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ORIGINAL PAPER

Studies on the ecology of actinomycetes in an agricultural soil amended with organic residues: II. Assessment of enzymatic activities of *Actinomycetales* isolates

Mokni-Tlili Sonia · Belguith Hafedh · Hassen Abdennaceur · Gargouri Ali

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Abstract The main objective of this investigation was to study the enzymatic activities of *Actinomycetales* strains isolated from an agricultural soil amended with different amounts of municipal solid waste compost (MSWC) or farmyard manure (FM). For this purpose, the hydrolytic activities of carboxymethyl cellulase, xylanase, pectinase, amylase, chitinase and protease were tested for 75 isolates of *Sterptomyces*, *Amycolatopsis* and *Nocardioides* from different sources (unamended soil, amended soil with FM or MSWC, FM and MSWC) at temperature ranging between 30 and 50°C. It was shown that the highest rate of enzymes producer's strains was registered at 30°C, and decreased gradually to annul at 50°C, with the exception of the MSWC strains origin. It was also shown that the percentage of strains producers of enzymes isolated from soil amended with MSWC appeared higher than the one registered for those isolated from control and amended with FM soils. Application of MSWC increases the number of enzymes producer-actinomycetes in the soil and then it improves its fertility.

Keywords *Actinomycetales* · Soil amendment · MSW compost · Farmyard manure · Hydrolytic activities

M.-T. Sonia (✉) · H. Abdennaceur
Laboratoire de Traitement et Recyclage des Eaux Usées, Centre des Recherches et des Technologies des Eaux, Technopole de Borj Cedria, BP 273, 8020 Soliman, Tunisie
e-mail: mt_sonia@yahoo.fr

B. Hafedh · G. Ali
Laboratoire de Génétique Moléculaire des Eucaryotes, Centre de Biotechnologie de Sfax, BP "K", 3038 Sfax, Tunisia

Introduction

Because are foremost producers of a large number of antibiotics, enzymes and several other bioactive compounds, *Actinomycetales*, Gram positive filamentous soil bacteria were commonly used in several industrial applications (Ben Ameur et al. 2006; Goshev et al. 2005; He et al. 2010; Kumar and Kannabiran 2010; Qiu et al. 2010). *Streptomyces*, major members of the bacterial order *Actinomycetales* (see Part I of this paper) which play an important role in soil ecology (Xu et al. 1996), are heterotrophic feeders. They utilize both simple and complex molecules as nutriment by the secretion of a hydrolytic enzymes variety. Among them, proteases (EC 3.4.21.19), α-amylases (EC 3.2.1.1), CMCase (EC 3.2.1.4), xylanases (EC 3.2.1.8), pectinases (EC 3.2.1.15) and chitinases (EC 3.2.1.14) have been widely studied over the past few years (Deng et al. 2010; Feng et al. 2010; Kolcuoglu et al. 2010; Lee et al. 2010; Li et al. 2010; Poosarla et al. 2010). These enzymes degrade plant polysaccharides such as cellulose, hemicellulose, starch, and animal compounds such as chitin.

Enzymes with high thermal capacity were particularly attracted the attention of most researchers because of their considerable potential for numerous industrial applications. One extremely valuable advantage of conducting biotechnological processes at elevated temperatures is reducing the risk of contamination by common mesophiles. It can also increase the efficiency of the degradation process of substrate. Indeed, the substrates and products solubility increased and the medium viscosity decreased allowing an increase of the diffusion coefficients of substrates and therefore the reaction rate (Krahe et al. 1996; Kumar and Swati 2001). For example, in the paper industry the wood used for the production of the pulp is treated at high temperature and basic pH, which implies that the enzymatic procedures require proteins exhibiting a high

thermostability and activity in a broad pH range (Jacques et al. 2000). Treatment with xylanase at elevated temperatures disrupts the cell wall structure. This, as a result, facilitates lignin removal in the various stages of bleaching (Haki and Rakshit 2003).

Such enzymes are widely employed in food (Van der Maarel et al. 2002), detergent (Abidi et al. 2008), animal feed (Silva and Smithard 2002), agriculture (Lorito et al. 1993), medicine (Emami and Diamandis 2007), biotechnology (Gupta et al. 2002), textile (Milagres and Prade 1994), pulp and paper (Chen et al. 1997) and waste management (Aloise et al. 1996) applications.

Considering the importance of these enzymes and knowing that cellular components of thermophilic organisms (enzymes, proteins and nucleic acids) are also thermostable (Haki and Rakshit 2003), there is ongoing interest in the isolation of new bacterial strains producing hydrolases suitable at high temperature for new industrial applications.

In this study and at light of our previous work concerning the identification of the *Actinomycetales* dominant groups in soil (Part I of this paper), the present study aims at first to determine and to understand the effect of bacteria origin (soil, municipal solid waste compost (MSWC) or farmyard manure (FM)) on their enzyme activities, and secondly to evaluate the capacity of *Actinomycetales* isolates to the decomposition of substrate that can be found in soil and organic amendments. This study was started by a screening of 75 *Actinomycetale*s isolates, originated of control or amended agricultural soil, producers of proteases, of amylases, of CMCase, of xylanases, of pectinase and of chitinases activities.

Materials and methods

Soil and organic amendments

The soil samples were collected from an open field in the experimental farm of the Agronomic National Institute of Tunis (see Fig. 1—Part I of this paper). We recall here briefly that soil was treated with MSWC applied at 40 t ha^{-1} (C40), 80 t ha^{-1} (C80) and 120 t ha^{-1} (C120) and FM at 40 t ha^{-1} (F40) and 120 t ha^{-1} (F120). Samples were collected from each plot, thoroughly and aseptically mixed to give homogenous samples, and stored directly at 4°C prior to use.

Isolation of actinomycetes

A total of 24 soil, MSWC and FM samples were suspended in sterile water (10%) and agitated for 30 min at 420 rpm. The supernatant were serially diluted and plated on the glycerol–arginine–agar which contained (g l^{-1}) glycerol—20, Arginine—2.5, NaCl—1, $CaCO_3$—0.1, $FeSO_4{\cdot}7H_2O$—0.1, $MgSO_4{\cdot}7H_2O$—0.1, Agar—20. All colonies showing distinctive morphological characters were selected, purified and cryopreserved at −80°C in the appropriate liquid medium supplemented with 20% of glycerol.

The morphological characterization of isolates was investigated basing to *Bergey's Manual of Determinative Bacteriology* information (Lechevalier 1989). The molecular characterization was done on the basis of PCR–RFLP-sequencing of 16S rDNA gene methods (see part I of this paper).

Revelation of hydrolytic enzymes on agar plates

Among 281 actinomycete strains isolated from agricultural soil, FM and MSWC, a collection of 75 strains were randomly isolated: 15 isolates from each treatment: soil T (untreated soil), soil C, soil F, MSWC and FM. Selected strains were spotted onto agar plates (20 g l^{-1}, pH 7) containing: soluble starch (Sigma S2630), chitin (Fluka 22719), xylan from oat spelts (Fluka 95590), CMC (Sigma C4146) pectin (Sigma P9135) or gelatin (Sigma G9391) (all 1%) and yeast extract (5 g l^{-1}). Enzymes activities were revealed, after 5 days of incubation, by the appearance of clear zones using iodine for amylase activity, Congo red for CMCase and xylanase activities and 1% CTAB for pectinase activity. Chitinase and protease activities were revealed directly by the appearance of a clear zone by producer colonies.

Different activities were tested at temperature ranging between 30 and 50°C. Then, thermophilic *Actinomycetales* producers of enzymes were selected.

Fermentation conditions

Cultures which were able to produce clear zones for different enzymes in the agar plates at different temperature were subjected to submerged fermentation. Selected cells were cultivated for 9 days (pH 7, 30°C) in 250-ml Erlenmeyer flasks, maintained under agitation (150 rpm), containing 50 ml of the growth medium composed of: carbon source (starch, chitin, xylan, gelatin, manure or compost), 1%; K_2HPO_4, 5 g l^{-1}; KH_2PO_4, 1 g l^{-1}; $(NH_4)_2SO_4$, 1.4 g l^{-1}; Tween 80, 2 ml; $MnSO_4{\cdot}H_2O$, 0.161 g l^{-1}; $ZnSO_4{\cdot}H_2O$, 0.21 g l^{-1}; $CuSO_4{\cdot}5H_2O$, 0.051 g l^{-1}; $CoSO_4{\cdot}7H_2O$, 5×10^{-4} g l^{-1} and $FeSO_4{\cdot}7H_2O$, 0.051 g l^{-1}. The pH was adjusted to 7 before autoclaving. After sterilization, the medium was completed by the addition of 6 ml of 5% $MgSO_4{\cdot}7H_2O$ and 10 ml of 3% $CaCl_2{\cdot}H_2O$ (Nisole et al. 2006).

Preparation of crude enzyme extracts and enzymatic assays

Culture media (1 ml) were harvested every 24 h during 9 days and centrifuged at 7,000*g* for 10 min. Supernatants were than used as crude enzyme preparation. Amylase, chitinase and xylanase activities were determined using a

standard assay at 50°C for 30 min by measuring released reducing sugars from 1% appropriate substrate (w/v) in 100 mM phosphate buffer, pH 7. The amount of released reducing sugars was determined by the dinitrosalicylic acid (DNS, Sigma D0550) method (Miller 1959). D-Glucose, *N*-acetyl-D-glucosamine and xylose were used as standards. The absorbance at 540 nm (A540) was measured using a spectrophotometer (UV–VIS Dualbeam Model 2700). One unit of enzyme activity was defined as the amount of enzyme that released 1 µmol of reducing sugars, expressed in glucose (for amylase), in *N*-acetyl-D-glucosamine (for chitinase) and in xylose (for xylanase) equivalents per minute under the specified conditions. Protease assay with azocasein (Sigma A2765-56) hydrolysis was performed according to the protocol described by Lee et al. (1995) with the modifications made by Abidi et al. (2007). The commercial azocasein (Sigma Chemical Co., St. Louis, USA) was dissolved at 5% (w/v) solution in 100 mM phosphate buffer, pH 7. Aliquots (150 µl) of suitably diluted enzyme solution were added to 50 µl of 5% (w/v) azocasein in reaction buffer (100 mM phosphate, pH 7) and the mixture was incubated at 50°C for 30 min. The reaction was stopped by adding 600 µl of 10% (w/v) trichloroacetic acid (TCA) and left for 15 min on ice, followed by centrifugation at 13,000 rpm for 5 min to remove the precipitated protein. Supernatant (600 µl) was neutralized by adding 700 µl of NaOH (1 M) and the absorbance at 440 nm (A440) was measured. One unit of protease activity (UP) was defined as the amount of enzyme leading to an increase in absorbance (A440) of 0.1 per hour under the assay conditions.

Results and discussion

Isolation of actinomycetes

It was shown in the part I of this paper that the *Actinomycetales* isolates collection is composed by three families: *Streptomycetaceae* (72%), *Pseudonocardiaceae* (23%) and *Nocardioidaceae* (5%). A number of 62 isolates from the above identified families and 13 *Actinomycetales* isolates unidentified by sequencing method were selected to perform the enzymatic activities tests (Table 1). Some of these isolates, especially those belonging to *Streptomycetaceae* family were known by their important metabolic activities (*Streptomyces coelicolor*, *Streptomyces longisporoflavus*) and can be involved in the degradation of several polymers present in the organic matter composition (antibiotic and enzymes; Bielen et al. 2009; Usuki et al. 2009).

Revelation of hydrolytic enzymes on agar plates

Enzymatic activity tests on agar plates were made for the 75 selected strains. i.e. Fig. 1 showed the test result for some isolates originated from compost. A good zone of clearance, appeared within 5 days of incubation at pH 7 and 30°C, indicated that these strains can produce the following hydrolytic enzymes: CMCase, xylanase, amylase, pectinase, chitinase and protease. The ratio of the zone of CMC, starch, pectin and gelatin hydrolysis to the colonies diameter is high, indicating a good diffusibility of the enzymes.

Figure 2 showed the enzyme-producers-strains rates versus the tested incubation temperatures (30, 37, 45 and 50°C) for different strains origin (soil, MSWC or FM) and tested enzyme. It was clearly deduced that the enzymes production was strongly dependent on the work temperature and on the isolates origin. Then, some either the tested enzyme:

- The highest enzymes-producers-strains rate was recorded at 30°C whatever the substrate and the origin of strains. It varied between 20%, in the case of soil F-strains producer of pectinase (Fig. 2d) and 100% for the soil T and C-strains producer of CMCase (Fig. 2a). For this same temperature of 30°C, tested *Strepromyces* strains were rather producer of CMCase (strains rate $> 70\%$) and xylanase (strains rate $> 50\%$) than the other enzymes (strains rate $< 60\%$), except for those obtained from FM and MSWC.
- A slight increase of temperature from 30 to 37°C reduces the enzyme-producers-strains. As function of the origin, this decrease of the producer-strains ratio can be more or less important. Activities of FM-Strains were sharply decreased and almost totally inhibited whereas they were maintained (Fig. 2a, b) or increased (Fig. 2c–f) in the case of MSWC-isolates. For strains isolated from T, C and F soils, their activities were slightly decreased.
- Beyond 37°C, only some strains isolated from MSWC continued to produce enzymes. For 45°C, the strain rate production decreased to reach nearly 50%. This rate does not exceed 15% for 50°C.
- The ratio of producer strains originated from amended soils (C and F) was more important than the one registered for soil T-strains.
- Whatever the work temperature, the enzyme-producer-strains ratio is almost the highest for the one isolated from MSWC than those isolated from FM and soils T, C and M.

The effect of temperature on reducing the strains producer enzymes number can be explained by the inhibition

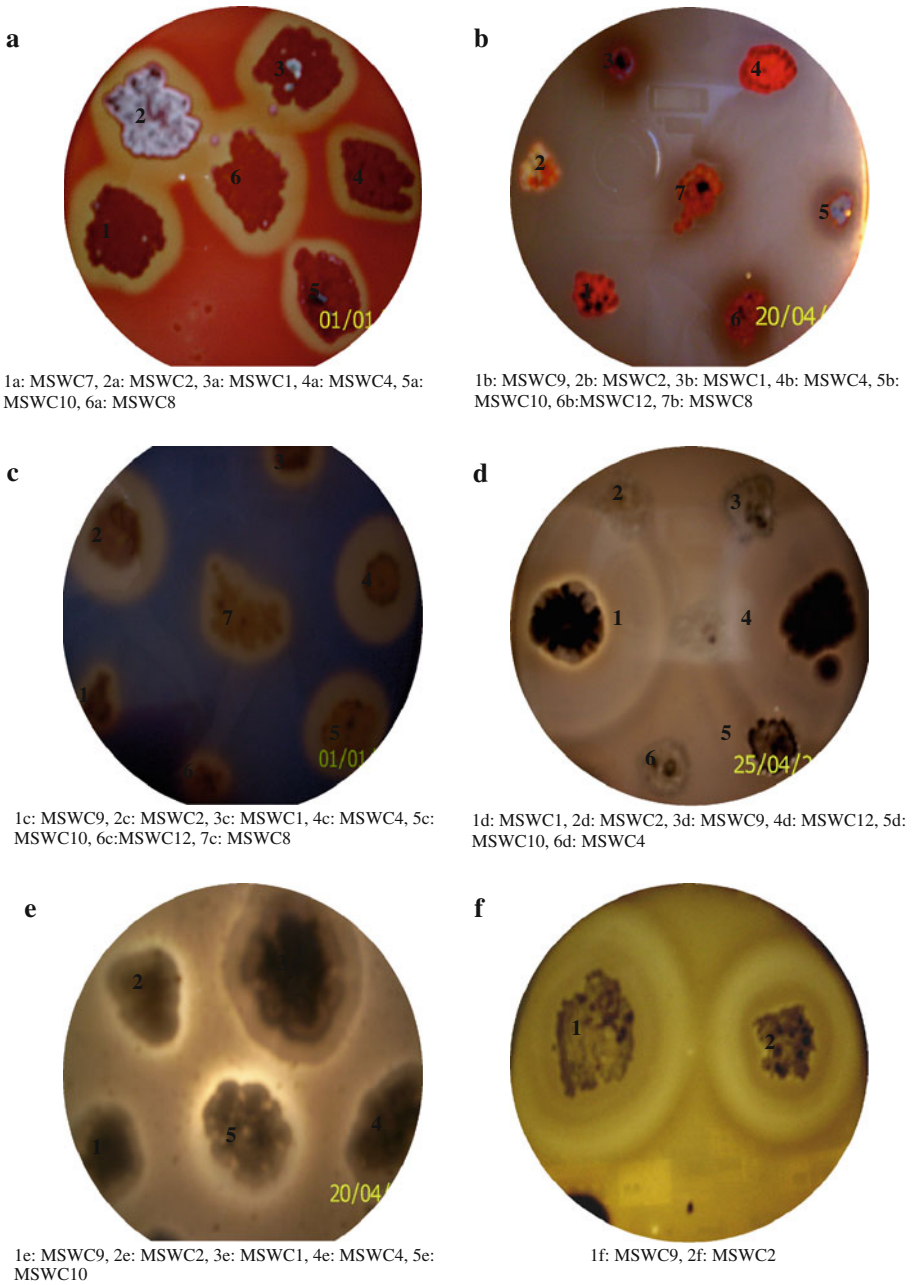
Table 1 Presentation of the species most related to tested isolates

References of tested isolates	Most related species	Phylogenetic group
T1	*S. venezuelae*	
T2	*S. globisporus-subsp.globisporus*	
T3, T4, T6, C5	*S. albidochromogenes*	
T5	*S. flavofuscus*	
T7	*Actinobacterium RG-51*	
T8	*S. narbonensis*	
T9, T14, T15, M14	*S. spiroverticillatus*	
T10	*S. exfoliates*	
M1, M15, FM15	*S. clavifer*	
M2	*S. fradiae*	
M3	*S. sp. B267*	
M4	*S. sp. Nm5*	
M5, M11, FM8	*S. coelicolor*	
M6	*S. sp. 3004*	
M7	*S. sp. AHW3*	
M9	*S. sp. P3562*	
M10, C2, C7	*S. spectabilis*	*Streptomycetaceae*
C1, C4, C14, FM12, MSWC15	*S. longisporoflavus*	
C3, FM2, FM3	*S. cavourensis*	
C22	*S. sp. L42*	
FM1, FM10, MSWC3	*S. collinus*	
FM4	*S. sp. 3004*	
FM5, FM7	*S. californicus*	
FM11, MSWC8, MSWC10	*S. sp. CHR28*	
FM14, MSWC5, MSWC6, MSWC7, MSWC14	*S. sacchari*	
MSWC1	*S. aureus*	
MSWC2, MSWC4	*S. sp. A528*	
MSWC9	*S. sp. AB654*	
MSWC11	*S. griseoaurantiacus*	
MSWC12	*S. azureus*	
FM9	*Amycolatopsis sp. WX001*	*Pseudonocardiaceae*
M8		
FM6		
FM13	*Nocardioides albus*	*Nocardioidaceae*
T11, T12, T13, M12, M13, C6, C8, C9, C10, C11, C12, C13, MSWC13	Unidentified	

of strains growth and/or activities (Fig. 2). Indeed, the data illustrated in the 9th edition of Bergey's Manual (Cross 1989) showed that the majority of *Actinomycetales* are mesophilic. They grow for temperature ranging between 25 and 30°C.

If temperature increases, only MSWC-strains maintain their activities (Fig. 2). Indeed, the MSW composting process occurred in a temperature ranging from 25 to 60°C. According to Albrecht (2007) the bacteria isolated from mature compost showed a large spectrum of growth and of activity in a wide range of pH and of temperature. Since, the actinomycetes appeared during the thermophilic phase as well as the cooling and maturation phase of composting process, these bacteria having a larger spectrum of temperature for the conversion of a wide range of natural substrates (cellulose, lignin) as compared to those from FM and soil (Chroni et al. 2009; Huang et al. 2010; Ryckeboer et al. 2003; Tuomela 2000).

On the other hand, the effect of the isolate source on the enzymatic production can be attributed to its chemical composition. Since the studied amendments contained a high proportion of organic matter with respect to soil



1a: MSWC7, 2a: MSWC2, 3a: MSWC1, 4a: MSWC4, 5a: MSWC10, 6a: MSWC8

1b: MSWC9, 2b: MSWC2, 3b: MSWC1, 4b: MSWC4, 5b: MSWC10, 6b:MSWC12, 7b: MSWC8

1c: MSWC9, 2c: MSWC2, 3c: MSWC1, 4c: MSWC4, 5c: MSWC10, 6c:MSWC12, 7c: MSWC8

1d: MSWC1, 2d: MSWC2, 3d: MSWC9, 4d: MSWC12, 5d: MSWC10, 6d: MSWC4

1e: MSWC9, 2e: MSWC2, 3e: MSWC1, 4e: MSWC4, 5e: MSWC10

1f: MSWC9, 2f: MSWC2

Fig. 1 Enzymatic activities of some *Streptomyces* isolates shown on agar plates containing CMC, xylan, starch, pectin, chitin or gelatin (all 5 g l^{-1}): yeast extract (5 g l^{-1}) and agar (20 g l^{-1}); pH 7.0, T 30°C. CMCase (**a**), xylanase (**b**), amylase (**c**), pectinase (**d**), chitinase (**e**) and protease (**f**) MSWC: strains isolated from municipal solid waste compost

(724 g/kg in manure, 404 g/kg in compost and 17.5 g/kg in soil; as reported by Ben Achiba et al. 2009), it is likely that such medium more favored the development of strains able to produce enzymes serving to their mineralization. This fact can explain the highest registered ratios of FM and MSWC originated strains producer enzymes, especially for 30°C (Fig. 2).

At the same temperature, it appears that the proportion of enzyme-producers strains from MSWC is the higher compared to the one isolated from soil (Fig. 2). This agree the results of Chroni et al. 2009, Huang et al. 2010 and Tuomela et al. 2000 showing that *Actinomycetales* originated from compost were considered among the bacteria responsible to the efficient degradation of cellulose and lignin. This was also observed for other bacteria originated from organic residues (Garcia-Gil et al. (2000); Madejon et al. (2003); Crecchio et al. (2004) and Benzarti et al. (2007)). In the case of CMCase and xylanase, the behavior of MSWC strains is due to the high content of cellulose, hemicellulose and lignin in the organic residues (Huang et al. 2010; Kumar et al. 2010; Paredes et al. 2002). Indeed, the microbial metabolism must be adapted to the environmental conditions.

In the same way, it was observed that the percentage of enzyme-producing strains originated from soil amended with MSWC is the higher compared to the one of the

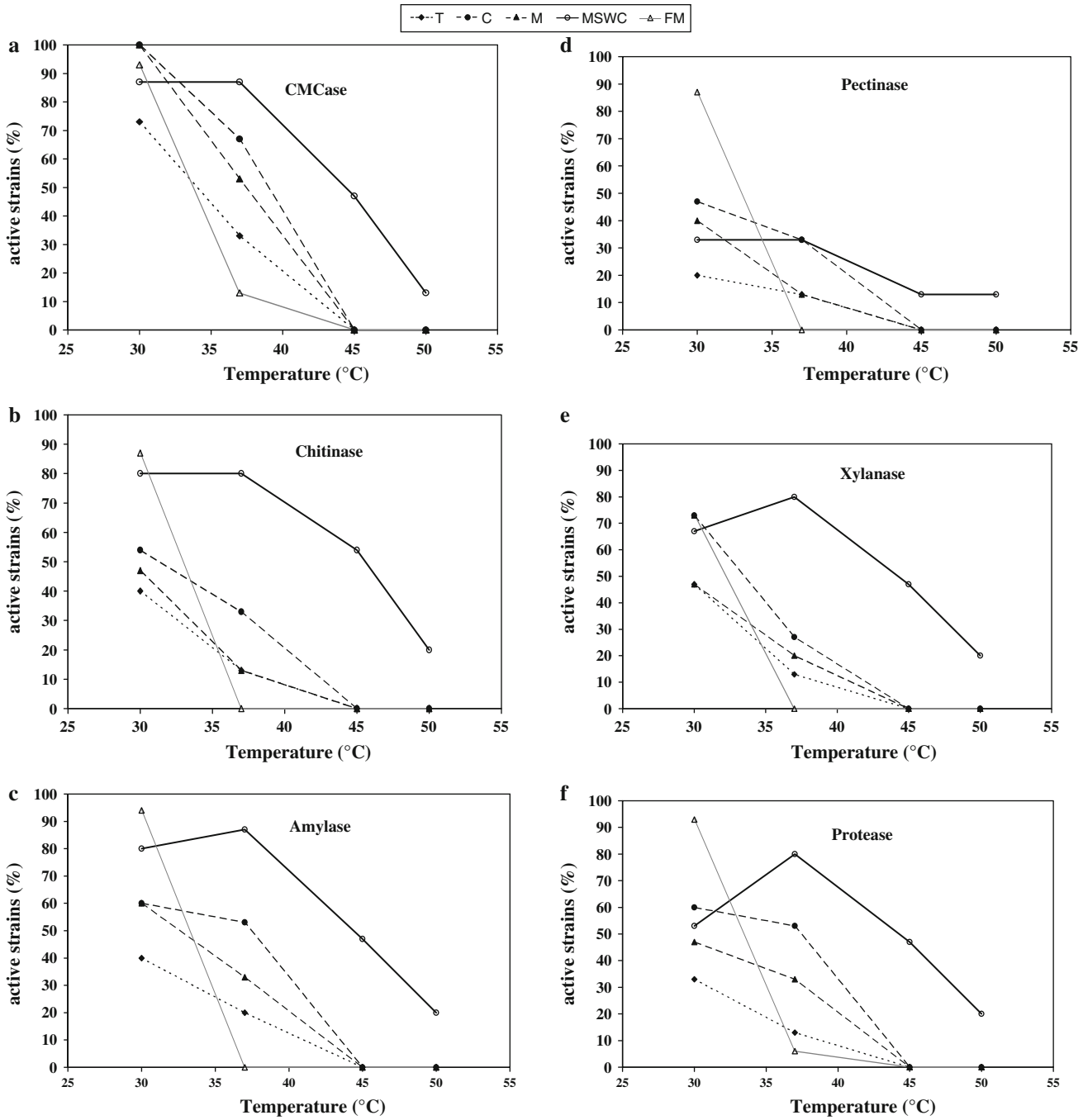


Fig. 2 Percentage of strains producer of CMCase (**a**), chitinase (**b**), amylase (**c**), pectinase (**d**), xylanase (**e**) and protease (**f**) isolated from soil T, soil F, soil C, MSWC and FM at different tested temperature

other soils. This finding was corroborated by several studies indicating that the enzyme activities were stimulated by the addition of organic amendments in soils (Crecchio et al. 2004; Serra-Wittling et al. 1996). Moreover, Pascual et al. (1998) showed that the organic amendment sufficiently added to a semi-arid soil increased significantly the enzymatic activity for at least 360 days. Therefore, the addition of mature compost to soil improves soil quality, promotes plant development and reduces the number of diseases caused by pathogens in soil (Cotxarrera et al. 2002).

For enzymatic production point of view, this comparative and qualitative investigation was often completed with a quantitative study by using broth culture (Ding et al. 2004; Techapun et al. 2002; Yue et al. 2008). Basing on the above presented results (Fig. 2), the strain MSWC1

Table 2 The maximum yield for tested enzymes

	Maximal enzymatic activity (IU/ml)		
	Adequate substrat	FM	MSWC
Chitinase	110	105	55
Protéase	290	44	40
Amylase	300	125	60
Xylanase	12,100	10,000	9,400

originated from compost and affiliated to *Streptomyces aureus* with similarity about 99% (see part I), was selected to determine its productive capacity of enzymes. Indeed, MSWC1 present the ability to produce enzymes in all tested temperature on agar plates.

Production of hydrolases in submerged cultures

Streptomyces sp. MSWC1 was cultivated on broth liquid medium under the above mentioned conditions and growth conditions yielding highest extracellular hydrolase activities were optimized. It was shown (Mokni-Tlili et al. 2010) that *Streptomyces* sp. MSWC1 produced a large amount of extracellular enzymes in growth medium with suitable substrate and reached the maximum level after 5 days in the case of amylase activity (300 IU ml^{-1}) and 3 days for chitinase (110 IU ml^{-1}), xylanase (12,100 IU ml^{-1}) and protease (290 IU ml^{-1}) activities. The originality of these findings was that the production values for xylanase and chitinase activities were more important compared to those found in other studies using other *Streptomyces* strains (Ding et al. 2004; Techapun et al. 2002; Yue et al. 2008). The substitution of the appropriate substrates with compost or manure decreases slightly the xylanase and chitinase and sharply the other enzymes production (Table 2). This was attributed to the toxic effects exerted by the heavy metals, especially present in compost (Mokni-Tlili et al. 2010). Despite this effect on enzyme production, the presence of these microorganism permit to valorize of biological wastes for the production of value-added products.

Conclusion

This work investigated the assessment of enzymatic activities of *Actinomycetales* isolated from an agricultural soil amended with MSWC and FM and amendments. It was clearly shown that the enzymes production was strongly dependent on the incubation temperature and on the isolates origin. Then, some either the tested enzyme:

- The highest enzymes-producers-strains rate was recorded at 30°C whatever the substrate and the origin of strains. The increase of temperature decreases the percentage of enzyme-producer-strains. For 45°C, the active strains rate decreased to reach nearly 50%. This rate does not exceed 15% for 50°C.
- The ratio of producer strains originated from amended soils (C and F) was more important than the one registered for soil T. In addition, whatever the work temperature, the enzyme-producer-strains ratio is almost the highest for the one isolated from MSWC than those isolated from FM and soils T, C and M. Therefore, MSWC appeared clearly as a stimulator of enzymatic activities and a potential source of a thermotolerant enzyme-producing *Streptomyces* having a wide industrial and biotechnological interest.

On the other hand, it was shown that *Streptomyces* sp. MSWC1 is able to produce hydrolases efficiently in the appropriate substrate, in manure and in compost. The high content of toxic heavy metals, especially in the compost, does not affect too much the hydrolase production by *Streptomyces* MSWC1 strain, which is especially efficient in producing xylanase and chitinase activity. These hydrolytic activities produced by *Streptomyces* are important in the regulation of the ecosystem. In fact, they catalyze several essential reactions for the life processes of micro-organisms in soils. Therefore, they play a key role in the stabilization of soil structure, in the decomposition of organic wastes, organic matter formation and in nutrient cycling.

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