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Partial purification and characterization of anti-leukemic L-Asparaginase produced by *Streptomyces hydrogenans* CA04 newly isolated in Algeria

Purification partielle et caractérisation de la L-Asparaginase anti-leucémique produite par Streptomyces hydrogenans CA04 nouvellement isolée en Algérie

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ABSTRACT

Introduction: In order to search for a new molecule of L-asparaginase with interesting industrial and analytical characteristics, we explored Lake Agulmim, located at 1700 meters' altitude in Mount Tikjda, part of Mountain range of Djurdjura (Algeria), for the isolation of actinomycete producing strain *CA04*. **Materials and methods:** After the molecular identification based in sequencing of 16S rDNA gene of our strain as *Streptomyces hydrogenans* CA04 and the demonstration of L-asparaginase activity, we extracted the extracellular interest enzyme at 90% ammonium sulphate followed by dialysis and separation by chromatography on Sephacryl S-200 gel. **Results:** We detected, therefore, two isoforms A and B of MW of 86 and 108KDa, eluted at 32min and 33min respectively, with a total protein level of 0.32mg/ml. An SDS-PAGE control was made showing the existence of the two isoforms with molecular weight mentioned. The L-asparaginase activity was maximal between pH 7 and 8, a temperature of 37°C, for 10min of reaction, with a specific activity of 7.28 IU/mg. On the other hand the activity is stable in the presence of Mg^{2+}, Cu^{2+}, Zn^{+} and EDTA, decreased by Fe^{3+} and inhibited by Mn^{+}. Finally, the L-asparaginase activity produced by *Streptomyces hydrogenans* CA04 has a high degree of specificity to the L-Asparagin substrate, with very weak relative activities, against the other nearby substrates, L-Glutamine and L-Aspartic Acid.

Keywords: Chromatography; L-Asparaginase; lake Agulmim in Algeria; SDS-PAGE; *Streptomyces*

RESUME

Afin de chercher une nouvelle molécule de L-Asparaginase ayant des caractéristiques industrielles et analytiques intéressantes, nous avons exploré le lac Agulmim, situé à 1700 mètres d'altitude dans le Mont Tikjda, en Algérie, pour l'isolement d'une souche d'actinomycètes *CA04* productrice. Après l'identification morphologique, biochimique et physiologique de notre souche et la mise en évidence de l'activité L-Asparaginase, nous avons extrait les enzymes d'intérêt à 90% en sulfate d'ammonium suivi d'une dialyse et d'une séparation par chromatographie sur gel de Séphacryl S-200. Nous avons détecté, de ce fait, deux isoformes A et B de PM 86 et 108KDa, élués à 32min et 33min respectivement, avec un taux de protéines totales de 0,32mg/ml. L'activité L-Asparaginase a été maximale entre pH 7 et

8, une température de 37°C, pendant 10min de réaction, avec une activité spécifique de 7,28UI/mg. D'autre part l'activité est stable en présence de Mg^{2+}, Cu^{2+}, Zn^{+} et EDTA, diminuée par le Fe^{3+} et inhibée par le Mn^{+}. Enfin l'activité L-Asparaginase produite par *CA04* présente un haut degré de spécificité au substrat L-Asparagine, avec des activités relatives très faibles, à l'encontre des autres substrats proches, L-Glutamine et Acide L-Aspartique. Ce qui est important pour sa propriété thérapeutique.

MOTS CLES : L-Asparaginase, Enzyme, Actinomycètes, Lac Agulmim en Algérie, purification partielle.

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Introduction

L-asparaginase (L-Asparagine amino hydrolase *EC.3.5.5.1.1*), was discovered forty years ago and recognized as an anticancer enzyme, particularly regard with lymphomas; when identified for the first time in pig serum [1]. Its mode of action based on the depletion of L-Asparagine, which inhibits the biosynthesis of proteins in cancer cells, thereby inhibiting their proliferation [2].

In contrast to normal cells in the body, the transformed lymphocytes show remarkable susceptibility to L-asparaginase because of their inability to produce L-Asparagine synthetase, an enzyme that catalyzes *De Novo* synthesis of L-Asparagine synthetase [3]. L-asparaginase of microbial origin is the most common; moreover, it is those produced by *E. coli* and *Erwinia* that are used in cancer chemotherapy protocols, as inhibitors of protein biosynthesis [4].

It has been reported, through experimental studies, that it is the LA of *E. Coli* that exhibits high immunotoxicity (including an immunosuppressive effect) [5]. This observation has led to the emergence of numerous scientific studies aimed at finding new LA molecules that are more effective and less toxic.

According to Warangkar and Khobragade [6], the majority of bacterial LA molecules is intracellular, and therefore depends on the biomass rate, which is highly dependent on physico-chemical growth conditions, namely the temperature and composition of the culture medium. For their part, Aly et al., [3], had certified that LA II located in the periplasmic space (between the plasma membrane and the cell wall) of actinomycetes, has a high affinity for cancer cells. This caught our attention, and motivated us to explore the power of our *CA01* strain, isolated from Algerian wheat bran and identified as belonging to actinomycetes, to produce an intracellular LA, and to study the effect of 3 selected factors on production yield, in order to optimize it.

Actinomycetes are microorganisms that are widely distributed in nature, including soils, waters and in association with plants [7], They are potential sources of L-Asparaginase (LA).

However, given the importance of side effects in treated patients, the search for new molecules of L-asparaginase less toxic is initiated through scientific works [8, 9]. In order to isolate the *CA04* strain, we thought of exploring a natural site known for its tourist vocation, in Algeria. This is Lake Agulmim, commonly known as Thamda Ougulmim, in the Kabyle language. It is a lake located at 1700 meters above sea level, in Mount Tikjda in the heart of the Djurdjura mountain range located at 36 ° 15 '53 "*North* and 4 ° 04' 26" *East* of the *Bouira* department (Algeria).

Material and Methods

1-2-Isolation of *CA04* strain

A dilution series of water from Agulmim Lake collected since November 2016 under aseptic conditions, was carried out until dilution 10^{-3}. Then 1 ml of this dilution was inoculated on ISP_2 agar [3] containing Nalidixic acid 50 µg/ml to inhibit the growth of Gram- bacteria and Nystatin 10 µg/ml, allowing inhibition of fungi [10]. The cultures are then incubated at 28°C for 7 days

[11]. After isolation, strain *CA04* was identified on the basis of these 16S rDNA sequence [12].

2.2. Molecular study

For performing molecular identification, we realized the sequencing of the 16S RNA gene of our strain in the *BIOFIDAL* laboratory (Lyon – France) according to the principle of Sanger et al. [13]. Sequences were aligned with *CHROMAS Pro* software to create the Contig Complete 16S gene.

1-3-Demonstration of L-asparaginase activity

Strain *CA04* was inoculated on optimized and modified Asparagin Dextrose Salt agar (ADS) [14] which contains Asparagine as the sole source of nitrogen. The culture was incubated at 25.8°C for 7 days. The result is considered positive if there are change of zone color from yellow to pink, around the bacterial colonies, caused by alkalinisation due to hydrolysis of L-asparagin. [15, 16].

1-4-Determination of total L-asparaginase activity

After centrifugation of bacterial culture realized in ADS broth, at 10000g for 20min at +4°C, the recovered supernatant served as a crude extract (CE) for assaying the enzyme activity. This is based on the detection of ammonia produced by hydrolysis of L-asparagin, using Nessler's reagent. To do this, we mixed 0.2ml of CE with 0.9ml of L-Asparagine *SIGMA* at 0.04M, solubilized in 0.5 M Tris/HCl, pH 7.2. The mixture is incubated at 37°C for 30min. We stopped the reaction by 0.5ml of 1.5M trichloroacetic acid. Then we added 0.2ml of the mixture to 1ml of distilled water and assayed with 0.5ml of Nessler's reagent. After 15min the absorbance was detected at 450nm [17, 18, 19]. A calibration curve was plotted using known solutions of ammonium sulphate as the source of ammonia [20].

1-5-Extraction and Partial Purification of Enzymatic Proteins

1-5-1-Recovery of the enzymatic crude extract

Large culture was launched in the same culture conditions at 500ml of optimized ADS broth, using an inoculum from a young culture grown on ISP_2 broth. After 7 days of incubation, the supernatant was recovered, following refrigerated centrifugation at 10000g (*SIGMA 4-16K*) for 20 min [21]. The supernatant was concentrated using a concentrating tube (*VIVASCIENCE MWCO 10,000 Da*). Protein assay was performed following the Bradford method [22].

1-5-2-Extraction by ammonium sulphate precipitation

Ammonium sulphate concentration ranging from 20% to 90% was added to the enzyme extract [23], and then incubated at +4°C, with gentle stirring until total solubilization of the salt [24]. Finally, a refrigerated centrifugation step at 10000 g for 20min was performed to recover the protein pellet after precipitation [21, 25]. The active fraction was dialyzed overnight at 4°C using membrane of 8Kda [26]. The dialyzed fraction was recovered for partial purification.

1-5-3-Separation by molecular exclusion chromatography on Sephacryl S-200

The dialyzed fraction was filtered on a 0.22µ membrane filter and deposited on a chromatography column (*Kontes™ Chromaflex™*) on *Sephacryl S-200*, previously stabilized with 0.02M Tris/HCl buffer, pH 8.4 [21]. After elution at a flow rate of 1 ml/min, all the collected fractions of 1ml each were subjected to the assays of the proteins and the L-asparaginase activity. The dead volume and the retention times of the active fractions were determined using Dextran Blue (*SIGMA Aldreich*). Absorption peaks were plotted and molecular weight (MW) were defined by plotting a correlation line of Log MW = f (retention time).

1-5-4-SDS-PAGE Electrophoresis Control

The eluted active fractions, corresponding to the enzymatic extract, were mixed and electrophoretically monitored (*Hoefler SE260 Vertical Vat*), according to the protocol of Leammli [27].

1-6-Characterization

In order to determine of the higher activity of the enzymatic isoforms, we studied the effect of temperature (30 at 80°C), pH (4 at 10), reaction time (5 at 60min) and effect of some co factors ($MgSO_4$, $CuSO_4$, $ZnSO_4$, EDTA, $FeSO_4$ and $MnCl_2$). In the other hand, we studied affinity of substrate, to do this, we replaced L-Asparagin by each of the two amino acids: L-Glutamine (0.04M) (*SIGMA*) and l-aspartic acid (0.04M) (*SIGMA*). The relative enzymatic activity to that recorded with the usual substrate has been noted and the affinity to the various substrates is thus estimated [28].

Results

In base of molecular study, the Contig performing study presented 99% of similarity with the *Streptomyces* genus and 99% of coverage of the 16S gene with *Streptomyces hydrogenans*. The complete 16S rRNA nucleotides sequence is available in NCBI GenBank with an accession number of: **SUB5200153 Contig_Souche-CA04_16S-Complet MK530175.** The figures 01 and 02 represent Sanger sequencing chromatogram and the taxonomic tree of our strain, respectively.

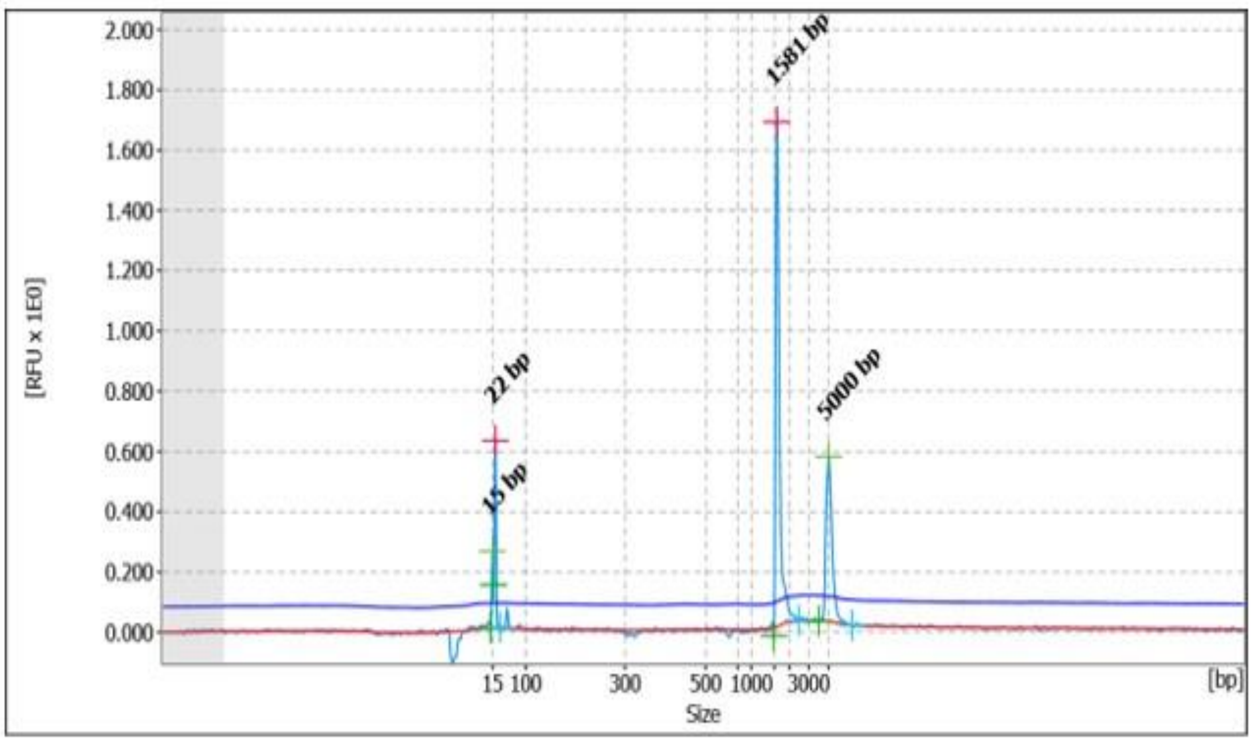

Figure 01: Sanger sequencing Chromatogram

After screening of our strain based in his production of L-asparaginase, the principle results of purification were discussed.

In the chromatographic separation of the obtained dialyzed fractions, we observe the existence of two peaks where the enzymatic activity sought, appeared (Figure 03). The retention times for both peaks are 33 min and 37 min, respectively. Since the two peaks are far apart, this indicates the existence of two L-asparaginase isoforms produced by the CA04 strain. On the other hand, the protein level as well as the enzymatic activity differs between the two isoforms. The isoform A_4 is more intense than the isoform B_4, which supposes that the rate of biosynthesis is different between the two molecules.

An SDS-PAGE electrophoretic migration was carried out in order to verify the presence of protein bands at the level of the MW found for the different isoforms. The figure 04 below shows the protein bands that appeared after SDS-PAGE analysis.

We also note, that the rate of proteins decreases through the various phases mentioned. The amount of final proteins obtained in the chromatography fractions is low: 0.07mg (for B_4 isoform). Nevertheless, the specific activity was mentioned at 2.31 IU/mg for isoform A_4 and 0.12IU/mg for isoform B_4. We note that the latter isoform is obtained with less important yield than 64.06% for the A_4 isoform. In fact, close yields in protein levels are commonly observed in the partial purification of L-asparaginase produced by actinomycete strains, as Selvam and Vishnupriya [29] recorded protein levels of 0.23 mg/ml in the crude extract, before extraction and purification and a purification yield of 12.05% of L-asparaginase produced by *Streptomyces acrimycini NGP*. However, the same authors had adopted molecular exclusion chromatography on Sephadex G-100 gel.

Figure 02: Taxonomic tree of the strain

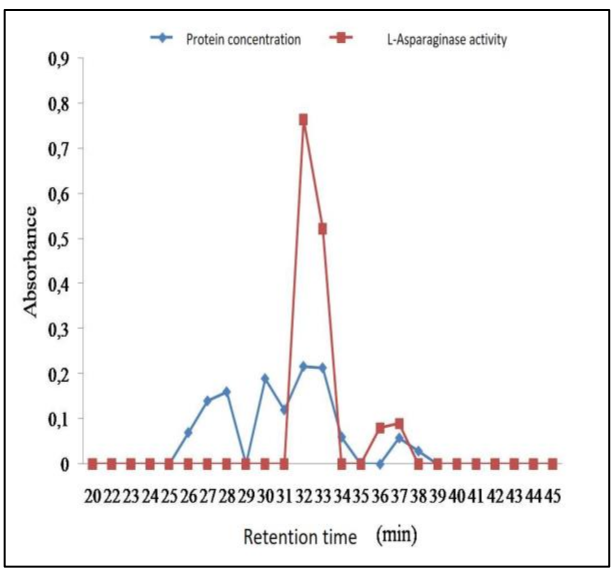

Figure 03: Chromatographic profile of L-asparaginase enzymatic isoforms produced by CA04 strain

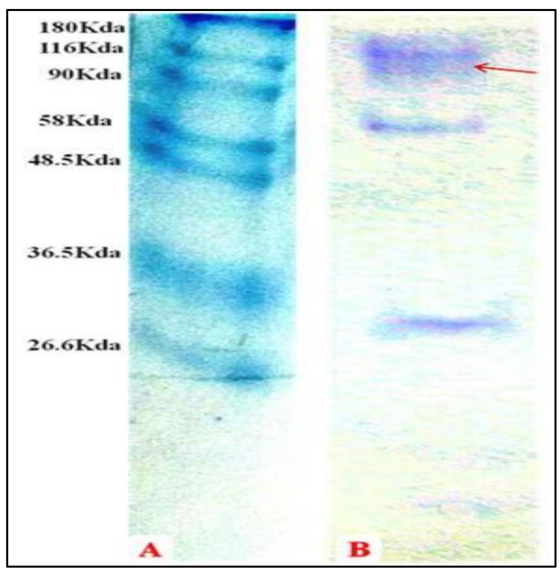

Figure 04: SDS-PAGE profiles of the two isoformes of L-asparaginase produced by *Streptomyces hydrogenans* CA04A: Standard proteins stained; **B:** Enzymatic active fraction of *S. hydrogenans* CA04

It appears that our A_4 enzyme presented the best activities at 37°C, pH8, 10min of reaction and in presence of all the co factors tested, except $FeSO_4$ and $MnCl_2$. We founded a complete disappearance of the activity in presence of the latter (shown in figures 05, 06, 07 and 08 respectively).

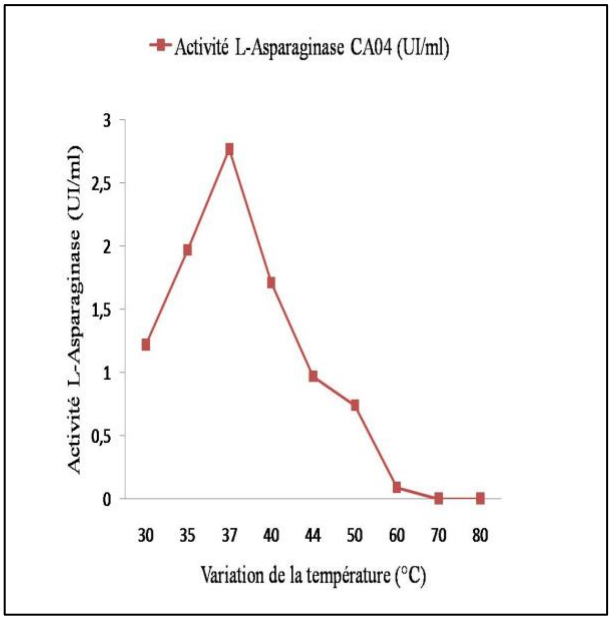

Figure 05: Effect of temperature variation in the L-asparaginase activity.

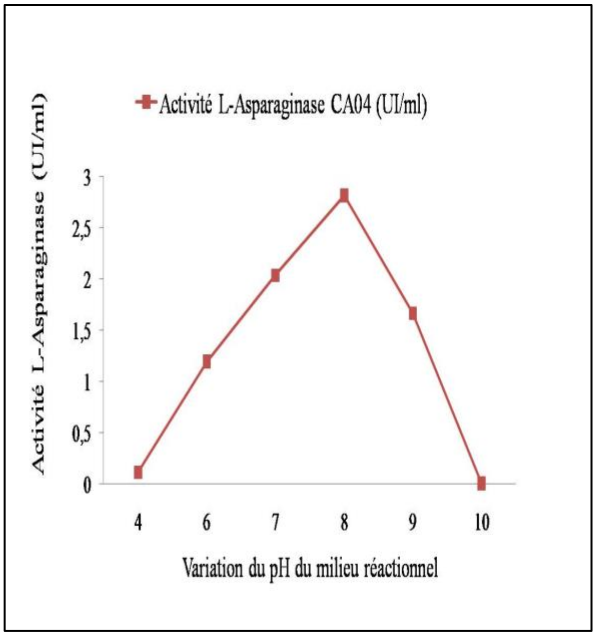

Figure 06: Effect of pH variation in the L-asparaginase activity.

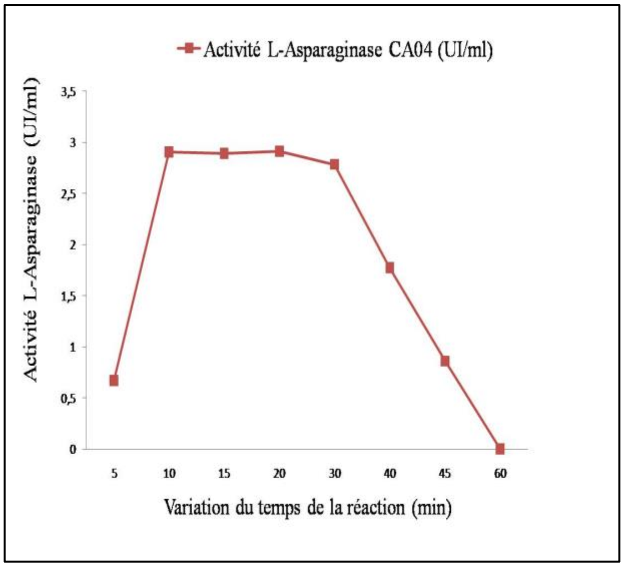

Figure 07: Effect of reaction time in the L-asparaginase activity.

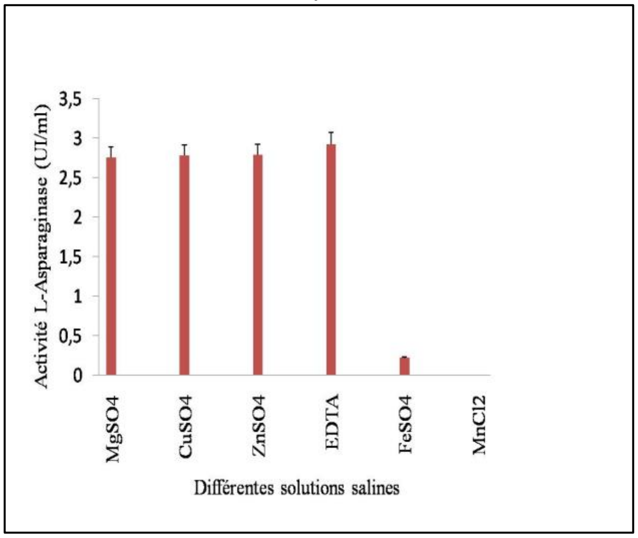

Figure 08: Effect of some co factors in the L-asparaginase activity.

In the other hand, the enzymes produced by CA04 have low hydrolytic activities with other substrates: L-Glutamine and l-aspartic acid, with relative activities of 3.24% against L-Glutamine and 0.43% against L-Aspartic acid. These results demonstrate that the enzymes produced have a high affinity for L-Asparagine, the usual substrate and very low affinities with respect to other substrates.

Table 1 : Summary of the affinity for the substrates used.

Substrates	L-Asparagine (IU/mL)	Relative activity (%)	L-Glutamine (IU/mL)	Relative activity (%)	L-Aspartic Acid (IU/mL)	Relative activity (%)
L-asparaginase of *CA04.*	**2,78**	**100**	**0,09**	**3,24%**	**0,012**	**0,43%**

Discussion

In order to find new interesting molecules of L-asparaginase, we started a research track on L-asparaginase produced by a *Streptomyces hydrogenans* CA04 strain newly isolated from Lake Agulmim at high altitude in Algeria.

This site has never been explored for similar scientific studies before. To do this, we isolated and identified the *CA04* strain and then demonstrated, extracted and partially purified the enzyme L-asparaginase. Finally, we proceeded to its physicochemical characterization. After the demonstration of the enzymatic activity sought, we proceed to its extraction. The activity was found at 90% saturation with ammonium sulphate.

Dharmaraj et al. [30] also concluded that the maximum of L-asparaginase activity produced by Actinomycetes strain, is localized to ammonium sulphate contents of 80% ; and detected a maximum of L-asparaginase activity produced by *Streptomyces sp*., at 80% ammonium sulphate. There is nothing to prevent that different salt concentrations may be advantageous, to result in a better extraction yield, where El-Sabbagh et al. [31] demonstrated that the best L-asparaginase activity is 70% ammonium sulphate. Lower concentrations of ammonium sulphate are used in some cases for the saline precipitation extraction of L-asparaginase. According to Basha et al. [14], the enzyme was extracted at only 45% salt saturation. An ammonium sulphate concentration range of from 40% to 60% has also been used by Amena et al. [20], when extracting L-asparaginase produced by an actinomycete strain, isolated from the marine environment. On the other hand, it was possible to carry out partial purification with only ammonium sulphate precipitation followed by dialysis of an L-asparaginase produced by *Streptomyces sp.* TA22, isolated in India, by the team of Mohana [25]. This proves that ammonium sulphate is a suitable salt for the extraction, by salting-out, of L-asparaginase, produced by the actinomycetes. The recovered fractions were dialyzed against 0.05M Tris/HCl buffer, pH8.4, and the dialysates were eluted along a low pressure chromatography column. Separation, molecular exclusion type, on Sephacryl S-200 gel, demonstrated the existence of two enzyme isoforms at respective MWs of 86Kda and 108Kda, confirmed by electrophoretic control [32].

On the other hand, the L-asparaginase currently used comes from the two bacteria *E. Coli* and *Erwinia*, where two isoenzymes in these two bacterial genera were found to be close to 100 Kda [33]. Borah et al. [34] purified an L-asparaginase having a MW of 153 Kda, produced by a strain of *E. coli* Isolated from the water. Other L-asparaginase with MW close to the isoforms that we obtained, were found in *Streptomyces sp.* PDK2, *S. albidoflavus* and *S. gulbargensis* with MW of 140, 110 and 85 kDa, respectively [35]. However, very large molecules have also been reported in *Chalmydomonas sp.* [36] with a MW of 275 kDa for purified L-asparaginase. In contrast, some smaller L-asparaginase molecules were recorded, such as the enzyme produced by a strain of *Bacillus*, with a PM of 45 Kda, reported in the work of Moorthy et al. [37]. Finally, it has been reported that, even after the discovery of bacterial L-asparaginase, different isoforms have been found in different strains of *E. coli*, with MWs ranging from 133 to 141 Kda [38], and according to Whelan et al. [39], all these isoforms were composed of 29Kda for each subunit (4 subunits).

On the other hand, the same approach for the partial identification of L-asparaginase produced by our strain has been adopted in previous, where molecular exclusion chromatography is often chosen as a means of identifying protein species present in the crude extracts of L-asparaginase or as partial purification.

Sivasankar et al. [40] have also localized a maximum of L-asparaginase activity produced by *Streptomyces sp.*, from precipitates obtained with 80% saturation with ammonium sulphate, followed by dialysis and partial purification by molecular exclusion chromatography on Sephadex G-100. El-Sabbagh et al. [31] identified an L-asparaginase produced by a strain of *Streptomyces halstedii*, isolated from Egyptian soil and have realized purification procedure in use of SDS-PAGE electrophoretic migration after series of chromatography revealed the existence of a single band of 100KDA.

After performing partial purification of the *CA04* enzymes, we detected a specific activity of 2.31 IU/mg protein for the best A_4 detected isoform, equivalent to a protein content of 1.42 mg/mL. Often, large variations are observed from one microorganism to another, in terms of amount of enzyme, in addition, the protein level is not dependent on the microbial group or the species, because Patro et al. [41] obtained a protein level very low to our result, with 0.4 mg/ml, during the purification of L-asparaginase produced by a mold strain of the genus *Penicillium*, using a chromatography column on Sephadex G-100. All the same, Basha et al. [16] obtained a much greater total enzyme activity than we obtained (3.28 IU/ml), with 49.2 IU/ml, yet the yield of protein was 21 times lower than ours, with 0.065mg/ml. This shows that the protein level alone does not influence the activity of the enzyme, but the affinity to the substrate is to be considered.

Finally, we obtained an L-asparaginase activity with a high specificity to its L-Asparagine substrate, expressing very weak activities with L-Glutamine (3.24%) and L-Aspartic acid (0.43%). This result shows that the L-asparaginase activity produced by our strain is targeted, and in consequence it hasn't toxicities caused by abolish of vital proteins rich in L-Glutamin [42].

For this, the enzyme produced by our strain may be less toxic to the body, given the low relative activity on L-Glutamine. Ashok et al. [43] have further specified that in any case certain L-asparaginase molecules currently used in cancer chemotherapy, in particular with regard to lymphomas, have low Glutaminase activities. According to Broome et al. [44], the toxicity is rarely due to the hydrolysis of L-Asparagine in normal cells, since the latter have resistance to L-asparaginase, in addition to the cancer cells are sensitive to enzyme attack at higher L-Asparagine concentrations than normal cells.

The notion of affinity of microbial L-asparaginase to differents substrates is discussed by researchers in the field, so according to Warangkar and Khobragade, [6], the microbial hope as well as its natural growth environment, play an important role in the affinity of the enzyme produced, against the different substrates. But most of the molecules currently used in cancer therapy have almost zero L-Glutaminase activities, with 0% for the enzyme isolated from *pig* serum, 2% for the isoforme produced by *E. coli*, 5% for *Serratia* enzyme and 10% for *Erwinia* enzyme [43]. But we think that immunological reaction against L-asparaginase which currently used in chemotherapy is one of the principle problems that we must take under consideration in search of new molecules.

Conclusion

We concluded that the *Streptomyces hydrogenans* CA04 strain isolated from the surface water in Lake of Agulmim (Djurdjura Mountains – Algeria) is the best source of anti leukemic L-asparaginase enzyme where it produces two extracellular isoforms. In perspective we'll complete procedure of purification by using HPLC and mass spectrometry identification, and in the other hand we plan to test the studied molecule against cancer cells in culture followed by his toxicological study.

Conflicts of interest

Authors do not declare any conflict of interest.

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