Short Communication

The growth and recovery of an exopolysaccharide-producing *Lactobacillus rhamnosus* culture on growth media containing apple juice or molasses

Claude P. Champagne* and Nancy J. Gardner[†]

Food Research and Development Center, Agriculture and Agri-Food Canada, 3600 Casavant, St. Hyacinthe, QC, J2S 8E3, Canada

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Probiotics can be defined as “Live microorganisms which, when administered in adequate amounts, confer a health benefit on the host” (Araya et al., 2002). Of the various lactobacilli being used as probiotics, *Lactobacillus acidophilus* has been the most widely studied (Champagne et al., 2005). Less is known, however, on the production and use of *Lactobacillus rhamnosus* in foods, even though some strains, particularly GG and R0011, have been shown to be valuable probiotics (Ouwehand et al., 2003). Strain *L. rhamnosus* RW-9595M is a very high exopolysaccharide (EPS) producer (Macedo et al., 2002) which is of interest to the food industry because of beneficial effects on texture and yields (Broadbent et al., 2001; De Vuyst et al., 2001) as well as for potential EPS-linked health benefits (Ruas-Madiedo et al., 2002). Additionally, the RW-9595M strain used in this study shows potential for the enhancement of the immune system (Chabot et al., 2001).

Culture manufacturers must propagate the strains in appropriate media, recover the cells and freeze or dry them. Commercial media such as Lactobacilli MRS

are expensive to use at the industrial scale (Garcia et al., 2006). Furthermore some consumers ask for products that do not contain meat or dairy ingredients as well as have Kosher certification. In response to these demands, producers of probiotic cultures are constantly challenged to develop novel growth media, particularly fruit or vegetable-based. In this study, we examined apple juice and molasses for this purpose and if it is possible to recover this EPS-producing culture by centrifugation.

Culture and media: *Lactobacillus rhamnosus* RW-9595M was obtained from the Agriculture and Agri-Food Canada’s Food R & D Centre. Cultures were prepared as described by Champagne et al. (2007). For the screening of growth media, three commercial substrates were used: molasses (65% total solids; Gramma, St-John, NB, Canada), apple juice concentrate (40% total solids; McCain, Florenceville, NB, Canada), and Lactobacilli MRS medium (Difco; Becton Dickinson, Sparks, MD, USA). Baker’s yeast extracts (YE) were FNI-100 (Lallemand, Montréal, QC, Canada) and Difco (Becton-Dickinson), while FNI-200 (Lallemand) was a brewer’s YE. Three peptones from Organotechnie (La Courneuve, France) were also assayed: N1 tryptone, E1 wheat and A3 soy peptone.¹⁾

* Corresponding author: Dr. Claude P. Champagne, Food Research and Development Center, Agriculture and Agri-Food Canada, 3600 Casavant, St. Hyacinthe, QC, J2S 8E3, Canada.

E-mail: ChampagneC@agr.gc.ca

[†] Present address: Canadian Food Inspection Agency, 3400 Casavant, St. Hyacinthe, QC, Canada.

¹⁾ The products used in this study were selected for research purposes only. Their selection does not constitute an endorsement of any nature by Agriculture and Agri-Food Canada.

Automated spectrophotometry (AS) and fermentations: Media were prepared at a carbohydrate content of 60 g/L. They were supplemented with various nitrogen sources as well as with 1 g/L of Tween adjusted to pH 6.1 and sterilized at 110°C for 10 min. These base media were supplemented with two mineral solutions prepared separately. The first contained 100 g/L K_2HPO_4 and 100 g/L KH_2PO_4 and was adjusted to pH 6.1 before sterilization, while the second contained 250 g/L $MgSO_4$ and 150 g/L $MnSO_4$. Both were sterilized at 121°C for 10 min, and then 1.0 and 0.1 ml were respectively added to 98.9 ml of the base media. For some AS assays, the growth media were diluted with a buffer solution containing 3 g/L K_2HPO_4 and 3 g/L of KH_2PO_4 , adjusted at pH=6.1 and sterilized at 121°C for 10 min. The AS was carried out as described in Potvin et al. (1997). The highest OD reading (OD_{max}), reached at the stationary growth phase, was recorded and maximum growth rate (μ_{max}) ascertained. For one series of assays, MRS was supplemented with 4% of lactose and this medium will be referred to as MRS-lactose. All media were supplemented with 1 g/L of Tween 80. The fermentations were carried out as described by Champagne and Gardner (2002). At least three independent repetitions were performed.

Analyses: Viscosity, ease of recovery to centrifugation and bacterial counts of the fermented broths were measured as described by Champagne et al. (2007). OD measurements were done at 600 nm using a Beckman (Fullerton, CA, USA) DU-7400 instrument. Some samples were diluted with unfermented media in order

to obtain readings under 1.0, and these values were multiplied by the dilution factor.

Selection of nitrogen supplements by AS: In the first series of assays, only 0.4 g/L of the nitrogen source was added in order to limit growth to OD values under 1.0; although this is insufficient to generate optimum biomass production, it nevertheless serves to compare the effectiveness of the supplements. In these preliminary assays 6 sources of peptides/amino acids were used alone or in combination for a total of 24 mixtures and data for 6 are presented in Table 1. Of the three YE sources, the Difco product gave the highest OD_{max} readings (data not shown). Fruits contain compounds inhibitory towards probiotic bacteria (Vindeola et al., 2002), and it cannot be assumed that fruit juices can always serve as good growth media. There was a good correlation ($R=0.91$) between biomass yields (OD_{max}) obtained in the 24 apple-based media and those obtained in the corresponding molasses-based media. This suggests that these substrates are similarly improved by the addition of peptones or YE. These observations add to those of date juice where YE and peptone supplementation markedly improved growth of *L. rhamnosus* (Nancib et al., 2004). The three peptones alone gave good biomass results in the AS assays (Table 1). However, from an economic standpoint, three reasons justify the use of blends of supplements: less effect of new ingredient lot (Potvin et al., 1997), YE (particularly brewer's YE) are generally less expensive than peptones, and blends may provide better yields than individual ingredients. Indeed, a

Table 1. Effect of nitrogen supplement mixtures on maximum biomass (OD_{max}) or growth rate (μ_{max}) of *Lactobacillus rhamnosus* RW-9595M in the automated spectrophotometry assays.

Supplement* added to either the molasses or to the apple juice bases (g/L)				Growth in apple juice		Growth in molasses	
Difco YE	Tryptone N1	Wheat E1	Soy A3	OD_{max}	μ_{max}	OD_{max}	μ_{max}
—	—	—	—	0.21	0.27	0.13	0.22
0.4				0.44	0.34	0.41	0.29
	0.4			0.47	0.31	0.42	0.28
		0.4		0.48	0.35	0.45	0.3
			0.4	0.44	0.36	0.43	0.29
0.2		0.2		0.51	0.39	0.47	0.35
0.2			0.2	0.31	0.33	0.2	0.25

* Difco YE are yeast extracts, while Tryptone N1, Wheat E1 and Soy A3 are peptones.

blend of baker's YE and wheat peptones gave the highest OD_{max} readings in apple juice as well as in molasses (Table 1). However, not all blends are successful as the Soy A3-YE data show (Table 1). These data resulted in the selection of the baker's YE and wheat peptone (YEWP) 50 : 50 blend for biomass optimization assays.

Screening of media by AS: Once the YEWP nitrogenous supplement was chosen, assays were carried out to ascertain the optimum supplementation level. In these assays, the media had to be diluted with buffer solutions and only the OD data under 1.0 were used in the calculations for the AS predicted OD levels (Table 2). MRS is a medium which constitutes a gold standard (Garcia et al., 2006) for the production of lactic cultures. The addition of 4% carbohydrate to basal MRS, for a final content of 6%, strongly improved OD_{max} values (Table 2). Since MRS is generally used in laboratories without pH control, its carbohydrate level is insufficient to enable the higher biomass levels obtained under pH control (Champagne and Gardner, 2002). The unsupplemented apple juice and molasses gave OD_{max} levels 25 times lower than the MRS-lactose. This showed that supplementation of these base media was critical in promoting extensive growth and confirmed the usefulness of the AS assays designed to

select nitrogen supplements. As had been found in the nitrogen selection assays (Table 1) the correlation between predicted OD data from molasses and apple were high ($R=0.97$). This suggests that, although the subsequent fermentation studies were limited to the apple juice base, it could be expected that the data would also apply to molasses. Molasses, a low-cost substrate used commercially for the production of baker's yeast (Chen and Chiger, 1985), has been proposed as base medium for streptococci (Rodrigues et al., 2006) as well as for a supplement to whey for *L. bulgaricus*, *L. acidophilus* and *L. casei* biomass production (Gandhi et al., 2000) but this is the first report of its potential for *L. rhamnosus*.

Biomass levels in fermentations: Two analyses were carried out for the evaluation of biomass: OD and CFU readings (Table 2). As the OD_{max} AS data had predicted, supplementation of the apple juice by YEWP improved yields and MRS-lactose was the best medium for biomass production. Apple juice is used for the production of *Leuconostoc* starters (Champagne et al., 1989) and this study shows that it is also appropriate for *L. rhamnosus*. There were good correlations between the AS-predicted OD_{max} data and OD values obtained in actual fermentation trials (Table 2), although AS systematically underestimated the OD level

Table 2. Effect of medium composition on growth of *Lactobacillus rhamnosus* RW-9595M in automated spectrophotometry assays, as well as in 2 L fermentors. Growth was estimated by optical density (OD) as well as with colony counts (CFU). The possibility of recovering the cells from the fermented broths by centrifugation (CF) was determined by examining the OD of the supernatant following a 10 min CF at 4,000 or 10,000 × g.

Medium*		Fermentations							
Base	YEWP	Spectrophotometry OD _{max}	CRV	OD before CF	OD after CF 4,000 × g	OD after CF 10,000 × g	Population (CFU/ml)	Average chain length	CFU/ml corrected for chain length
Apple	0	0.3 a	1.3 a	0.5 a	0.21 a	0.02 a	1.2 × 10 ⁸ a	1.0 a	1.2 × 10 ⁸ a
Apple	2.5	2.1 b	2.1 b	4.8 b	2.03 b	0.06 a	1.4 × 10 ⁹ b	1.5 ab	2.1 × 10 ⁹ b
Apple	5	3.0 bc	2.3 bc	7.3 c	2.46 c	0.54 b	3.6 × 10 ⁹ b	1.2 a	4.3 × 10 ⁹ bc
Apple	7.5	4.2 cd	2.5 bc	6.5 bc	2.73 cd	1.08 c	5.2 × 10 ⁹ bc	1.3 ab	6.8 × 10 ⁹ c
MRS	—	5.9 e	2.8 cd	8.2 c	2.60 c	0.02 a	4.1 × 10 ⁹ bc	2.4 c	9.8 × 10 ⁹ cd
MRS + lactose	—	8.4 f	3.6 d	10.2 cd	2.82 cd	1.87 d	5.1 × 10 ⁹ bc	2.4 c	1.2 × 10 ¹⁰ d
MRS 50% : apple 50%	—	4.9 de	2.6 bc	10.9 d	2.87 d	0.78 bc	6.6 × 10 ⁹ c	1.2 a	7.9 × 10 ⁹ c

* YEWP=yeast extracts wheat peptone 50 : 50 blend; MRS=commercial *Lactobacillus* MRS medium.

abcdef=In a given column, values which are followed by the same letter are not significantly different ($p>0.05$).

achieved in fermentations. CFU counts underestimate the true number of cells when cultures produce chains, which occurs for many probiotic and starter cultures. Contrary to streptococci, ascertaining the number of cells in a chain is difficult with lactobacilli. The analyst must rely on chain length to characterize the biomass rather than establishing a true "number of cells per chain" reading. Apparent chain length varied as a function of the growth medium (Table 2). In response, attempts were made to ascertain if corrected CFU counts would give better relationships with OD readings. A good correlation was detected in the regression analysis of OD_{max} in fermentors and corrected CFU data ($R=0.89$), and it was even higher with the AS-predicted OD_{max} data ($R=0.97$). This suggests that correcting CFU values may better represent population levels, and improve correlations with other methods of ascertaining biomass yields.

Recovery of growth media on viscosity: *Lactobacillus rhamnosus* RW-9595M is a very high exopolysaccharide (EPS) producer (Macedo et al., 2002). The EPS increases the viscosity of the medium and makes it difficult to recover the cells by centrifugation (Champagne et al., 2007). This was the case since centrifugation at $4,000 \times g$ for 5 min only removed half the biomass. As a rule, the greater the biomass, the more viscous was the medium. Indeed, statistical analysis showed that there was a significant ($p=0.03$; $R=0.70$) relationship between OD readings from the fermentations and capillary relative viscosity (CRV; comparative flow rate, in a capillary, of the fermented medium to that of water). Previous data with this strain also showed that medium composition had an effect of EPS synthesis (Macedo et al., 2002). The relationship between the CRV and the residual OD after centrifugation at $10,000 \times g$ was judged to be significant ($p=0.02$) but the correlation coefficient was not high ($R=0.74$). This suggests that viscosity is not the only factor which influences the ease by which cells are recovered by centrifugation.

Conclusions: Scientific publications contribute two ways to knowledge: they provide confirmatory data to enhance that already published, and provide new findings. This study confirmed numerous previous observations: 1) fruit-based media can be used for the production of lactobacilli but require nitrogen supplementation, 2) mixtures of peptones and yeast extracts may be better than single sources for biomass production, 3) AS is a valuable tool to screen media for

biomass production, 4) taking into account chain length of lactic cultures may improve links between CFU and other biomass assessment methods, 5) growth medium composition affects EPS production by lactobacilli, 6) the viscosity of the fermented broths affects the ability to recover the biomass by centrifugation, 7) there is low correlation between maximum growth rate and the maximum biomass attained in the fermented media, 8) used under pH control, MRS can be improved as a growth medium for lactobacilli under pH control simply by adding 4% sugar. This study also provides new findings: 1) effect of a variety of nitrogen supplements and plant-based media specifically on growth of the *L. rhamnosus* species, 2) molasses and apple juice are equivalent as base growth media for *L. rhamnosus*, 3) in AS, using readings from multiple dilution factors in the 0.3 to 1.0 OD zone improves the calculation of predicted biomass, 4) it can be economically attractive to blend the high-cost MRS medium with apple juice for biomass production. In addition to providing tools and media formulations for research teams in the probiotic and starter lactic acid bacteria field, these data could be of interest for two industrial bases: suppliers of probiotics, and manufacturers of probiotic-enriched functional foods who prepare their own cultures rather than direct inoculation procedures.

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