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EXECUTIVE SUMMARY OF THE THESIS

Printability assessment of edible bioinks for cultured meat extrusion-based 3D bioprinting

TESI MAGISTRALE IN FOOD ENGINEERING – INGEGNERIA ALIMENTARE

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1. Introduction

Proteins — large macromolecules composed of amino acids — are the building block of our metabolism. To this day livestock meat is still the major source of proteins for the world population that is in continuous growth. Livestock farming, however, is one of the main causes of Green House Gasses emissions — accounting for 14% of the world's overall emissions [1] [2] — water depletion and land use [3]. The emerging sector of protein alternatives aims to substitute the consumption of meat from livestock origin. The amount of resources absorbed by the livestock meat supply chain and the resulting environmental impact represent nowadays one of the most urgent global problems. In this scenario, the industrial scale production of protein alternatives is not only an economic challenge, but also an environmental must in order to substitute the consumption of meat from livestock origin.

Protein alternatives can be classified into three different categories: plant-based, microorganism-based, and cellular-based. The last category, commonly referred as cultured meat (CM), is the only one with the potentiality to substitute completely the livestock meat offering a product with the same organoleptic, textural, and nutritional properties.

The production of cultured meat follows two main approaches that aim to produce a 3D biological tissue. Both of them derive from the Tissue Engineering (TE) for biomedical applications. The first and most common is Scaffolding, a process where cells are seeded into a porous substrate that guarantees their proliferation in three dimensions. The second is 3D Bioprinting (3DBP), which represents a novel promising alternative to scaffolding [4]. 3DBP is an Additive Manufacturing (AM) process where a cell-laden bioink is deposited in a three-dimensional pattern.

The main advantages of 3DBP are the possibility to process multi-material and to skip the cell seeding step. This procedure is in fact the main limiting factor of scaffolding due to the low diffusion depth of cells in their substrate, that is in the order of the microns [5], meaning that scaffolds once seeded need to be layered to reach a desirable thickness.
Despite its potentiality, few research is dedicated to the development of 3DBP for CM, mainly due to novelty of the process and the low availability of materials that satisfy the constraints of edibility, biocompatibility, economic feasibility, and printability. In this sense, hydrogels are promising materials: they are biocompatible polymers commonly implemented in 3DBP for biomedical applications [6]. Alginate hydrogels in particular are edible materials that can be suitable for CM applications when integrated with gelatin to enhance their biocompatibility [7]. Moreover, these polymers have a wide availability and are economically feasible for large-scale productions.
This thesis aims at presenting the first empirical printability model that identifies the optimal window of printing parameters considering the model's uncertainty, in order to guide the experimenter according to any objective functions. The focus will be on two edible hydrogels — namely pure alginate 6%, and alginate 6% gelatin 4% — used as bioinks for an extrusion-based 3D bioprinting process.

2. Materials and methods

Materials

To produce the two bioinks, alginate 8% and gelatin 16% stock solutions were prepared with sterile PBS as solvent (sodium alginate and gelatin type A powders were purchased from Sigma Aldrich). To produce the alginate 6% bioink, the first stock solution was diluted with PBS, while to produce the alginate 6% and gelatin 4% bioink the two stock solutions were mixed in a ratio 3/1.
The Bioprinter used in this work was the BioX from Cellink, equipped with the pneumatic extrusion-based printhead and the HD camera tool. The nozzle adopted was a 0.410 mm conical nozzle form Cellink.

Design of experiment

An in-situ image analysis approach was pursued in order to assess the printability: for each repetition of the experiment a picture of the final print was gathered and analyzed. The test was based on a two-factors factorial Design of Experiment (DoE). The two factors analyzed were the pressure of extrusion (P), and the scanning speed (v) (Figure 1).

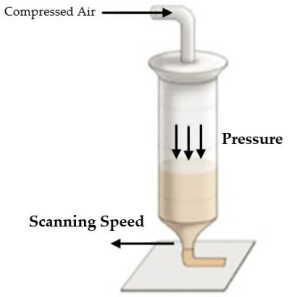

Figure 1: Influence of the two factors on the extrusion process (Source: modified from [8]).

6 levels of for both the factors were explored — $P \in [5, 10, 15, 20, 25, 30]\ kPa$ and $v \in [5, 10, 15, 20, 25, 30]\ mm/s$ — for 3 repetitions of each combination.

Data collection

The measured variable for the assessment of the printability was the Printability Index, evaluated on the voids of the printed object [9]:

$$PI = \frac{A_{real}}{A_{ideal}} \quad (1)$$

The A_{ideal} is the ideal area of the printed void, while A_{real} is the real area of the printed void, measured by the image analysis algorithm. The printed object was designed to have a net-like geometry with squared voids, using a CAD software . The known ideal area was used for the evaluation of the PI (Figure 2).

Figure 2: The input model from the design (left) to the g-code (center), to the final print picture (right).

After each print, the printed object was photographed with the HD camera tool, and the images were imported on MATLAB R2022a. An image analysis algorithm was run on all the different images (Algorithm 1). Through image segmentation, the real area of the 4 voids was measured, and an averaged value of PI was estimated and collected for each print, called Average Printability Index (API) (Figure 3).

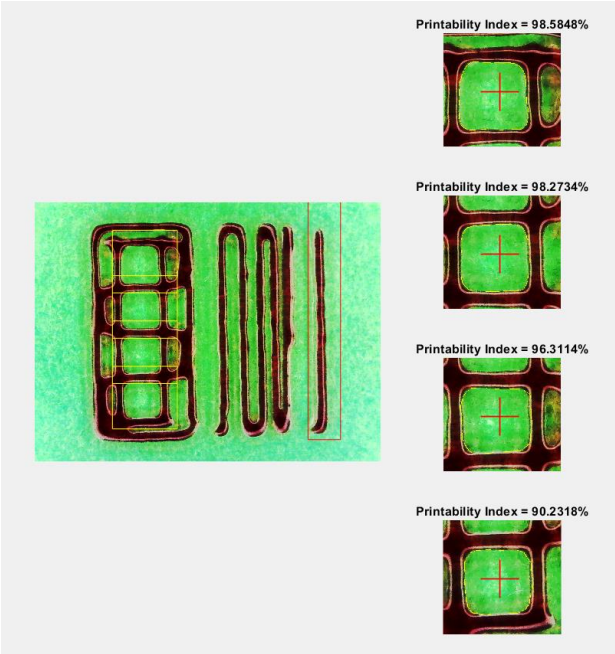

Figure 3: Estimation of the PI through image segmentation.

3. Results

The collected values of API were reorganized and analyzed both with Minitab and Excel. With the data regarding the two bioinks were created two datasets namely Alg for the pure alginate 6%, and Alg+Gel for the alginate 6% gelatin 4% hydrogel.

Linear Regression Models

Since the two datasets belonged to the same phenomena, but with different materials, it was forecasted to have similarity between the two models. In fact, following an iterative approach, from the two databases were generated two regression models with the same regressors both with high significance and that respected the hypothesis (see "*Hypothesis of the models*") (Table 1).

Table 1: Regression coefficients, and model significance indicators.

Regressors	Significance	Alg+Gel	Alg
1		2.541	4.914
P		-0.2518	-0.4049
v		0.1460	0.1810
*P*P*		0.005186	0.00773
*v*v*		-0.00151	-0.00174
*P*v*		-0.00248	-0.00318
	R-sq adj	93.26%	93.09%
	S	0.2152	0.2162

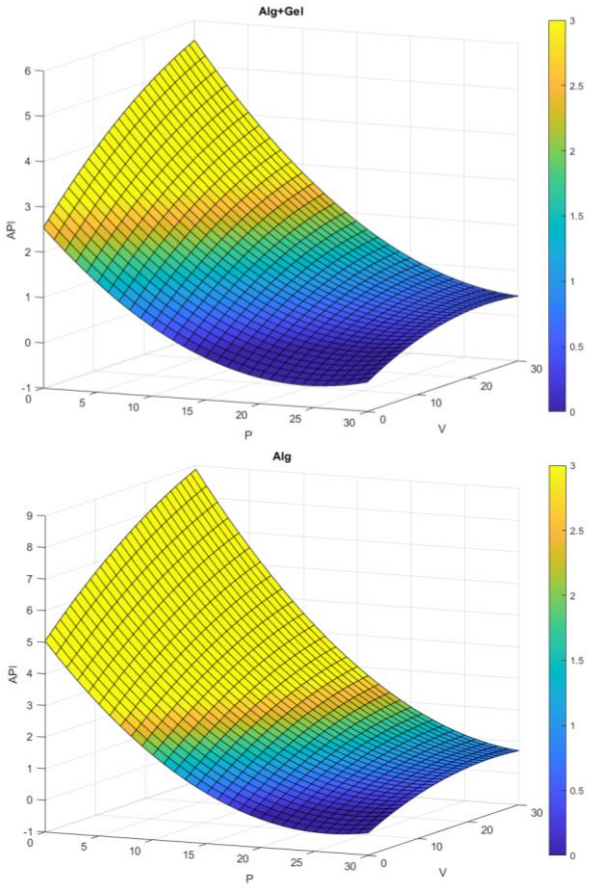

Figure 4: Surface plots of the regression equations of the Alg+Gel dataset (top), and Alg dataset (bottom).

Probability maps

From the regression equations it is possible to predict the values of API from the process parameters, however the models provide a punctual prediction, lacking in information on the prediction uncertainty. Therefore, the probability maps were produced considering both the regression model and the prediction uncertainty, defining the probability of having the prediction in a certain interval.

Considering the optimal quality interval, where $API \in [0.75; 1.25]$, the probability maps followed the relation:

$$F_{T,df_E}\left(\frac{\alpha_{sup} - \hat{y}(\boldsymbol{x})}{\Delta(\boldsymbol{x})}\right) - F_{T,df_E}\left(\frac{\alpha_{inf} - \hat{y}(\boldsymbol{x})}{\Delta(\boldsymbol{x})}\right) = P \quad (2)$$

where $\alpha_{sup} = 1.25$ and $\alpha_{inf} = 0.75$, and $\Delta(\boldsymbol{x})$ is the prediction uncertainty term evaluated as:

$$\Delta(\boldsymbol{x}) = \sqrt{MS_E(1 + \boldsymbol{x}^T(\boldsymbol{X}^T\boldsymbol{X})^{-1}\boldsymbol{x})} \quad (3)$$

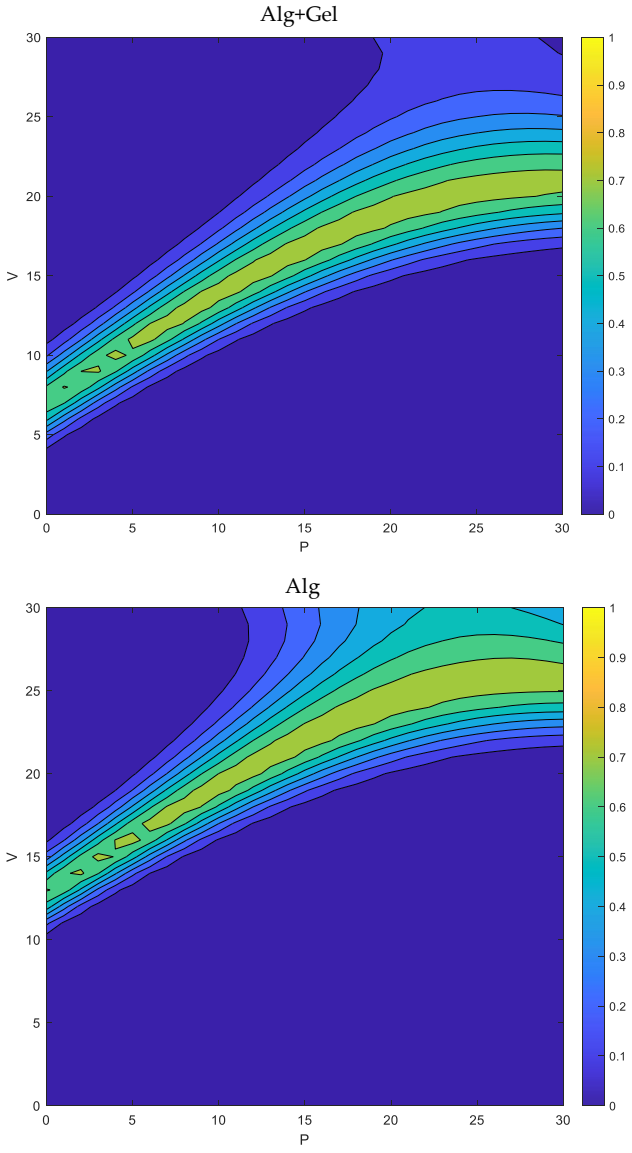

Figure 5: Probability maps of Alg+Gel (top) and Alg (bottom) for the interval [0.75; 1.25].

4. Conclusions

The output of this thesis aims to open the doors of process scalability to the 3DBP of CM. The probability maps are strong tools in the process optimization, and in the subsequent reduction of processing costs with the aim to reach the time of parity before 2035 (the predicted year of cost equalization between livestock meat and CM) [10]. Due to the sector of application, the output of the probability maps can be interpreted and used for different optimization purposes. Firstly, it can be used to identify the optimal pressure of operation in order to minimize the time of printing (maximizing the scanning speed). This will achieve the reduction in processing time, and consequently a cost reduction. The reduction of processing time will also affect the cellular activity: in fact, the longer cells are kept in a "open" environment without growth and the lower will be the final viability of the cellular population.

An alternative is to identify the energy consumption function that links pressure and velocity, and together with the probability map resolve a minimization problem to achieve minimal energy usage. This is a fundamental step in the cost reduction of the process.

Eventually, the probability map can be used to achieve the highest robustness and identify the optimal conditions for the quality reliability of the process. In fact, pressure is a parameter that can suffer from fluctuations during the processing, while velocity is more stable and controllable. If we focus on robustness, it is possible to fix the velocity or pressure that identifies the widest region of high probability on the map.

5. Future developments

Focusing on the in-situ monitoring, the proposed data analysis and model regression can be adopted in the formulation of a closed-loop controller by the implementation of a closed-loop controller algorithm.

Another interesting development may be represented by the analysis of two additional dimensions: the Filament Fusion, and the Diameter expansion. The 3D model adopted for this thesis was designed in order to also measure these dimensions, but the focus was given only on the PI. These additional dimensions are two important features to consider for 3DBP for CM.

The former accounts for the fusion of parallel filaments that are deposited adjacent to each other, which is particularly interesting for cultured meat applications, since in order to resemble the fibrous texture of meat, it is often bioprinted with parallel adjacent filaments.

The latter refers to the final diameter of the extruded filament. This dimension intends to quantify the expansion of the filament when extruded, that reaches a diameter larger than the diameter of the nozzle. It is an important measure because it influences both the filament fusion and the printability index.

6. Algorithms

Algorithm 1 Image segmentation algorithm.

```
PR=InputCroppedImage;
GM=PR(:,:,2)>160;
BM=PR(:,:,3)>180;
RM=PR(:,:,1)>170;
RGBM=GM-(BM+RM);
GrayPR=rgb2gray(PR);
RegionGrowth=
regiongrowing(im2double(GrayPR));
Reg=RegionGrowth(:,:,1);
Reg=Reg.*RGBM;
se=strel('disk',9);
RegCL=imclose(Reg,se);
se=strel('disk',10);
RegOP=imopen(RegCL,se);
Area=regionprops(RegOP,'Area');
if isempty(Area)==1
     Porosity=0;
else
     Porosity=Area.Area/(170^2);
end
```

7. Hypothesis of the models

Regression models follow the structure:

$$\hat{\boldsymbol{y}} = \boldsymbol{X}\hat{\boldsymbol{\beta}} \quad (4)$$

Where $\hat{\boldsymbol{y}}$ is a $(n \times 1)$ vector of the responses, $\boldsymbol{X}$ is an $(n \times p)$ matrix of the levels of the independent variables and $\hat{\boldsymbol{\beta}}$ is a $(p \times 1)$ vector of the estimated regression coefficient.

The regression model followed two hypothesis:

- The residuals are normally distributed, with mean $\mu = 0$.
- The residuals follow a random pattern.

If at least one of the above hypothesis is rejected, the whole model has to be rejected.

8. References

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