ORIGINAL RESEARCH

Culture of *Bacillus subtilis* and *Saccharomyces cerevisiae* from byproducts of the enzymatic extraction of *Atlantoraja castelnaui* oil

Andrea L. Salomone[1, †] and Daniela L. Lamas[1, 2, *]

[1]Instituto Nacional de Investigación y Desarrollo Pesquero (INIDEP), Paseo Victoria Ocampo Nº 1, Escollera Norte, B7602HSA - Mar del Plata, Argentina. [2]Instituto de Investigaciones Marinas y Costeras (IIMyC), Universidad Nacional de Mar del Plata (UNMdP), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Rodríguez Peña 4002, B7602GSD - Mar del Plata, Argentina.
ORCID *Daniela L. Lamas* https://orcid.org/0000-0001-6009-2478

ABSTRACT. The growing trend in global fish consumption has led to the generation of a large amount of waste and byproducts with negative economic and environmental impacts. The production of fish oil from fish processing residues seems to be a sustainable and future opportunity to provide valuable fatty acids for animal and human consumption. In parallel, this alternative valorization of fish waste has increased the demand to explore sustainable extraction methods. Enzymatic hydrolysis is an efficient, rapid and reproducible method for the extraction of oils from fish guts, producing an aqueous phase rich in proteins and soluble compounds. In this work, a highly soluble aqueous phase obtained from the enzymatic extraction of the liver oil of the ray *Atlantoraja castelnaui* was tested as a source of nutrient for the growth of *Bacillus subtilis* and *Saccharomyces cerevisiae*. Culture media were supplemented with the aqueous phase at a final concentration of 10 mg ml^{-1} total protein. The growth pattern and biomass yield of yeasts cultured in yeast extract peptone-dextrose (YPD) medium did not show statistically significant differences (p: 0.05) with the diluted medium and the aqueous fraction (AF) supplemented medium. Similar results were obtained for *B. subtillis* and its positive control in Luria Bertani (LB) medium. In both cases, the ability of these inexpensive media to support the growth of microorganisms was demonstrated. Results suggested that the remaining aqueous phase of the discards from *A. castelnaui* oil production can be used as an alternative substrate for microorganism culture purposes. In this way, a residue destined for disposal could become a product with added value achieving a good result in the context of circular economy.

Key words: Cartilaginous species, fish liver, oil extraction, culture media, microbial growth, circular economy.

*Correspondence:
dlamas@inidep.edu.ar

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Cultivo de *Bacillus subtilis* y *Saccharomyces cerevisiae* a partir del descarte de la extracción enzimática de aceite de *Atlantoraja castelnaui*

RESUMEN. La creciente tendencia en el consumo mundial de pescado ha llevado a la generación de una gran cantidad de desechos y subproductos con impactos económicos y ambientales negativos. La producción de aceite de pescado a partir de residuos del procesamiento de pescado parece ser una oportunidad sostenible y de futuro para proporcionar ácidos grasos valiosos para el consumo animal y humano. Paralelamente, esta valorización alternativa de los desechos de pescado ha aumentado la demanda de explorar métodos de extracción sostenibles. La hidrólisis enzimática es un método eficiente, rápido y reproducible para la extracción de aceites de las vísceras de pescado, produciendo una fase acuosa rica en proteínas y compuestos solubles. En este trabajo, una fase acuosa altamente soluble obtenida de la extracción enzimática del aceite de hígado de la raya *Atlantoraja castelnaui*, se probó como fuente de nutrientes para el crecimiento de *Bacillus subtilis* y *Saccharomyces cere-*

visiae. Los medios de cultivo se suplementaron con la fase acuosa a una concentración final de 10 mg ml^{-1} de proteína total. El patrón de crecimiento y el rendimiento de biomasa de las levaduras cultivadas en medio extracto de levadura peptona-dextrosa (YPD) no mostraron diferencias estadísticamente significativas (p: 0,05) con el medio diluido y suplementado con fracción acuosa (AF). Resultados similares se obtuvieron para *B. subtillis* y su control positivo en el medio Luria Bertani (LB). En ambos casos, se demostró la capacidad de estos medios de cultivo económicos para el crecimiento de microorganismos. Los resultados sugirieron que, la fase acuosa restante de los descartes de la producción de aceite de *A. castelnaui*, puede usarse como sustrato alternativo para fines los mismos fines. De esta manera, un residuo destinado a la eliminación, podría convertirse en un producto con valor agregado, logrando un buen resultado en el contexto de la economía circular.

Palabras clave: Especies cartilaginosas, hígado de pescado, extracción de aceite, medio de cultivo, crecimiento microbiano, economía circular.

INTRODUCTION

The fishing industry generates a large number of byproducts or wastes that have a negative economic, social and environmental impact. Up-cycling them into value-added products is a major challenge to achieve a circular economy through the efficient use of integral natural resources (Mirzapour-Kouhdasht et al. 2021). This approach is relevant because the production and processing of fish products results in the disposal of more than 50% of the biomass annually (Mozumder et al. 2022). Solid fish waste, consisting of heads, tails, skin, viscera, fins and bones (Nawaz et al. 2020), is an important source of biocompounds of commercial interest, such as fatty acids, hydrolyzed peptides, enzymes, among others (Lamas and Massa 2019, 2022, 2023). In the fishing industry, the extraction of oils from the livers of cartilaginous species generates waste byproducts such as sludge and aqueous fractions rich in proteins, macro- and micronutrients (Ghaly et al. 2013; Lamas and Massa 2019, 2024; Salomone and Lamas 2024). There is a growing trend towards their use in the context of circular economy goals. One of the lines of research focuses on the cultivation of microorganisms of biotechnological interest using fishing waste as a source of energy for direct production or for obtaining final or intermediate products with high added value (Martone et al. 2005; Rebah and Milled 2013; Vázquez et al. 2020). This residue is usually rich in peptones, a set of proteins, peptides, and free amino acids useful as ingredients acting as a source of organic nitrogen in complex media necessary for the grow of microorganisms (Vázquez et al. 2020). In culture broths, peptones are usually the most expensive ingredient (Ramkumar et al. 2016). Although fish peptones are hardly commercialized, several fish byproducts have successfully supported the growth of various microorganisms (Deraz et al. 2011; Shi et al. 2018). Furthermore, virtually any substrate that is a source of organic carbon can be used to grow microorganisms, as long as it is capable of meeting all their nutritional requirements (Ghaly et al. 2013; Fallah et al. 2015; Kogje and Ghosalkar 2016).

Bacillus subtilis and *Saccharomyces cerevisiae* are two microorganisms of great interest for biomass production. The yeast *S. cerevisiae* is one of the species generally regarded as safe (GRAS) microorganism, and is therefore approved for use as a food additive (FDA 2018). In addition to its commercial use in the bakery industry, temperature-inactivated yeast is used as a source of nutrients with antioxidant properties in animal feed and human food, both as whole yeast and yeast derivatives (Czerucka et al. 2007; Yalçm et al. 2011; Datta et al. 2017). Another application of biotechnological interest is their cultivation for unicellular protein production, where their biomass is used directly in animal feed without any protein extraction or purification process and as a dietary supplement (Schultz et al. 2006; Garimelas et al. 2017; Jiaqian et al. 2018).

Bacillus subtilis is a Gram-positive bacterium that has been widely used in bioremediation as an anionic dye scavenger in industrial landfills (Binupriya et al. 2010; Jabeen et al. 2015), for the synthesis of lipopeptides used as biosurfactants for oil degradation in various industries and contaminated soils (Fernandes et al. 2016), and for the control of phytopathogens in agriculture (Soto Deza et al. 2012; Mercado Flores et al. 2014), and the production of unicellular proteins (Kurbanoglu and Algur 2002; Gomashe et al. 2010; Aldedayo et al. 2014), among other applications.

In the present work, a highly soluble aqueous phase obtained from the enzymatic extraction of the liver oil of the ray *Atlantoraja castelnaui* has been tested as a source of nutrients for the growth of *Bacillus subtilis* and *Saccharomyces cerevisiae*. Results obtained were compared with commercial control media to evaluate the feasibility of this biotechnological approach to valorize fish liver oil waste. This is the first approach dealing with the application of this type of residues from *A. castelnaui* to the growth of microorganisms of technological interest.

MATERIALS AND METHODS

Biological samples

Livers from three different batches of seven *A. castelnaui* rays each were used in this study. Samples were obtained from research surveys carried out by the Instituto Nacional de Investigación y Desarrollo Pesquero (INIDEP, Mar del Plata, Buenos Aires, Argentina). The extracted livers were conditioned at -25 °C until use.

Enzymatic oil extraction

For the extraction of the oil, livers were thawed and cut into cubes of approximately 2 to 3 mm^3 in size. They were then ground in a household grinder for 30 s and homogenised in distilled water at 50°C in a 1:1 ratio. Enzymatic hydrolysis was carried out in a thermostated reactor with constant stirring for 1 h using Purazyme AS 60L enzyme (alkaline serine protease, *B. licherniformis)* at an enzyme/homogenate ratio of 2%. The reaction was inactivated at 85 °C for 10 min. The hydrolysate was centrifuged at 13,000 rpm for 30 min at 4 °C and then frozen at -20 °C in polypropylene tubes. The sludge, oil phase and aqueous fraction (AF) rich in proteins were separated by cutting the frozen contents of the tubes.

Conditioning and protein characterisation of the AF

The AF was centrifuged again (20 min; 4,000 rpm; 20 °C) to remove any remaining insoluble and stored at -20 °C until use. For protein extraction and denaturation, 0.1 ml of the AF was treated with 5X Seeding Buffer (2.5 ml SDS 10%; 0.2 ml β-mercaptoethanol; 0.5 ml bromophenol blue 0.05%; 0.5 ml Tris 0.5 M; pH 6.8) and heated at 100 °C for 5 min and immediately cooled on ice. Electrophoresis was performed under denaturing conditions, 15% for the separator gel and 4% for the concentrator gel (acrylamide 30%; bisacrylamide 0.8%; SDS 10%; ammonium persulphate 100 mg ml^{-1}; TEMED; Separator Gel Buffer: 1M Tris; pH 8.8; concentrating gel buffer: 0.5 M Tris; pH 6.8). Electrophoresis was performed using Tris-glycine buffer (glycine 14.4 g ml^{-1}; Trizma base 3 g l^{-1}; SDS 10%) for 2 h at 120 V. The gel was stained for 1 h (Coomasie Blue 0.25 g; methanol 45%; acetic acid 10%) and developed overnight with several washes in decolourisations solution (methanol 10%; acetic acid 40%) (Laemli 1970).

Effect of AF on the growth of *S. cerevisiae* and *B. subtilis*

Saccharomyces cerevisiae yeast was obtained from a commercial bakery product. To obtain a metabolically stable yeast culture, 1 g of lyophilisate was inoculated into 50 ml of complete YPD

medium (yeast 1%; peptone 2%; glucose 2%; pH 4.5). The *B. subtilis* strain used was obtained from a commercial product used for the bioremediation of cesspools and septic tanks. To obtain a metabolically stable culture of this bacterium, 1 g of lyophilizate was inoculated into 50 ml of complete LB medium (0.5% yeast; 1% peptone; 1% sodium chloride; pH 7).

In order to evaluate the capacity of AF as a nutrient supplement for growth, tests were carried out using two culture media (Table 1). The modified medium is the one obtained by adding the AF obtained from enzymatic oil extraction. For both microorganisms, cultures were grown in 250 ml Erlemeyer flasks for 24 h at 30 ± 1 °C with shaking at 150 rpm. The inoculation volume of 1 × 104 CFU (colony forming units) was determined by measuring the absorbance at 620 nm for yeasts and at 600 nm for bacteria. Culture methods were monophasic.

For the preparation of growth curves, media were inoculated with 1×10^4 CFU in a final volume of 10 ml, incubated in an oven at 30 ± 1 °C with shaking at 150 rpm. The absorbance was determined at 620 nm for yeasts and at 600 nm for bacteria with aliquots being taken at different times during 48 h. Biomass yield was determined after 24 h of culture by harvesting the cells by centrifugation at 4,000 rpm at 4 °C. They were then oven dried at 60 ± 1 °C to constant weight and expressed as dry weight litre^{-1} of culture medium. Negative controls were included for the different culture media used, without inoculum (contamination control). In parallel with each culture, cell viability was determined by plate count assays on agar-YPD or agar-LB, as appropriate, by plating different dilutions of 24-h cultures in duplicate. Plates containing between 50 and 200 CFU were counted.

Statistical analysis

All assays were performed in triplicate. Data were expressed as mean ± SD. The analysis of variance (ANOVA) using a 95% confidence interval ($p < 0.05$) (GraphPad Prism) was applied to results. For the comparison of the biomass yield of each trial, the Newman-Keuls test for multiple comparisons was used, which allowed comparing the means of the t-levels of a factor after rejecting the null hypothesis of equality of means using the ANOVA technique.

RESULTS AND DISCUSSION

Enzyme-treated liver produced peptides smaller than 3.5 kDa (Figure 1). This gives them properties as a culture medium, since their composition of mainly low molecular weight peptides can be metabolised by yeast.

Growth curves of *S. cerevisiae* in both YPDc and modified medium (YPDd + AF) showed the same kinetics up to 48 h of culture (Figure 2 A), and were consistent with the result of biomass yield per litre of culture (Figure 2 B). Similar results were observed with *B. subtilis* when grown on LBc and modified (LBd + AF) media (Figure 3 A and 3 B). Both microorganisms did not show acceptable growth on the culture media used as negative control (1/4 diluted media) compared to the complete media (YPDd and LBd, respectively) ($p > 0.05$), due to the rapid depletion of glucose provided by the diluted media.

Biomasses produced with the respective modified media supplemented with AF were not significantly different from the corresponding positive controls (Figures 2 B and 3 B) ($p < 0.05$), indicating that the aqueous fraction of the enzymatic oil extraction method is capable of supplementing with YPDd and LBd media for use as culture media for *S. cerevisiae* and *B. subtilis*, respectively. When the most energy-rich carbon source for microbial growth, simple sugars such as glucose, is absent or limited, transcriptional regulation of genes involved in the metabolism of alternative carbon sources occurs. In this way, the low mo-

Table 1. Composition of culture media used for the growth of *Saccharomyces cerevisiae* and *Bacillus subtilis.*

Culture medium	Composition
Saccharomyces cerevisiae	
YPDc (Control +)	YPD complete
Medium modified (YPDd + AF)	1/4 YPD supplemented with AF to a final concentration of 10 g l^{-1} protein
YPDd (control -)	YPD diluted in 1/4
Bacillus subtilis	
LBc (Control +)	LB complete
Medium modified (LBd + AF)	1/4 LB supplemented with AF to a final concentration of 10 g l^{-1} protein
LBd (control -)	LB diluted in 1/4

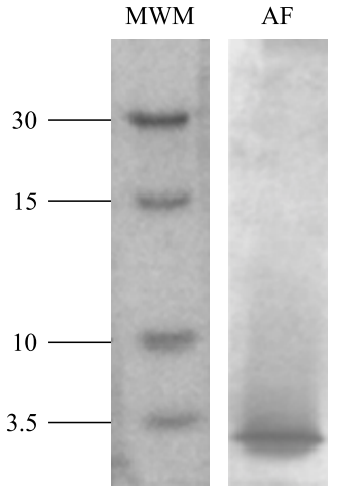

Figure 1. SDS-PAGE separation profile with Coomassie blue staining. MWM represents the molecular weight marker (kDa). AF corresponds to the aqueous fraction of *Atlantoraja castelnaui* liver enzyme hydrolysate.

lecular weight peptides and free amino acids that make up the protein hydrolysate can be taken up and metabolised by these microorganisms, compensating for the low availability of carbohydrates in the medium (Turcotte et al. 2010; Paulo et al. 2015; Melnykov 2016). Hydrolysates from silver carp waste treated with an alkaline protease have been used to culture *Staphylococcus aureus*, with higher growth rates obtained with the hydrolysate than with commercial media. By supplementing culture media with fish waste hydrolysates, proteases from *Pseudomonas aeruginosa* MN7 and *B. subtilis* were obtained (Triki-Ellouz et al. 2003). Also, strains of *S. aureus* and *Salmonella enteriditis* have been tested in microbiological culture media prepared from peptone-based fish protein hydrolysates and have shown similar or better growth than industrially prepared reference media (Petrova et al. 2021).

The viability of *S. cerevisiae* and *B. subtilis*, determined after 24 h of culture in all assays, correlated with data obtained from the absorbance measurements of growth curves. Average cell counts for the biomass determination study (Figures 2 B and 3 B) confirms that the biomass produced was viable in all cases, at least up to 24 h of culture (Table 2).

In this study, the final fish protein concentration was 10 g l^{-1}. Previous reports indicated that mixed populations of microorganisms from food (beef, egg and milk) and selected pure microorganisms (*Aspergillus flavus*, *B. subtilis*, *S. cerevisiae*, *Escherichia coli* and *S. aureus*) grown on liquid media containing 5 g l^{-1} test peptones from cowtail ray viscera performed similar or even better than commercial peptones as nitrogen sources for microorganism growth (Poernomo and Buck-

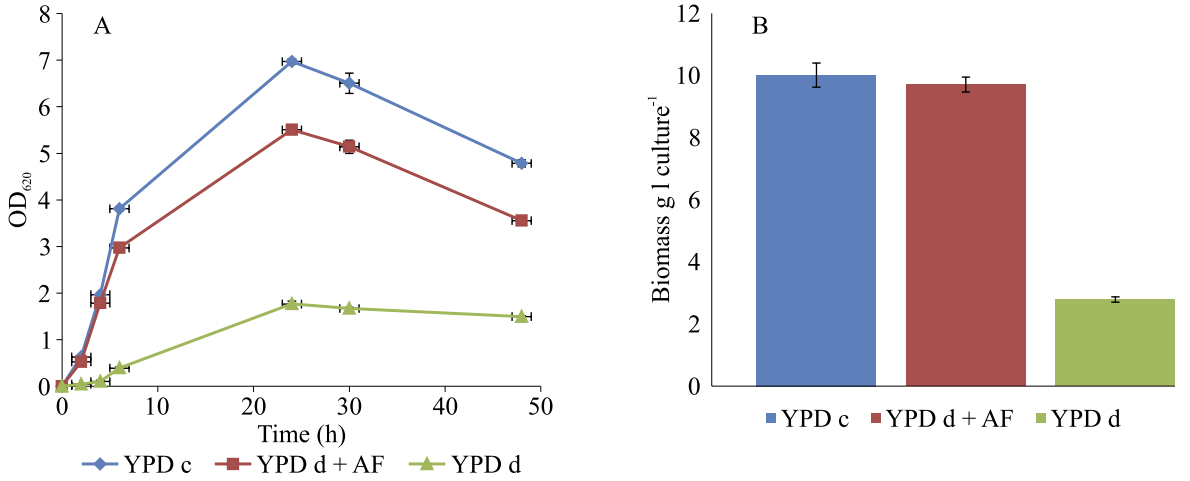

Figure 2. A) *Saccharomyces cerevisiae* growth curves obtained by absorbance (OD_{620}) measurement as a function of time. B) Biomass yield as dry weight of cells in each litre of culture medium. YPDc: positive growth control. YPDd: negative growth control. YPDd + FA: 1/4 diluted YPD supplemented with AF.

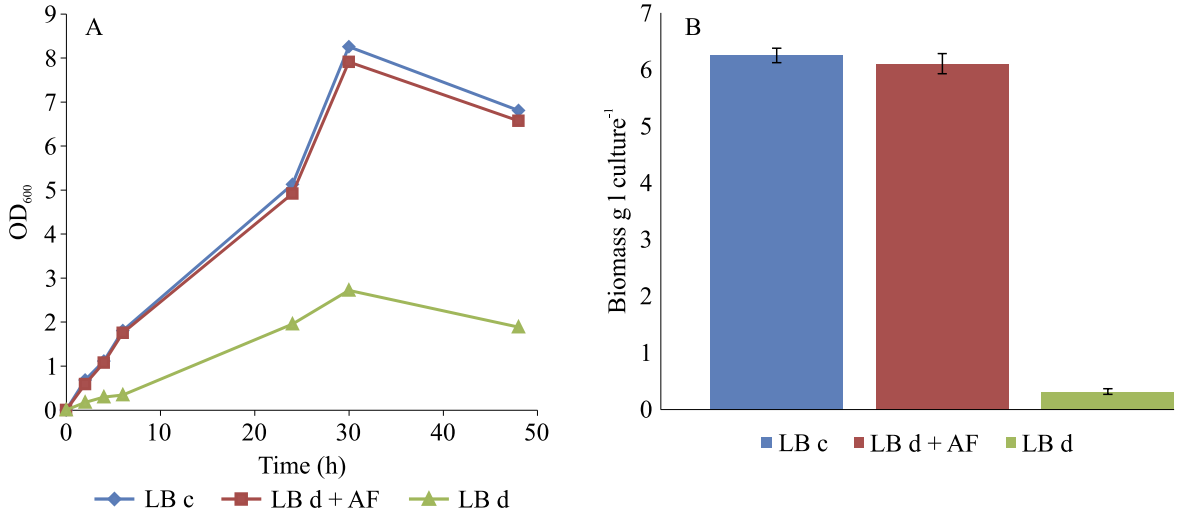

Figure 3. A) *Bacillus subtillis* growth curves obtained by absorbance (OD_{600}) measurement as a function of time. B) Biomass yield as dry weight of cells in each litre of culture medium. LBc: positive growth control. LBd: negative growth control. LBd + FA: 1/4 diluted LB supplemented with AF.

le 2002). Safari et al. (2012) reported that 18 g l^{-1} meat and casein peptone replaced by 10 g l^{-1} fish peptone gave better results for LAB growth rates compared to the commercial sample. Aspmo et al. (2005) used low (5 g l^{-1}) fish peptone instead of the 22 g l^{-1} peptones found in commercial media and found that LAB could grow well on this amount of fish peptone (5 g l^{-1}), but not as on the commercial media (22 g l^{-1}). Similar results have been observed using hydrolysate produced from protein-rich waste from Icelandic scallop processing (Mukhin et al. 2001). Other works reported that peptones from aquaculture waste (turbot, salmon, trout, etc.) produced by thermal extraction

Table 2. Number of viable microorganisms after 24-h culture. CFU: colony forming units. YPD: standard medium for *Saccharomyces cerevisiae*. LB: standard *Bacillus subtilis* medium. AF: aqueous fraction of enzyme hydrolysate.

	YPDc	YPDd + AF	YPDd	LBc	LBd + AF	LBd
UFC mL^{-1}	2.1×10^9	1.7×10^9	5×0^8	1.5×10^9	1.4×0^9	4×10^8

and enzymatic proteolysis of waste (heads, trimmings and frames) gave maximum biomass values (Vázquez et al. 2020). Collagen hydrolysates from shark, tuna, salmon and turbot skins also showed increased growth of a marine probiotic bacterium, *Phaeobacter* sp. DIFR 27-4 (Vázquez et al. 2023).

Results suggest that these waste fractions are a good source of nutrients for the growth of *B. subtilis* and *S. cerevisiae*, since they provide an alternative source of proteins and carbon, as well as essential microelements required by these microorganisms, which makes their use feasible in the production of these microorganisms. Regarding the use of fish residues and byproducts, there are many low-cost raw materials that could be used for various purposes, such as providing nutrients for the growing of microorganism in the present study. As a result, there is good potential for the use of the side flows in the production of a more cost-effective, more effective medium for microbial growth, preventing environmental contamination due to the disposal of fishery waste. It could therefore be considered as an economically viable and environmentally sustainable alternative with prospective applications in biotechnological processes. Finally, it is important to mention that this study is in line with several Sustainable Development Goals (SDGs) proposed by the United Nation.

INIDEP contribution no 2391.

Declaration of conflict of interest

The authors declare that they have no conflicts of interest.

Author contributions

Andrea L. Salomone: conceptualization; data curation; formal analysis; writing. Daniela L. Lamas: conceptualization; data curation; formal analysis; investigation; visualization; writing-review and editing.

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