
Increasing the efficiency of screening *Streptomyces* able to produce glucanases by using insoluble chromogenic substrates

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Abstract

The lignocelluloses biomass represents an unexploited resource. An increased interest for the hemicelluloses wastes bioconversion used in order to obtain unconventional energy and food resources was observed.

Nowadays, the screening method of the high active micro organisms, which are able to produce the enzymatic degradation of polysaccharides is the use of the plate assay method. This technique is very useful for the pre-selection and for the semi-quantitative selection of strains.

The screening methodology using the insoluble chromogenic substrates based on azurine-cross linked (AZCL) provides a specific, reliable and a rapid identification with an increased potential of polysaccharides (cellulose and xylan) bioconversion.

The aim of this study was to test the method of the plate assay the streptomyces screening which shows a high glucanase activity, using common and insoluble chromogenic substrates.

An increased efficiency of screening streptomyces able to produce glucanases was obtained by using the agar screening media supplemented with 0.05% AZCL-Cellulose or 0.05% AZCL-Xylan, comparing with common plate assay method which is more difficult to detect the active strains.

Keywords: *Streptomyces sp., strains selection, plate assay for screening, azurine-cross linked (AZCL), insoluble chromogenic substrates*

Introduction

The lignocelluloses biomass, as organic substrate, comprises various wastes such as:

- From agriculture (wheat straw, oat, barley, corn cobs, remnant vegetables)
- From food industry (as by-products from the sugar, fruits and vegetables industry)
- From wood industry (wood waste, sawdust)

Regarding the complexity of the substrates organic composition, the molecular mass of the polymeric compounds and solubility – properties responsible for the hemicelluloses wastes bioavailability – the controlled conversion presupposes the changing of the macromolecular structure using conventional techniques (chemical hydrolysis, physical treatments) or by using enzymes and to result free simple compounds (mono-disaccharide that can be transformed through diverse fermentative processes in products with economical value such as: single cell proteins, bio diesel, alcohols, solvents, other chemicals) (Figure 1) [3].

Actually, it is considered that the lignocelluloses biomass represents an unexploited resource. An increased interest for the hemicelluloses wastes bioconversion used in order to obtain unconventional energy and food resources was also noticed [7].

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Microorganisms (bacteria and fungi) able to produce polysaccharide-degrading enzymes are widespread in nature. The filamentous bacterial species included in the *Streptomyces* genus (widespread in soil) are able to produce different enzymes, associated in enzymatic complexes with hydrolytic activity on celluloses and hemicelluloses substrates. The biosynthesis potential as well as the catalytic properties of these enzymes depends on the strains capacity and on the biotechnological conditions of cultivation [4].

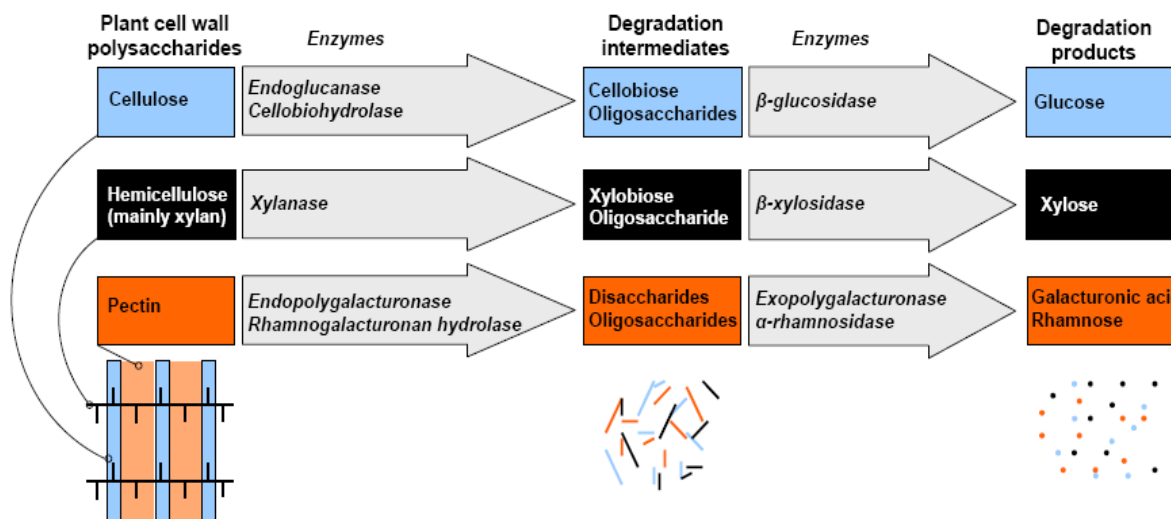


Figure 1. The structure of a plant cell wall and the most important enzymatic reactions involved in the degradation of its polysaccharides. Cellulose micro fibrils are cross-linked by hemicellulose chains within a matrix of pectin [6]

For the qualitative and semi quantitative screening of microorganisms able to produce cellulases and xylanases using the classical method, the results are not conclusive because of the difficult substrates consumption evaluation during their cultivation on agar media with cellulose or xylan in Petri dishes.

The increased efficiency of the selection criteria, the costs reduction and the investigated stages minimization are a result of the continuous improvement of the active strains selection methods [8].

A few recent researches about the screening methods based on the insoluble chromogenic substrates utilization revealed an increased fidelity of the results that offer the possibility to detect the high active strains in the primary screening directly. Sometimes because of the high prices of the insoluble chromogenic substrates their utilization is only as supplements in small concentration (0.05% - 0.2%) for the specific screening media [7]. During the hydrolysis, the substrates cross linked macromolecules are attacked together with the colorant particles and through polymer hydrolysis the colorant particles are free to become soluble and are able to form a blue zone around the microorganisms tested able to produce extracellular enzymes. The intensity of the blue zone depends on: colorant concentration, enzymes concentration and their catalytic properties.

The screening techniques application leads to the following advantages [2]:

- Allows the testing of a large number of strains and provides a rapid answer, easy to evaluate, regarding the selection of the high active strains.
- Enlarges the economical efficiency through the reduction of working stages, manual labor and also the reduction of the requirement of reagents and utensils.

- c) The commercial media are also available on Petri films thus being easier to manipulate, use and store.

The aim of this study was to increase the qualitative screening efficiency of the streptomycetes able to produce cellulases and xylanases by using a specific media supplemented with 0.05% insoluble chromogenic substrates based on AZCL. This method was compared with the common plate assay method that used a difficult criterion of the active strains identification.

Materials and Methods

Insoluble chromogenic substrates. Commercial chromogenic substrates were purchased from Megazyme International Ltd such as: AZCL-HE Cellulose, AZCL-Xylan (Birchwood), AZCL-Xylan (oat splets).

Microorganisms. A number of 97 pure cultures classified as *Streptomyces sp.*, which were isolated from Romanian soils during November 2007 – March 2008. For the selective isolation of the streptomycetes strains the soil samples were treated first with 1% CaCO₃ and after were incubated for 7 days at 25-28⁰C. Pure cultures were acquired by applying the specific isolation on Gauze media [2].

Culture media and screening procedures

Common media. The qualitative screening based on cellulases and xylanases production was made on basal Gauze agar media containing (g/L): K₂HPO₄ - 0.5; MgSO₄ 7 H₂O-0.5; KNO₃- 1.0; NaCl-0.5; FeSO₄ 2H₂O-0.01 and agar – 25.0 supplemented with 1% carboxymethyl cellulose or 1% oat xylan as a single carbon or nitrogen source.

Insoluble chromogenic plates. The used basal medium was prepared from: 0.5 yeast extract, 0.5 peptone, 0.5 casamino acids, 10.0 NaCl, 0.3 sodium piruvat, 0.3 KH₂PO₄, 0.3 MgSO₄ 7H₂O, 0.5 soluble starch and 15.0 agar and after that was supplemented with 0.05% insoluble chromogenic substrate functional on AZCL (the commercial products, AZCL-HE Cellulose and AZCL-Xylan powder, have been transferred in 96% ethanol and added into the basal media in the concentration of 0.05%). To abide the particles dispersed, autoclaved medium was agitated gently being poured into plates.

Semi-quantitative plate agar screening assay. To screen polymers-degrading streptomycetes by using classical methods, the *Streptomyces sp.* cells were inoculated “in point” on common agar plate surface in Petri dishes and were incubated for 7 days at 28⁰C.

To quantify the hydrolytic potential, the colourless zone on substrates with celluloses or xylan was evaluated. On the media supplemented with 0.5% chromogenic substrates the cultivation conditions were preserved and the hydrolysis zones were more visible due to the blue zones around each active colony.

The evaluation of the semi-quantitative screening was based on Substrate Hydrolysis Index (S.H.I) that was calculated as the ratio between the average diameter of the hydrolysis zone of substrate (d) and the average diameter of the bacterial colony (D), expressed in millimeters.

Results and Discussions

A number of 97 streptomycetes strains were tested for their capacity to biosynthesize active enzymes on cellulose and xylan through cultivation on the Gauze basal media

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supplemented with 1% celluloses and 1% xylan as a unique carbon source. After 168 hours of cultivation at 28°C, the active strains had a clear hydrolysis zone around the colonies when compared with the rest of the opaque media (Figure 2a).

It has been proved that the soil streptomycetes have a high capacity to biosynthesize enzymes able for celluloses and xylan bioconversion, as follows with: xylan activity – 13 strains; cellulose activity – 24 strains; combined xylan and cellulose activity – 55 strains and no xylan and cellulose activity – 2 strains.

By using the classical techniques, the results regarding the biosynthesis potential are not clear because the only evaluation criterion of the hydrolytic potential is the colony's diameter. However, the use of the insoluble chromogenic substrates based on azurine cross-linked, AZCL-HE Cellulose and AZCL-Xylan, displayed the clear hydrolysis zone of the substrate. In this situation the enzymatic activities of strains are more efficient evaluation (Figure 2b).

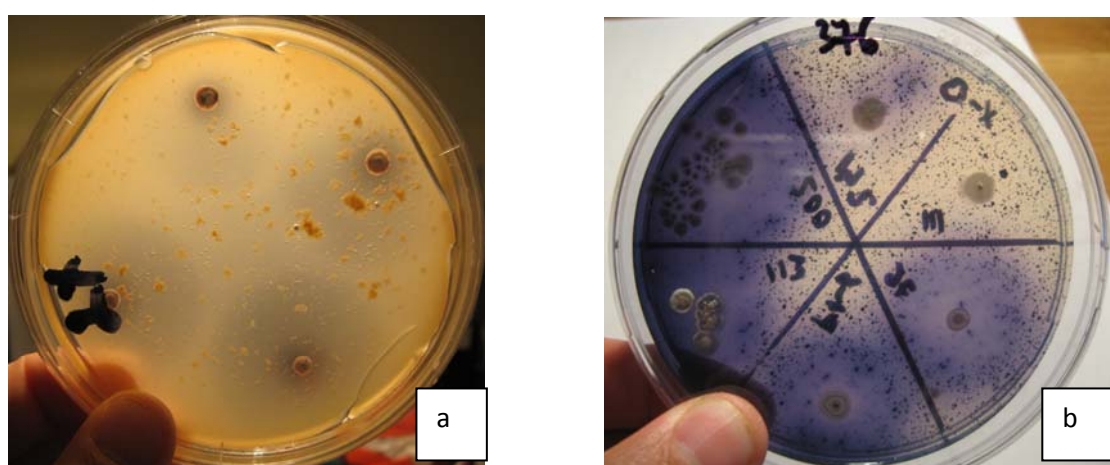


Figure 2. Enzymatic polymers hydrolysis zones development at *Streptomyces* sp. active strains with xylan and cellulose activities

a) classical technique; b) insoluble chromogenic substrates plate assay

After the “in point” inoculation and incubation during the 168 hours at 28°C, around the colonies' active strains (able to produce enzymes with activity over cellulose and/or xylan) a blue zone was developed. This blue zone was a result of substrate hydrolysis that forms oligomer fragments with various dimensions when the chromophore compounds are released in the agar medium. The blue zone's diameter was direct correlated with the concentration and the activity of the extracellular enzymes released by streptomycetes. A semi-quantitative evaluation of the Substrate Hydrolysis Index (S.H.I) can be achieved.

The performance of the most active selected strains through the classical screening method on Czapek basal media supplemented with 1% cellulose and 1% xylan it has been observed for 18 high active strains. These active strains were identified and coded as follows: active cellulases producers P6 – 30, P6 – 25; complex activity on xylan and/or cellulose P6 – 24; P7 – 50, P1 – 9, P4 – 51 and active xylanases producer with strains coded P7 – 72 (Figure 3 and Figure 4).

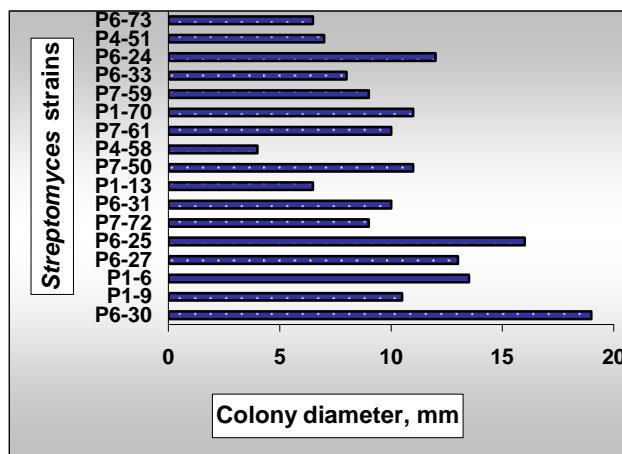


Figure 3 *Streptomyces sp* active strains able to cellulases biosynthesis

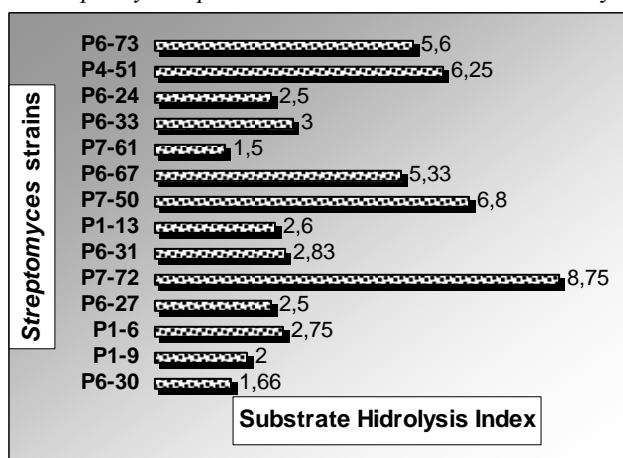


Figure 4 *Streptomyces sp* active strains able to produce xylanases biosynthesis

The enzymatic potential of the selected strains quantified by the Substrate Hydrolysis Index criteria by using direct cultivation of the streptomycetes on specific media supplemented with insoluble chromogenic substrates are depicted in Figure 5, Figure 6a and Figure 6b. A clear delimitation of the high activity strains was observed and also the variation of the biosynthesized enzymes spectrum with the substrate type was noticed.

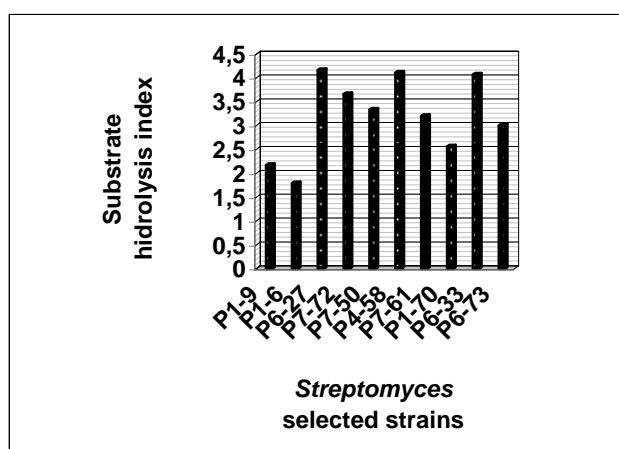


Figure 5. Cellulase activity potential of *Streptomyces sp.* selected strains, by cultivation on agar medium supplemented with AZCL – HE-Cellulose

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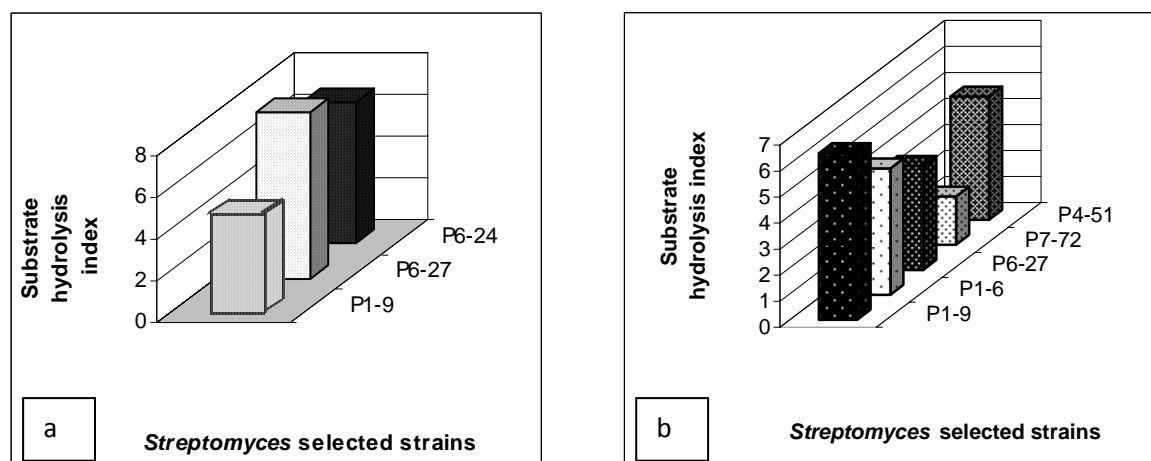


Figure 6. Xylan activity potential of *Streptomyces* sp. selected strains, by cultivation on agar medium supplemented with AZCL – Xylan (oat splets) (a) and AZCL – Xylan (Birchwood) (b)

Conclusions

1. Classical methods of pre-selection strains by using celluloses and xylose media as a unique carbon source are inefficient in the absence of specific detection criteria for the high enzymatic activity strains.
2. The media supplemented with insoluble chromogenic substrates increases the qualitative screening efficiency and allows the using of semi-quantitative evaluation (S.H.I defined as the ratio between the visible substrate hydrolysis zone's diameter and the colony's diameter).
3. The use of the agar media supplemented with azurine cross-linkage and specific substrates improves the fidelity of the screening criteria with a rapid detection of the high active strains (xylan, cellulose and combined xylan-cellulose hydrolyses activities).
4. From economical point of view the use of chromogenic substrates in the extra cellular enzymes selection is profitable because is easy to handle and no extra cost with reagents and equipments are necessary.

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