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Cells of Yeasts Adhered in Corn Grains and the Storage Perspective for Use as Probiotic

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ABSTRACT

Yeasts were applied to corn grains, containing 16 or 20% moisture, at concentrations of 1 and 2%. The cellular viability was assayed at 0, 15, 30, 90 and 110 days. The cellular viability did not differ statistically among the treatments up to 30 days of storage, with the median viability of 89.10%. The average viability found at 90 days (72.20%) was lower than in the first three storage periods. After 110 days, the average viability was 61.14%. In conclusion, since yeast cells were viable up to 110 days in storage on corn grains, these can be used as a vehicle for the application of yeast as a probiotic.

Key words: Cellular viability, *Saccharomyces cerevisiae*, storage, corn grain

INTRODUCTION

The search for biologically active products to feed living organisms has increased lately. This is due to the constant and increasing concern with reducing the use of certain products, such as antibiotics, while simultaneously, maintaining the development of substitute products. As an alternative, there are derivative compounds, which are products that act in a harmonious manner to obtain a total balance, which are found in probiotic products (Fuller, 1989). These are given to individual as viable cells, and when they reach the gastrointestinal tract, they cause a rebalance in the microbiota, thus improving the utilization of the digested food by providing essential metabolites, complexing toxins or their inducing agents (Baptista, 2001).

Live yeast (*Saccharomyces cerevisiae)* is recommended for animal feed at 1 g for every 100 kg live weight. At this amount, yeast has a metabolic or probiotic effect instead of a protein source (Machado, 1997). It is essential that yeast be live and/or metabolically active to be functional. Yeast stimulates growth and bacterial activity at degrading fiber and increasing the production of acetic, propionic and lactic acids, which causes improved food degradation, thus providing more energy and protein for consumption (Dawson, 1993). The use of live yeast cells as a mycotoxin detoxifying agents was reported by Stantey et al. (1993) and Baptista et al. (2002). Moreover, other studies showed that the severity of organ damaging caused by diets with aflatoxins was lowered in the presence of *S. cerevisiae* due to its ability in decreasing animal stress, providing vitamins and a non-identified growth factor as well as being a source of enzymes and proteins (Phillips and Von Tungeln, 1984; Crumplen et al., 1989; Krause et al., 1989).

Theoretically, the availability of yeast after fermentation is in excess of 2 kg of dry yeast per

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hectoliter of ethanol produced (Desmonts, 1966). Therefore, during the 1997 harvest, in which the Brazilian ethanol production was about 15 billion liters, approximately 300,000 tons of yeast could have been used for human and animal nutrition. Hence, the utilization of this microorganism as a probiotic additive could be an excellent alternative for Brazil due to the large production of yeast, which is a subproduct of etanol and beer production (Butolo, 1997).

The application of yeast as a probiotic in animal feed has been widely reported in the literature (Leedle, 2000). However, its application and the techniques developed for its use recommend adding it in the ration only before feeding. Due to this requirement, the application may not be accepted because the diet needs to be formulated just before it is consumed. Wienken (1990) utilized anhydrobiotic organisms, such as yeast, which had the capacity to stay alive longer due to its dehydrated state, thus, making them resistant to cold, heat and drought.

Therefore, basic research was conducted with the objective to study yeast cell viability when applied to corn grain at different concentrations and stored under different moisture contents. Additionally, the use of these coated corn grains as a vehicle to apply yeast as a probiotic was also investigated.

MATERIAL AND METHODS

This study utilized a live dehydrated ***Saccharomyces cerevisiae*** strain Y904 obtained from 'Burns Philp Brasil Ind. Co.'. It was grown in sugar cane molasses and dried by extrusion and had 93% dry matter, 98% viability, (11.61%) trehalose, and (12.21%) glycogen. Trehalose content was estimated as described by Trevelyan and Harrison (1956) and the glycogen concentration was determined according to Leão et al. (1984).

Corn was used as a substrate to store the yeast. The moisture content of the corn was corrected to 16% or 20% by spraying water over the grains and protecting them with a plastic cover for 24 h to homogenize the moisture content throughout the grain mass. Afterwards, a 0.5% sucrose solution (w w^{-1}) was sprayed over the substrate to work as an adhesive agent for the yeast as well as nourishment. The humidity content was estimated by gravimeter based upon Brasil (1992).

Four treatments were conducted after preparing the substrate. Treatments 1 and 2 had substrates with 16% moisture content and live dehydrated yeast cells at concentrations of 1 and 2% (w w^{-1}) per mass of grain, respectively. Treatments 3 and 4 had substrates with 20% moisture content and live dehydrated yeast cells at concentrations of 1 and 2% (w w^{-1}) per mass of grain, respectively.

The inoculation of live dehydrated yeast was conducted by applying live cells at desired concentrations for each Treatment through a mini rotatory barrel to obtain a uniform and homogeneous distribution of the yeast cells over the corn grains. Afterwards, the yeast-coated corn grains were stored in 2.5-liter plastic container, closed and sealed with silicone rubber to avoid gas exchange with the exterior environment. Coated substrates were stored in these containers for 0, 15, 30, 90 and 110 days to analyze the yeast cell viability and mycotoxins.

Yeast cellular viability was estimated by analyzing a representative 5-g sample from each treatment. Each sample was surface washed with a 0.1% peptone water solution based upon Silva et al. (1997) to remove the cells adhered on corn grains. After, a serial dilution was conducted and the dilution that allowed observing 40 to 60 yeast cells at 400X magnification was standardized. Cell viability was determined by 0.1% methylene blue staining according to Pierce (1970). Cells were considered alive only if they produced, microscopically, a light blue color due to the synthesis of an enzyme that degraded the stain. On the contrary, dead cells did not show the active enzymatic complex, did not degrade the stain, and therefore, were microscopically stained as dark blue color.

The determination of aflatoxins was done by thin-layered chromatography according to the method described by Soares and Rodrigues-Amaya (1989) with modified quantification, which was done with bi-directional thin-layer chromatography with a detection limit for the four aflatoxins of 0.5 µg kg^{-1}.

The experimental design was a three-factor arrangement (2x2x5) completely randomized with four repetitions for each treatment. The experiment had two concentrations of yeast cells; substrate with two moisture contents, and five periods of storage, for a total of 80 samples for analysis.

Results were evaluated by analysis of variance (ANOVA) and the comparison of the means was done by the Tukey test ($p \leq 0.05$). Data produced

were analyzed through the "SANEST" software (Silveira Junior, 1989).

RESULTS AND DISCUSSION

At zero time, the data for the mean percentage of yeast cell viability (93.91%) from all four treatments did not show any significant differences (Table 1). This indicated that the distribution of yeast cells was uniform and homogenous, and after the yeast was inoculated on the corn grains, the cellular viability was similar in all treatments. Moreover, the mean values for cell viability from those treatments that had different yeast cell concentrations and from those substrates with different moisture content were not statistically different up to 30 days. This suggested that at 15 and 30 days of storage, the different atmospheric conditions did not cause changes in the environment that could affect yeast cell viability. These results corroborate with those found by Petterson and Schnürer (1995), who reported that yeast cells inoculated on wheat grain showed high viability after 14 days.

At 90 days, no significant differences were found between the mean values for the yeast cell viability at concentrations 1 and 2% on substrates with 16% moisture content. However, in substrates with 20% moisture content, the mean values for cell viability were greater in the treatments with 1% added yeast cells than with 2%. Moreover, the mean values for cell viability did not differ between the treatments that had 1% yeast and 16 and 20% moisture content. On the other hand, the treatment with 2% yeast on substrate with 16% moisture content showed a greater mean value for cell viability than the Treatment that had 2% yeast on a substrate with 20% moisture content (Table 1). A hypothesis to explain such data could be that the sucrose solution used as an agent for adherence and carbon source to inoculate yeast cells onto corn grain could be used up faster in those substrates that had higher moisture content.

Table 1 - Influence of the concentration of yeast cells inoculated and the substrate moisture content on the percentage of viable yeast cells.

	Substrate moistures content	
	16%	**20%**
Yeast cell concentration	Period 1 (zero time)	
	% viable yeast cells	
1% yeast cell	92.89 Aa	93.80 Aa
2% yeast cell	94.20 Aa	94.75 Aa
Yeast cell concentration	Period 2 (15 days)	
	% viable yeast cells	
1% yeast cell	90.63 Aa	86.59 Aa
2% yeast cell	94.57 Aa	90.60 Aa
Yeast cell concentration	Period 3 (30 days)	
	% viable yeast cells	
1% yeast cell	92.29 Aa	88.10 Aa
2% yeast cell	90.74 Aa	85.27 Aa
Yeast cell concentration	Period 4 (90 days)	
	% viable yeast cells	
1% yeast cell	72.77 Aa	73.47 Aa
2% yeast cell	80.53 Aa	62.00 Bb
Yeast cell concentration	Period 5 (110 days)	
	% viable yeast cells	
1% yeast cell	82.42 Aa	79.85 Aa
2% yeast cell	75.90 Aa	39.00 Bb

Mean values followed by distinct uppercase letters in the same column and at the same period differ statically by Tukey test at 5%.
Mean values followed by distinct lowercase letters in the same row and at the same period differ statically by Tukey test at 5%.

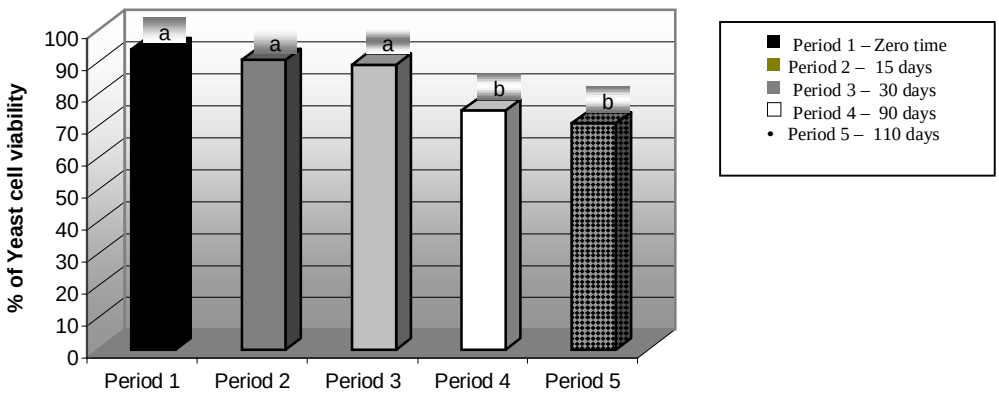
These substrates might have favored the development of competitor microorganisms, thus causing environmental changes, which was showed by a higher yeast cell mortality rate probably due to the competition for space and energy. This same fact probably did not occur in the Treatments that had 1% yeast on substrates with 16 and 20% moisture content due to the lowest number of cell per area. This hypothesis was based upon studies conducted by Crowe et al. (1998), who stated that the amount of disaccharides available for yeast, as in the case of sucrose, could allow the cells to survive for a longer period under adverse conditions. Moreover, according to Diniz-Mendez (1999), sucrose could prevent or reduce adverse effects on the viability of yeast cells.

At 110 days storage, the mean values for yeast cell viability did not differ statistically between the treatments with 1 and 2% yeast cells on substrate that had 16% moisture content. However, in the treatments with 20% moisture content, the mean values for cell viability was greater in the treatment with 1% yeast than with 2% yeast. With regard to substrate moisture content at concentration of 1% yeast, no significant difference was found between the mean values for cell viability in substrates with 16 and 20% moisture. Nevertheless, significant differences were observed between the mean values for cell viability in substrates with 16 and 20% moisture inoculated with 2% yeast. A similar hypothesis to that mentioned for the data obtained from 90 days of storage may explain these results.

Fig. 1 showed that during the three first periods of storage (0, 15 and 30 days), yeast cell viability was not affected since no significant differences were documented between the mean values found. However, a reduction in the mean values for Periods 4 and 5 (90 and 110 days) were observed when these values were compared to those found for the initial Periods, thus suggesting that the reduction in yeast cell viability was gradient and limited during the tested Periods. Despite the yeast cell mortality rate found, the mean values for cell viability were closer to 70%, which could be considered a high rate due to the conditions where yeast cells were stored and the type of substrate used, since it was not a natural habitat for yeast. Another hypothesis to explain these results could be the fact that a depletion of carbohydrates occurred during storage and that these were responsible for maintaining the integrity of cell membranes and therefore, cell viability. This explanation corroborated with the studies conducted by Leslie et al. (1994), whose findings showed a clear relationship between the amount of trehalose in viable cells of *S. cerevisiae* and their capability of surviving during a storage period.

Table 2 illustrates the results on yeast cell viability found in substrates with 16 and 20% moisture content in relationship to storage period. No significant differences were found between the mean values for yeast cell viability from treatments with substrates at 16 and 20% moisture and stored for 0, 15 and 30 days.

Figure 1 - Yeast cell viability evaluated during the experimental periods.



Different letters represent mean values that are statistically different at 5% through Tukey test.

Table 2 - Influence of the storage period and substrate moisture content on the percentage of yeast live cells.

	% viable yeast cells	
Storage Period	**Substrate with 16% moisture content**	**Substrate with 20% moisture content**
Period 1 (zero time)	93.55 a	94.28 a
Period 2 (15 days)	92.60 a	88.60 a
Period 3 (30 days)	91.51 a	86.69 a
Period 4 (90 days)	79.16 b	67.74 b
Period 5 (110 days)	76.65 b	59.89 b

Mean values followed by distinct lowercase letters in the same column differ statically by Tukey test at 5%.

These data indicate that at these moisture contents, and the substrates stored during 30 days, the differences in moisture did not cause significant changes to affect yeast cell viability. Moreover, yeast cell viability was affected as the storage period was extended, since the mean values found were lower at 90 and 110 days when compared to those obtained at the 0, 15 and 30 days at both substrate moisture contents. These results might be a consequence of alterations caused in the atmospheric conditions inside of the storage container and a reduction in the storage carbohydrates used by the yeast cells.With regard to concentration of yeast cells (1 and 2%) inoculated on the substrates, the mean values for cell viability found in all treatments did not show significant differences for 0, 15 and 30 days of storage at both cell concentrations (Table 3). This shows that at 30 days of storage the differences in yeast concentrations did not influence the values found for cell viability. On the other hand, storage for 90 and 110 days at both cell concentrations resulted in lower mean values for cell viability during the storage period, independent of the cell concentration. These results were expected since it is common that long periods of storage cause unfavorable conditions besides the fact that yeast cells could undergo modifications, thus favoring cell mortality.

Table 3 - Influence of the storage period and concentration of yeast cells inoculated on the substrate on the percentage of yeast live cells.

	% viable yeast cells	
Storage Period	**1% inoculed yeast**	**2% inoculed yeast**
Period 1 (zero time)	93.35 a	94.47 a
Period 2 (15 days)	88.61 ab	92.59 a
Period 3 (30 days)	90.20 ab	88.00 a
Period 4 (90 days)	73.12 c	71.27 b
Period 5 (110 days)	81.17 bc	58.88 c

Mean values followed by distinct lowercase letters in the same column differ statically by Tukey test at 5%.

These results showed that for 30 days the corn grain could be used to store yeasts without risking their cell viability, wich indicates this substrate could be used as a vehicle for yeasts until the moment of their utilization, when the aim is to use it in a probiotic form.

The presence of aflatoxins was not detected. However, it is important to emphasize the necessity of new studies to evaluate the presence of others mycotoxins, that could occur on the corn grain with the moisture contents used in this work or with humidity levels lower than 12.5%, to prevent the occurrence of mycotoxins.

CONCLUSIONS

1. The substrate moisture contents at the time when yeast cells were inoculated influenced the cell viability cells during storage.
2. Substrates with higher moisture content had a tendency to promote yeast cell death during prolonged storage periods.
3. The viability of yeast cells varied only slightly during the first 30 days of storage. After 90 days, there was a reduction of approximately 30% in the number of live cells.

4. Yeast cells were viable up to 110 days on stored corn grain.
5. The use of stored corn grain as a medium for yeast cells was a viable approach and raised the possibility that they could be used as a vehicle for the application of yeast as a probiotic.

RESUMO

O comportamento da viabilidade celular de leveduras é essencial quando se almeja utilizá-la como um probiótico e o veículo que assegure esta propriedade até o momento do consumo é particularmente. Sobre grãos de milho, contendo 16 ou 20% de umidade, foram aplicadas leveduras desidratadas vivas, nas concentrações de 1 e de 2%. A viabilidade celular foi avaliada nos períodos de 0, 15, 30, 90 e 110 dias de armazenamento. A viabilidade celular nas leveduras não diferiu estatisticamente entre os tratamentos aos 30 dias em armazenamento, com a viabilidade média de 89,10%. A viabilidade média encontrada aos 90 dias de 72,20% foi menor do que aquelas nos três primeiros períodos de armazenamento. Após 110 dias de armazenamento foi de 61,14%. Conclue-se que leveduras apresentam viabilidade celular até 110 dias em armazenamento sobre milho e que estes podem ser utilizados como um veículo para a aplicação da levedura como um probiótico.

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