African Journal of Microbiology Research Vol. 6(47), pp. 7377-7382, 11 December, 2012
Available online at http://www.academicjournals.org/ajmr
DOI: 10.5897/AJMR12.243
ISSN 1996-0808 ©2012 Academic Journals

Full Length Research Paper

Novel complex organic substrates for cultivation of bacteria like *Bacillus mojavensis* A21

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Accepted 6 September, 2012

The media containing new complex organic substrates suggested that *Mirabilis jalapa* tuber powder (MJTP), shrimp wastes powder (SWP) and sardinella peptone could be used as an alternative source of cheaper carbon and nitrogen substrates for production of enzymes by *Bacillus mojavensis* A21. The use of 40 g/L MJTP as a sole carbon and nitrogen sources resulted in a proteases and α-amylases production of 730 and 3 U/ml, respectively. A two fold higher production of proteases by A21 strain was achieved with 4 g/L of sardinella peptones added in the MJTP medium. More interestingly, SWP supplementation in a medium containing of MJTP resulted in maximum growth and highest α-amylase production.

Key words: Complex organic substrates, proteases, α-Amylases, *Bacillus mojavensis* A21.

INTRODUCTION

The expansion of biotechnology has created an increasing demand for new and inexpensive microbial growth media. The growth medium accounts for approximately 30 to 40% of the production cost of industrial enzymes (Joo and Chang, 2005). Considering this fact, the use of cost-effective growth medium can significantly reduce the cost of enzyme production (Gessesse and Gashe, 1997). The most important industrial enzymes in use today belong to hydrolase group, including proteases and amylases enzymes. Amylolytic enzymes find applications in food, baking, textile, paper and detergent industries (Pandey et al., 2000). Proteolytic enzymes are used in the various industrial processes such as detergent, dehairing, decomposition of gelatin on X-ray films and peptide synthesis (Joo et al., 2005).

Several studies have been undertaken to define ideal culturing and nutritional conditions for obtaining higher yields of α-amylase and/or proteases. Hmidet et al. (2010) reported the co-production of a thermostable α-amylase and alkaline proteases by *Bacillus licheniformis* NH1 strain grown on chicken feathers as carbon and nitrogen sources. For this purpose, powder prepared from *Mirabilis jalapa* tubers was recognized as a potentially useful and cost-effective medium ingredient. *M. jalapa* is cheaply cultivated in Tunisia. Chemical analysis showed that tubers are rich in organic and inorganic compounds, suggesting it as a good candidate for culture media. On the other hand, marine by-products has also been used to a minor extent as a fermentation substrate for protease production, despite its availability in large quantities and its low cost. The use of sardinelle fish powders as bacterial substrate for the production of the protease by *Bacillus cereus* BG1 was reported by Sellami-Kamoun et al. (2011). Fish visceral waste are an excellent source of proteolytic activities (Murado et al., 2009), marine peptones for supporting bacteriocin production (Vázquez et al., 2004a) recovery of carotenoid from shrimp biowaste (Bhaskar et al., 2007), lactobacillus growth (Aspmo et al., 2005; Horn et al., 2005), biomass production from marine bacteria (Vázquez et al., 2004b), hyaluronic and lactic acid production (Vázquez et al., 2009), recovery of oil from viscera (Raia et al., 2010) and also microbial enzyme production (Vázquez et al., 2006; Rebah et al., 2008)

This paper describes the preparation and the use of powders from *M. jalapa* tubers, shrimp wastes and sardinella peptone as a new complex microbial growth sub-

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Table 1. The main chemical composition of MJTP and SWP (%).

Components	MJTP	SWP
Proteins	17.3 ± 3	39.6 ± 1.5
Ash	20 ± 2	30.5 ±
Lipids	0.5	2 ± 0.04
Chitine	-	19 ± 0.5
Starch	32.6 ± 2	-
Polysaccharides without starch	20.3 ± 2	-
Lignin	6 ± 0.5	-
Ca^{2+}	5.04	5.5
K^{+}	0.3	0.22
Na^{+}	0.78	0.3
PO_4^{2-}	0.56	-
Cl^{-}	0.24	-
Mg^{2+}	-	0.19

substrate for the co-production of protease and α-amylase by *Bacillus mojavensis* A21.

MATERIALS AND METHODS

Bacterial strain

B. mojavensis A21 producing a detergent-stable alkaline proteases was isolated from a marine water sample (Haddar et al., 2009). The strain is available in the strain collection of "Center of Biotechnology, Sfax-Tunisia" and assigned an accession number CTM 50616.

Cultivation and media

Inocula were routinely grown in Luria-Bertani (LB) broth medium composed of (g/L): peptone, 10; yeast extract, 5; NaCl, 5 (Miller, 1972). The initial medium used for protease and α-amylase co-production (M1) was composed of (g/L): carbon source, 10; ammonium sulfate, 2; K_2HPO_4, 0.1; KH_2PO_4, 0.1; $CaCl_2$, 0.5; pH 8.0. Media were autoclaved at 120°C for 20 min. Cultivations were conducted in 25 ml of medium in 250 ml conical flasks maintained at 37°C. Incubation was carried out with agitation at 200 rpm for 24 h. The cultures were centrifuged and the supernatants were used for estimation of proteolytic activity.

Cell growth determination

The growth of the microorganism was estimated by the determination of colony-forming units (CFU/ml). This procedure involves making decimal serial dilutions of the sample in sterile physiological water. Nutrient agar plates were then incubated at 37°C for 24 h.

Preparation of powder substrates

To obtain *Mirabilis jalapa* tuber powder (MJTP), raw material was peeled, grinded then dried at 80°C for at least 5 h. The dried preparation was minced again to obtain a fine powder, and then stored in glass bottles at room temperature. In order to obtain shrimp wastes powder (SWP), raw material collected from the marine food processing industry was washed thoroughly with tap water and then cooked for 20 min at 100°C. The solid material obtained was dried and minced to obtain a fine powder.

Sardinella meat (*Sardinella aurita*) (500 g) were first minced then cooked to inactivate endogenous enzymes. The cooked meat sample was mixed with an equal amount of distilled water and homogenized in a Moulinex® blender for about 2 min. The pH of the mixture was adjusted to the optimum activity value for the proteolytic enzyme. After the required digestion time, the reaction was stopped by heating the solution at 80°C for 20 min to inactivate the enzyme. The fish hydrolysate was centrifuged at 5000 g for 20 min to separate insoluble and soluble fractions. Finally, the soluble phase was lyophilized. Samples were stored as sardinella peptones.

Protease and α-amylase activities

Protease activity was measured by the method of Kembhavi et al. (1993). One unit of protease activity was defined as the amount of enzyme required to liberate 1 µg of tyrosine per min under the experimental conditions. α-Amylase activity was estimated by determination of the reducing sugars liberated from starch hydrolysis, determined by the dinitrosalicylic method (Miller 1959). One unit of α-amylase activity was defined as the amount of enzyme that released 1 µmol of reducing end groups/min.

Detection of enzyme activity by zymography

Protease activity staining was performed on SDS-PAGE according to the method of Garcia-Carreno et al. (1993). α-Amylase activity staining was performed by layering the SDS-PAGE gel on a thin 2% agarose-1% soluble potato starch gel and incubated the sandwich for 30 min at 50°C. Upon staining the agarose gel with iodine solution, protein bands with amylolytic activity became visible as a white band against a dark blue background.

RESULTS AND DISCUSSION

Chemical composition of complex substrates

The main chemical composition of MJTP and SWP is given in Table 1. These data show that MJTP is relatively rich in both organic and inorganic materials. Notably, it contains the essential substances required in microbial media such as sources of carbon, nitrogen and minerals. The powder has a relatively high starch content 32.6 ± 2%. The protein content is between 14 and 20%, by weight. The SWP contained higher protein content (40%), relatively high ash and chitin contents (30.5 and 19%, respectively), and lower lipid content (2%). The powders are very stable and may be conserved several years. The stability of MJTP and SWP could be explained by the low lipid content and particularly by the presence of antioxidants.

The chemical composition of elaborated peptones and defatted undigested fish protein were determined. All peptones had approximately the same composition (Table 2). Low moisture content (2 to 5%) might significantly contribute to stability of peptones. However, since sardinella is a fatty fish, the peptones had relatively high lipid content.

Table 2. Composition of raw material and fish protein hydrolysates.

Hydrolysate	Composition (%)			
	Dry weight	Ash	Protein	Lipids
Defatted undigested fish proteins	28.07 ± 1.96	1.075 ± 0.045	18.81 ± 0.877	2.98 ± 0.74
Peptone 1	98 ± 0.92	12 ± 1.3	75.7 ± 2	7.2 ± 0.65
Peptone 2	95.4 ± 0.43	13.5 ± 0.34	68.41 ± 0.07	9.28 ± 0.03
Peptone 3	94.28 ± 0.44	10.56 ± 0.4	69 ± 0.43	10.65 ± 0.13
Peptone 4	95.56 ± 0.32	12.3 ± 0.34	68.3 ± 0.53	11.56 ± 1.23

Peptones 1, 2, 3, and 4 were obtained using Alcalase® and crude extracts from *B. pumilus*, *B. mojavensis* and *B. subtilis*, respectively.

Table 3. Effect of different carbon sources on the co-production of α-amylases and proteases by *B. mojavensis* A21. Cultivations were performed for 24 h at 37°C in media consisting of (g/L): carbon source 10, ammonium sulphate 2, KH_2PO_4, 0.1 K_2HPO_4 0.1 and $CaCl_2$ 0.5.

Carbon source	Final pH value	Biomass ($\times 10^7$ CFU/ml)	Protease activity (U/ml)	α-amylase activity (U/ml)
Glucose	6.6	57	10	0.3
Starch	8.15	150	95	0.6
Hulled grain of wheat	7.85	263	87.5	1.54
Feather meal	9.0	38	70	2.9
MJTP	6.45	287	130	1.34
SWP	8.52	140	100	3.86

Effects of carbon sources on enzymes production

Proteases and α-amylases co-production were first tested in medium M1 containing different carbon source at a concentration of 10 g/L. As shown in Table 3, *B. mojavensis* A21 exhibited higher growth and productivity of alkaline proteases in culture media containing MJTP as carbon source (130 U/ml) followed by SWP (100 U/ml). Higher α-amylase activity was achieved with SWP (3.86 U/ml).

Since MJTP was the best carbon source for proteases synthesis by A21 strain, the effect of its concentration on alkaline proteases production was tested. The production of proteases was mainly favoured in the presence of MJTP concentration ranging from 20 to 60 g/L, while maximum enzyme activity (544 U/ml) was achieved with 40 g/L MJTP (data not shown). It also noted that bacterial growth increased with the increase of MJTP concentration. Few works reported the use of MJTP as carbon source for enzyme production. The growth substrate represents more than 30% of the industrial enzyme production cost (Hinman, 1994). Considering this fact, the use of MJTP in medium could significantly reduce the cost of proteases production. Protease production from *Bacillus* species using various agricultural residues (such as soybean meal, rice bran and wheat flour) was widely described in literature. Naidu and Devi (2005) reported the production of thermostable alkaline protease by *Bacillus* spp. K30 utilizing rice bran. Joo and Chang (2005) showed that maximum protease synthesis by alkalophilic *Bacillus* spp. I-312 was obtained when the bacterium was grown in a medium containing wheat flower and soybean meal as carbon and nitrogen sources, respectively. Soybean meal was also used as a nitrogen source for protease production by *Bacillus* spp. L21 in low cost producing media (Tari et al., 2006). The flours of different grains such as wheat, barley and rice have also been used in the fermentation medium to increase the productivity of α-amylase (Mamo and Gessesse, 1999). In the same context, processing by-products such as potato peels (Mukherjee et al. 2008) or low cost substrates such as hulled grains of wheat (Zouari et al., 2002) were suitable for metabolite production.

Effect of nitrogen sources on enzyme production by B. mojavensis A21 grown on MJTP

B. mojavensis A21 was grown in liquid medium in the presence of various nitrogen sources supplemented to MJTP. Ammonium sulphate, casein peptone, yeast extract and different sardinella peptones (P1, P2, P3 and P4) were screened as additive nitrogen sources. Protease and α-amylase activities were assayed after 24 h incubation. In these experiments, MJTP was taken at a constant level of 40 g/L. According to the results shown in Table 4, production in the absence of nitrogen source was relatively high about, 727 U/ml. This indicates that the strain can grow well on MJTP as the sole organic complex substrate and can obtain its carbon and nitrogen requirements directly from this material. Maximum protease production (1378 U/ml) and biomass (141×10^9 CFU/ml) increased with increasing P1 concentration and reached a maximum at 4 g/L (data not shown). *Bacillus*

Table 4. Effect of different nitrogen sources supplemented to the MJTP on the co-production of proteases and α-amylases by *B. mojavensis* A21. Cultivations were performed for 24 h at 37°C in media consisting of (g/L): MJTP 40, K_2HPO_4 0.1, KH_2PO_4 0.1, $CaCl_2$ 0.5 and different nitrogen sources at 2 g/L concentration.

Nitrogen source	Final pH value	Biomass (×10^9 CFU/ml)	Protease activity (U/ml)	α-Amylase activity (U/ml)
None (control)	7.38	50	727	2.91
$(NH_4)_2SO_4$	5.73	11	540	2.49
Yeast extract	7.71	83	945	4.04
Casein peptone	7.66	65	879	2.59
P1	7.66	120	1295	3.28
P2	7.80	116	1100	3.30
P3	7.68	103	1083	3.24
P4	7.66	115	1198	3.81

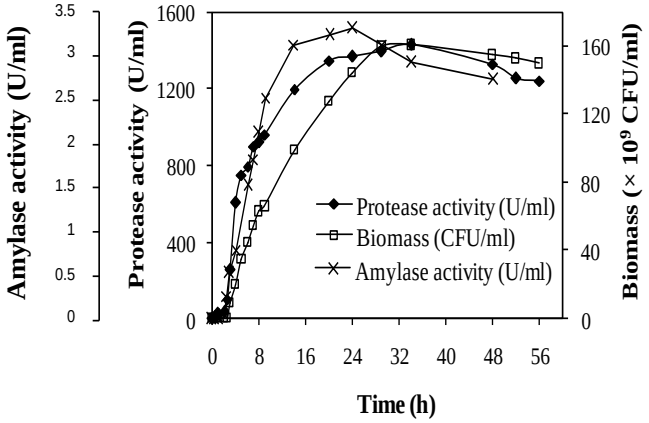

Figure 1. Time-course of proteases and α-amylases co-production and growth of *B. mojavensis* A21. Shaking cultivation was carried at 37°C. Protease and α-amylase activities were determined in culture filtrate obtained after removal of cells by centrifugation.

clausii produced 1520 U/ml of protease under optimized culture conditions (Oskouie et al., 2008) and maximum protease production (1939 U/ml) was obtained in the batch fermentation of a *Bacillus* sp. (Puri et al., 2002). However, protease production by *B. mojavensis* A21 is much better than protease production by *Bacillus* sp. L21 (306.5 U/ml) (Tari et al., 2006), *Bacillus* sp. (410 U/ml) (Patel et al., 2005) and *Bacillus* sp. RKY3 (939 U/ml) (Reddy et al., 2008).

Protein hydrolysates from different fish species such as tuna, cod, salmon and unspecified fish have been used as nitrogenous sources for microbial growth (Dufossé et al., 2001). Soluble fish protein hydrolysate from extensive hydrolysis comprises free amino acids and low molecular weight peptides, which can be used as excellent nitrogen source for microbial growth and thus can be used as microbial peptone. The peptides with the molecular weight of 6,500 Da, dipeptides and amino acids from tuna treated with Alcalase served as a suitable nitrogenous source in microbial media (Guerard et al., 2001). Protein hydrolysates from yellow stripe trevally have been produced successfully using Alcalase and Flavourzyme (Klompong et al., 2007a, b). Degree of hydrolysis (DH) can affect the functional properties and antioxidative activity of resulting protein hydrolysate (Klompong et al., 2007a). DH influencing peptide chain length may have the impact on the ease of utilization by microorganisms. In addition, different peptides produced by different proteases can be used by microorganisms at varying degrees. Recently, Klompong et al., (2012) descibed the efficacy of protein hydrolysate from yellow stripe trevally as microbial nutriaent varied with DH, enzyme and amount of hydrolysate used.

Pattern of proteases and α-amylases co-production by B. mojavensis A21 grown on MJTP and sardinella peptone

The pattern of proteases and α-amylases co-production, as well as bacterial growth, was followed with time. As reported in Figure 1, the strain grew well in the medium. The biosynthesis of proteases and α-amylases by the strain appeared to be growth-related, since activity was detected during the early stages of the growth, which exponentially increased at the end of the exponential phase of growth and then decrease during the stationary phase. These results are in line with those of Joshi et al. (2008), which showed that alkaline protease production by the S-20-9 strain started at the beginning of the stationary phase and increased during the middle of the stationary phase. However, many previous studies reported that protease production ends before the stationary phase (Kembhavi et al., 1993).

To give more information about the diversity of extracellular proteases and α-amylases secreted by *B. mojavensis* A21, zymogram analysis was done as described earlier in “Materials and methods”. Many enzymes, such as proteases and α-amylases, have proven to be renaturable after electrophoresis in the presence of SDS. *Enzymes of B. mojavensis A21* were renatured by the removal of SDS after polyacrylamide gel electrophoresis, and proteolytic and amylolytic activities were visualised

Table 5. Effect of combination of MJTP and SWP on the co-production of proteases and α-amylases by *B. mojavensis* A21. Cultivations were performed for 24 h at 37°C in media consisting of: MJTP, SWP, P1 (4 g/L), K_2HPO_4 (0.1 g/L), KH_2PO_4 (0.1 g/L) and $CaCl_2$ (0.5 g/L).

Parameter	MJTP (40 g/L)	MJTP (30 g/L) + SWP (10 g/L)	MJTP (20 g/L) + SWP (20 g/L)	MJTP (10 g/L) + SWP (30 g/L)	SWP (40 g/L)
Protease activity (U/ml)	1343	1556	1119	1004	1052
α-Amylase activity (U/ml)	3.25	4.1	5.17	5.42	5.53
Biomass ($\times 10^9$ CFU/ml)	135	210	250	255	142
Final pH value	7.48	8.12	8.82	9.09	9.17

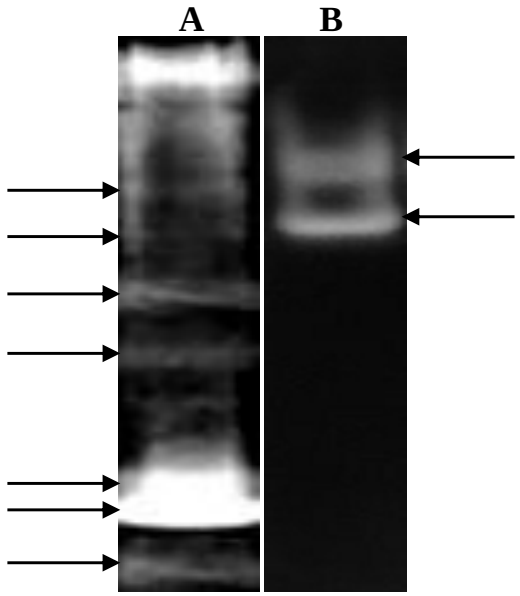

Figure 2. Zymogram activity staining showing proteolytic (A) and amylolytic (B) activities of the crude enzyme of *B. mojavensis* A21 cultivated in MJTP medium.

as a clear zone. Seven proteases were observed in the proteolytic activity profile of the cell-free enzymatic preparation of A21 strain (Figure 2A). However, starch zymography revealed the presence of a two bands of amylase activity, suggesting the presence of at least two amylases in the A21 crude enzyme (Figure 2B).

Effect of combination of MJTP and SWP on proteases and α-amylases co-production by B. mojavensis A21

As it was already shown, the higher level of protease synthesis was obtained with 40 g/L of MJTP and the production of α-amylases was mainly favoured in the presence of SWP (Table 3). The effect of addition of SWP in a medium containing of MJTP on improving proteases and α-amylases synthesis by the strain A21 was also studied. According to the results shown in Table 5, α-amylase activity was stimulated by adding of SWP, which showed growth-associated enzyme production. The induction of α-amylase activity, in medium containing SWP, may be due to the effect of inducible peptides or free amino acids resulting from shrimp proteins degradation by extracellular proteases produced by the strain. The higher co-production of both enzymes by *B. mojavensis* A21 strain was achieved in the presence of MJTP supplemented with SWP.

ACKNOWLEDGEMENT

This work was funded by the Ministry of Higher Education and Scientific Research, Tunisia.

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