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PRODUCTION AND OPTIMIZATION OF EXTRACELLULAR AMYLASE FROM A NEWLY ISOLATED STRAIN OF *Bacillus mycoides*

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Abstract

Microbial enzymes are known to be superior to enzymes obtained from other organisms, particularly for applications in industries on commercial scales. The species of the genus Bacillus are known to be producers of enzymes of industrial interest. Among them, amylolytic enzymes have got great biotechnological applications and economic exploitations. Amylases are known to be produced by a variety of bacteria and fungi and their applications at industrial level have stimulated interest to explore their amylolytic activity in several microbes to be used as bioresources.
A newly soil-isolate, identified as a Bacillus mycoides strain, was tested for its ability to produce extracellular amylase in liquid media, using multiple carbon sources and starchy substrates. Chip electrophoresis was used to obtain the electrophoretic profile of proteins derived from the bacterial isolate and a molecular weight of 60 kDa, characteristic for amylase produced by Bacillus genus, was obtained in two experimental media. Bioprocess optimization was designed using L9 and L16 Taguchi orthogonal arrays and analyzed by ANOVA statistical methods. A maximum enzymatic activity (10.44 U/ml) was determined when malt extract and ammonium sulphate, as starchy substrate and nitrogen source, were used. Optimum growth conditions were identified to be 32°C, 220 rpm and 48 hrs fermentation time, while the inoculation volume was 2%. A positive effect for amylase production was observed for citric acid and $CaCl_2$ interaction in the culture media.

Key words*: Bacillus mycoides, amylase, biosynthesis, optimization, Taguchi.*

INTRODUCTION

Enzymes are considered an indispensable component in biological reactions; these catalysts are highly specific, faster than chemical catalysts and environmentally safe. They can be produced using natural sources and this is a valuable fact in order to achieve a sustainable development (Sundarram et al., 2014). Microbial enzymes generally meet the industrial demands (Naidu and Saranraj, 2013); they are easy to obtain in high amounts with a low-cost and short time production (Gopinath et al., 2017). Nowadays, amylases are one of the most studied groups of enzymes in biotechnology. A large number of amylases are commercially available and they have been industrially exploited for many decades, due to their biodegradability, which makes the processes cleaner for the environment. (Monteiro de Souza and Magalhaes, 2010, Naidu and Saranraj, 2013; Sundarram et al., 2014; Singh et al., 2015). The history of amylases dates since 1811, when Kirchhoff discovered the first enzyme of this type. In 1930, Ohlsson made the first suggestion regarding the amylase classification and he

grouped them in a- and b-amylase, respectively according to the sugars type produced by the starch digestive enzyme reaction (Naidu and Saranraj, 2013).

Microbial amylases were predominantly used in scientific research and in industrial sectors. Apparently, the primary enzyme produced for industrial level (in 1984) was an amylase which had a fungal basis; its role was to treat digestive disorders (Shanmugasundaram et al., 2015). A wide range of bacterial and fungal species were isolated and used in various industrial applications. Terrestrial isolates, such as *Aspergillus* sp. (*A. oryzae*, *A. niger*, *A. awamori*, *A. fumigates*, *A. flavus*) and *Penicillium* sp. or species belonging to the *Bacillus* genus (*B. subtilis*, *B. coagulans*, *B. polymyxa*, *B. mesentericus*, *B. megaterium* and *B. cereus*) are very well known for their commercial uses in this field (Oyeleke et al., 2010; Gopinath et al., 2017).

Amylases constitute approx. 25% of the world enzyme market; the scientific world recognizes the great biotechnological significance of these starch-amylolitic degrading enzymes, which almost completely replaced the chemical hydrolysis of starch in industrial processes (Monteiro de Souza and Magalhaes, 2010; Naidu and Saranraj, 2013). Amylase applications have expanded in many other fields, such as: clinical, pharmaceutical, analytical chemistry, foods, detergents, textile, paper and distilling industries (Monteiro de Souza and Magalhaes, 2010; Gopinath et al., 2017). All amylases act on starch and yield small units of glucose and maltose (Gopinath et al., 2017), but the most widely used amylases are α - and β- subtypes, which act on α -1,4-glycosidic bonds. α-amylases catalyze starch hydrolysis through the cleavage of α -1,4-glycosidic bonds (they are able to cleave those bonds which are present in the inner part of amylase or in the amylopectin chain); the end products are oligosaccharides with variable length (mixtures of maltose, maltotriose and glucose units) (Monteiro de Souza and Magalhaes, 2010; Riaz et al., 2013). β-amylases act on the nonreducing end of a polysaccharide chain and yield successive maltose units (Sundarram et al., 2014).

Mesophilic microorganisms are the most popular producers used in industrial enzymes application and the *Bacillus* genus is known as producer for amylase and protease with a significant industrial importance.

The production of bacterial amylases is usually carried out by submerged fermentation (SmF), in which natural and cheap substrates are preferred. Also, this type of process permits to optimize parameters like pH, temperature, incubation time, nutrient concentrations in an easier manner. Microbial amylases which are commercially available don't require complex downstream processing, because they can be used as crude preparation (except medical and pharmaceutical fields) (Monteiro de Souza and Magalhaes, 2010).

They are indispensable biological components in hydrolysis reactions with numerous advantages (especially for the starch industry). Therefore, new microorganisms with biotechnological potential regarding amylase production with new or improved properties are required.

The present study is focused on the *Bacillus mycoides* amylase production, starting from common methods for screening and continuing with process optimization. SmF is advantageous, because is an easily controlled process to control and also, depending on the strain and culture conditions, the enzyme can be inducible showing different producing patterns.

MATERIALS AND METHODS

Isolation of the bacterial strain

Bacterial isolate was obtained using standard pour plate method into nutrient agar (NA). 1 g of each freshly collected soil sample, from Dambovita County, was mixed with 9 ml of sterile distilled water for serial dilutions. The poured plates were incubated at 32°C for 48 hrs. Pure isolate was maintained on NA slants and stored at 4°C, for further studies.

Screening for amylase production

The bacterial strain was tested for amylase activity by employing zone clearing technique using nutrient agar medium supplemented with 1% soluble starch. The use of starch nutrient agar and iodine for detecting amylase producing microorganisms and the presence of clear zones surrounding microbial colonies as

evidence of starch hydrolysis have been reported since 1974 by Iverson and Fogarty, respectively (Singh et al., 2015). Inoculated plates were incubated at 32°C for 48 hrs and then they were checked for amylase production by addition of Lugol's solution (1% iodine in 2% potassium iodide, w/v). Clear zones of starch hydrolysis surrounding the colonies were measured (blue colour was evidence of negative results) (Lamabam et al., 2010; Sirohi and Prakash, 2015), two colonies showing large halo zones were selected for further studies.

Identification of the bacterial isolate

The microbial strain was identified using MALDI-TOF mass-spectrometry (MS) – a "shotgun" type proteomics technique for direct protein fingerprinting of bacteria. Based on the specificity of the mass spectrum for a large number of bacteria, fungi and yeasts, the dedicated MALDI Biotyper software identifies microorganisms by analysing the expression of the most abundant ribosomal proteins from the acquired mass spectra. The pattern of ribosomal protein expression is automatically compared by the software with a large number of reference patterns from its database. MALDI Biotyper analysis generates a characteristic mass and intensity distribution of those proteins and uses them to identify unknown samples by comparing their "fingerprint" with the patterns included in its open database (Tomulescu et al., 2015).

Amylase production using submerged fermentation (SmF)

Inoculum preparation – the isolate, 48 hrs actively growing culture, was used to inoculate 50 and 100 ml of IA medium (nutrient broth with 2% soluble starch), which were prepared in 500 ml Erlenmeyer flasks. IPS medium composition (g/L) was used for submerged fermentation: glucose 10.0, yeast extract 2.0, KH_2PO_4 2.0, citric acid 1.0 and $MgSO_4*7H_2O$ 0.5 and pH 7.5. After inoculation, the flasks, were incubated at 32°C for 24 hrs on a rotary shaker (220 rpm).

Fermentation media contained various sources of carbon (cornflour, starch, glucose, maltose, malt extract) and nitrogen (ammonium sulphate and peptone) in different concentrations. Also, calcium chloride was used as an enhancer for amylase production. Inoculation volume was tested for 1, 2, 3, 10 and 20%. Cell growth and enzyme activity were studied taking into account the incubation period and the content of reducing sugars in production media. The classical method for medium optimization – changing one independent variable at a time and statistical experimental designs - L9 and L16 Taguchi orthogonal arrays, were applied to investigate the effects of different factors and interactions, on the amylase enzymatic activity; the significance of the effect of each factor and interaction was determined by ANOVA analysis. Trials were performed in duplicates or triplicates and the averages of amylase activity results were treated as responses.

Amylase assay. The extracellular enzyme solutions were obtained by centrifugation at 5000 rpm for 20 minutes at 4°C, using a high speed centrifuge. The cell free supernatants were collected and used to demonstrate the amylase activity, which was assayed as described by Bernfeld – the liberated reducing sugars were estimated by DNS method, with 3,5 – dinitrosalicylic acid. The reaction mixtures containing enzyme solutions and soluble starch as substrate (prepared in phosphate buffer, pH 6.9) were incubated at 30°C for 10 minutes. The reactions were stopped by adding 3,5 – dinitrosalicylic acid solution and then, these mixtures were heated again in a boiling water bath for 5 minutes and cooled at room temperature to develop brown colour. Absorbance of each sample containing the brown reduction product was measured at 546 nm in a UV-visible spectrophotometer. One unit of amylase activity was defined as the amount of the enzyme required to produce one µmole of maltose from starch, under the assay conditions.

Chip electrophoresis was used to obtain the electrophoretic profile of proteins derived from the bacterial isolate. The protein assay was performed on the chip in conjunction with Agilent 2100 Bioanalyzer, which allows software integration and multiple steps automate of electrophoresis (sample handling, separation, staining, discoloration and detection) combined with digital processing of data. Agilent Protein 80 Kit ladder was used to ensure the separation of the proteins (in the

range 5-80 kDa). The free supernatant was recovered by centrifugation of fermentation medium, at 6000 rpm for 20 min at 4°C. Samples were prepared with 4µl of supernatant and 2µl protein denaturing solution; the mixtures were incubated in a water bath at 95°C for 5 minutes and then diluted with 84µl double distilled water. Each sample was injected sequentially in the capillary, in which the protein separation was performed on the basis of their size; the protein detection was performed using a fluorescence detection system, at 630 nm.

RESULTS AND DISCUSSIONS

The soil environment is a good source for valuable microorganisms. Amylases are widely distributed and are ones of the most studied enzymes; they have a wide scale of applications, being capable to digest the glycosidic linkage found in starch or glycogen while, under aqueous conditions, liberating glucose, maltose and maltotriose. Nowadays, the renewed interest in the exploration of extracellular amylase production in bacteria and fungi is due to various industrial applications ranging from food to effluent treatment (Sankaralingam et al., 2012).

Identification of bacterial isolates

Based on the morphological, physiological and the most important, by the MS analysis, the bacterial isolate was identified as *Bacillus mycoides*. Fig. 1 shows the mass spectra of the identified isolate.

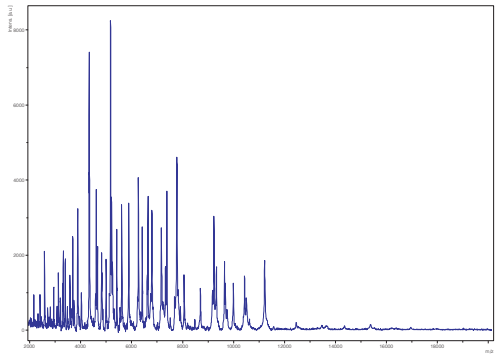

Fig. 1. The mass spectra of isolated bacterial strain identified as *Bacillus mycoides* (image generated with flexAnalysis v 3.3)

Enzyme production

Members of the genus *Bacillus* produce a large variety of extracellular enzymes of which amylases are of particularly significant industrial importance (Divakaran et al., 2011). The composition and concentration of media can greatly affect the growth and production of extracellular amylase in bacteria (Viswanathan et al., 2014). In this background, the production of amylase by submerged fermentation was investigated using over 30 biosynthesis media, in order to select the most suitable medium composition and to optimize it. To assess the optimal growth conditions, various sources of carbon (corn flour, wheat bran, rice flour, soluble starch, wheat starch, corn starch, malt extract, dextrin) and nitrogen (soy peptone, bacto peptone, yeast extract, soy flour, tryptone and NH_4SO_4), in different concentrations, were tested. Other factors, such as citric acid, $CaCl_2$ and inoculum volume, which could influence the enzyme production, were also investigated. The results of a first experiment showed a maximum amylase activity of 2.43 U/ml, using the *B. mycoides* as producing strain and medium F (corn flour - 10% as substrate, NH_4SO_4 1% as inorganic nitrogen source and 2% inoculum volume), under the following incubation conditions: 32°C, 220 rpm, and 48 hrs, fermentation time.

Chip electrophoresis

The bacterial amylase obtained in the experimental culture media, was subjected to an investigation with the Agilent 2100 Bioanalyzer to perform its electrophoretic profile (Fig. 2). For the assay, 5 variants of the medium composition were selected, in which enzyme activity was observed, namely: 4, 8, 15, 16 and 20, each consisting of two samples per chip. As a result of the electrophoretic profile of the 5 samples, the presence of amylase in the 15 and 16 medium was found, with 4% malt extract and NH_4SO_4/soybean meal 1%. The use of the bioinformatics resources of the European Informatics' Institute - EBI, in particular the UniProtKnowledeBase, allowed identification of the characteristic amylase for *Bacillus* sp. Based on the estimated molecular masses, the amylase from *B. mycoides* has shown a molecular weight of approx. 60 kDa.

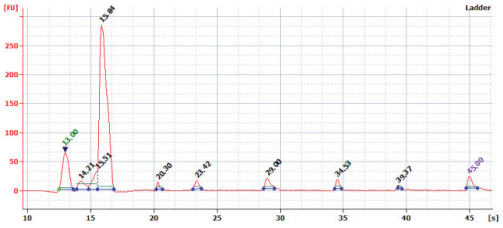

Fig. 2. a) Ladder of electrophoretic analysis

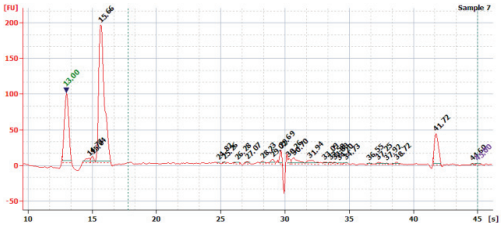

Fig. 2 b) Electrophoretic profile for sample 7: medium 15

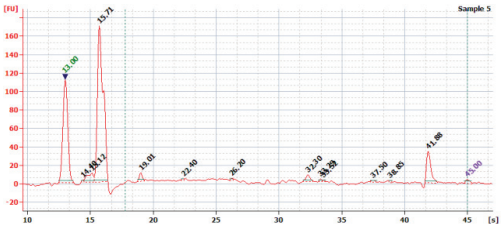

Fig. 2 c) Electrophoretic profile for sample 5: medium 16

Bioprocess optimization

Optimization of culture conditions is very important for maximum enzyme production. Due to the mesophilic nature of *B. mycoides*, 30-32°C was considered as optimum growth temperature, at pH 7.

It is known that high temperatures and pH values could inactivate the expression of the gene responsible for starch degrading enzyme (Nananganuru et al., 2012).

Amylase production depends on the characteristics of the culture and growth rate (Viswanathan et al., 2014).

Incubation time is an essential parameter to analyze. Fig. 3 shows a time growth experiment of *B. mycoides* using medium 15 (malt extract - 4%, NH_4SO_4 - 1%) and 2% level of inoculum, under optimum cultivation conditions.

A gradual increase was seen in cell growth from 4 to 36 h, after which a gradual decrease was observed.

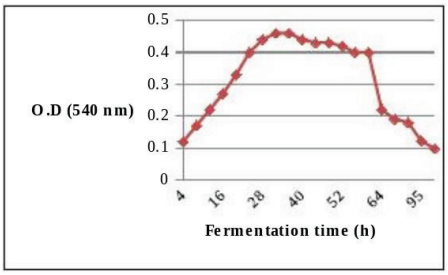

Fig. 3. Growth of *B. mycoides* with respect to time (hrs)

Thereby, incubation time is also a crucial factor for enzyme production and in all our experiments, 48 hrs was the best time for amylase activity, reaching its maximum (8.36 U/ml). Further increase in fermentation period did not have a positive effect on amylase production, rather it was decreased (4.84 U/ml); this fact could be happening due to the accumulation of other products in the cultivation medium (Hiteshi et al., 2014). Fig. 4 shows the amylase productivity, correlated with the biomass and fermentation period.

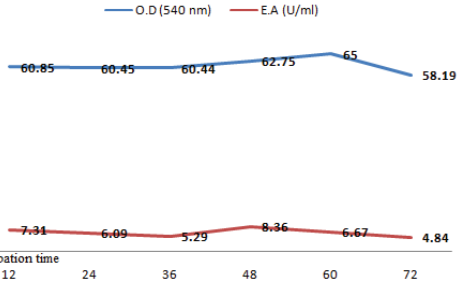

Fig. 4. Enzymatic activity in relation with incubation time and biomass

Unlike the conventional optimization method (changing one independent variable), using the Taguchi approach and ANOVA analysis as statistical optimization method offers a more balanced overview on factors influence and interactions, which also shows a targeted response(Sreenivas et al., 2004; Abel-Nabey and Farag, 2016).

In this paper, we describe the optimization of two selected fermentation media for alpha-amylase production, with the help of factorial design using Taguchi method; the experiments were planned to obtain a model consisting of 9, and 16 trials respectively.

The range and levels of the independent variables are presented in Table 1 and 2.

Table 1. Factors and levels for L9 Taguchi optimization model

Factor	Level I	Level II	Level III
A (malt extract)	2%	4%	6%
B ($(NH_4)_2SO_4$)	0.5%	1%	1.5%
C (inoculum volume)	1%	2%	3%
D (citric acid)	0.1%	0.2%	0.3%

Table 2. Factors and levels for L16 Taguchi optimization model

Level	Factor				
	A (1) Corn flour (%)	B (2) Ammonium sulphate (%)	C (3) $CaCl_2$ (%)	D (4) Citric acid (%)	E (5) Inoculum Volume (%)
1	12	0.5	0.05	0.15	5
2	15	1	0.1	0.2	10
3	18	1.5	0.15	0.25	15
4	21	2	0.2	0.3	20

Fig. 5 shows the significance of the effect of each factor and interaction, determined by the ANOVA analysis. Experimental results for L9 orthogonal array trials suggest that the most significant influence on amylase activity is given by malt extract, followed by ammonium sulphate: 84.67% and 12.06%, respectively. The maximum activity (4.97 U/ml) was found for trial 7, which contained malt extract 6% and $(NH_4)_2SO_4$ 0.5%.

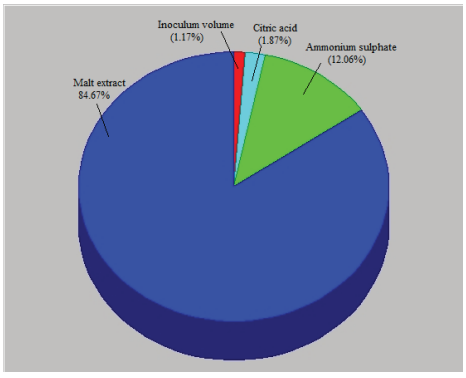

Fig. 5. ANOVA interpretation for L9 orthogonal array: main effects on amylase activity

Enzymatic activity was subjected to a multiple linear regression and the optimum conditions of the experiments were: malt extract 6 g/L, ammonium sulphate 0.5 g/L, inoculum volume 2 % and citric acid citric 0.1 g/L.

Fig. 6 shows the average values and standard deviation, obtained in all of the 16 experimental trials. Higher amylase activities were observed in medium 9 and 16, with 10.87 U/ml and 10.44 U/ml, respectively. The lowest activities, 5.76 U/ml and 6.17 U/ml were registered for medium 11 and 13.

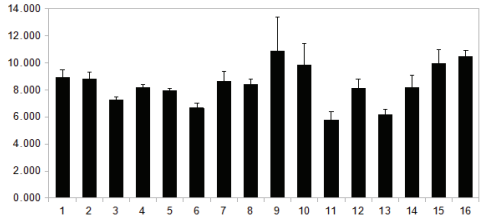

Fig. 6. L16 experimental design model: average values together with average standard deviation

Fig. 7 shows that the most significant contribution (55.54%) was observed for inoculation volume, followed by citric acid (22.83%) and ammonium sulphate (10.03%). An increased substrate concentration (corn flour) had not a relevant contribution to a higher enzyme activity. Following the ANOVA analysis, an interesting fact was observed: some interacting factor pairs, such as $CaCl_2$ - citric acid and NH_4SO_4 - citric acid, depending on their concentrations in fermentation medium, could influence the enzymatic activity. In our experiment, the influence was under 50% (43.64 and 36.91%), but a higher one could be obtained by using different factor levels, such as: 1 (0.05% $CaCl_2$) and 3 (0.25% citric acid) and 4 (2% $NH4SO_4$) and 3 (0.25% citric acid).

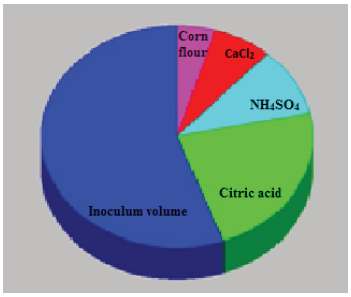

Fig. 7 L16 experimental design model: graphical representation of factors contribution to amylase activity

CONCLUSIONS

The production of economically-important amylases is essential for the conversion of starches into oligosaccharides; these enzymes are extensively used in the specific industry. Isolating and characterizing new strains, from different environments is a promising direction from the point of view of their biotechnological potential (Bozic et al., 2011).

The optimization of cultural conditions is very important to maximize the productivity and, in this regard, the present study focused on finding suitable conditions to optimal production of the required bio-product. The ability of the isolated strain to produce extracellular amylase was examined on solid and liquid media, with different inductors. Different carbon sources as starchy substrates, nitrogen sources and other medium components, such as citric acid, calcium chloride were adjusted in order to find the most suitable formula and to optimize it.

Further experiments will be done to purify the secreted enzyme, because the experimental results revealed an interesting perspective for this new soil isolated *Bacillus mycoides* strain, as amylase producer.

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FOOD SAFETY