thuringiensis israelensis* achieved 8.99 log CFU.mL^{-1} in 24 hours and pH 5.34.

pH is considered an important indicator of microbial metabolism, as it is influenced by the consumption of nutrients and consequent secretion of molecules during the fermentation process [7]. The medium containing *Bacillus subtilis* presented an initial pH close to neutral, which may have influenced greater cell growth compared to other microorganisms. The fermentation medium containing Bacillus megaterium suffered a greater pH reduction than the other fermentative processes, which may have led to lower bacterial cell synthesis and, therefore, lower cell growth [7]. A small increase in pH values at the end of fermentation can be justified by the consumption of acetates and organic acids present in the medium when nitrogen and carbon sources may have been limited [12].

4 CONCLUSION

In this way, yeast residue from the brewing industry can become a viable component for the development of alternative cultivation media for *Bacillus* spp. growth. This promotes the reduction of costs in waste treatment and in bioprocesses, which promotes aspects of waste valorization, circular economy, and responsible production.

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