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Enhanced L-lysine Production by UV-irradiated and S-2-aminoethyl-L-cysteine Resistant Mutants Derived from *Bacillus species* Using Agricultural Products as Carbon and Nitrogen Sources

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

L-Lysine is essential for human and animal nutrition and may be added to food and feed materials to improve the protein quality. It is utilized in human medicine, in cosmetics and in the pharmaceutical industry, particularly in the formulation of diets with balanced amino acid

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concentration and in amino acid infusions. Enhanced L-lysine production by UV irradiated and S-2-aminoethyl-L-cysteine resistant (AEC^R) mutants derived from *Bacillus* species, using agricultural products as carbon and nitrogen sources was studied. The L-lysine producing bacteria had already been isolated from Nigerian soil. They were purified and identified as *B. subtilis* PR13, *B. subtilis* PR9 and *B. pumilus* SS16, using cultural and biochemical characteristics. UV irradiated mutants were obtained by exposing the parent strains which include *B. subtilis* PR13, *B. subtilis* PR9 and *B. pumilus* SS16, to UV light while, AEC^R mutants were derived by exposing the parent strains to various concentrations of AEC. The mutants were screened for L-lysine production by cultivating in fermentation media. The results obtained showed that, UV mutants PRMU-8 and PRMU-14 derived from *Bacillus subtilis* PR13 accumulated higher lysine yield of 2.47 and 2.55 mg/ml compared to the wild type. Also, UV mutant MUPR-4 derived from *Bacillus subtilis* PR9 recorded a higher lysine yield of 1.77 mg/ml while, enhanced lysine yields of 1.96 and 2.34 mg/ml were observed in UV mutants SSMU-4 and SSMU-9 respectively. AEC^R mutant PRT-2 derived from *Bacillus subtilis* PR13, accumulated higher lysine yield of 2.35 mg/ml compared to the wild type. Enhanced lysine production of 1.47 and 1.32 mg/ml was observed in mutants ART-5 and ART-8 derived from *B. subtilis PR9* while, mutant SRT-4 derived from *B. pumilus SS16* recorded enhanced lysine accumulation of 2.18 mg/ml. The results obtained in the study showed that L-lysine production by some UV irradiated and AEC^R mutants was enhanced.

Keywords: Bacillus species; L-lysine; UV irradiation; AEC; mutants.

1. INTRODUCTION

"Amino acids are major industrial products derived by fermentation, covering a world market of more than 5 million tons per year. As the building blocks of life, amino acids have long played an important role in both human and animal nutrition and health maintenance" [1]. Among the amino acids is the L-lysine, that is one of the leading biotechnological products and the global L-lysine production is estimated to reach 3.0 million tons in 2022. This corresponds to 5.6 billion USD of market value according to the current L-lysine market report [2], with a current production of 2.2 tons per year [3]. "L-Lysine is essential for human and animal nutrition. In addition, it has pharmaceutical applications both in the formulation of diets with a balanced amino acid composition and in the infusion of amino acids. Lysine supports bone health by ensuring adequate absorption of calcium and therefore prevents osteoporosis. L-Lysine cannot be synthesized biologically in the body and its breakdown is irreversible" [4], but "may be added to food and feed materials to improve the protein quality" [5]. "Children and growing animals have a high requirement of lysine, since it is needed for bone formation" [4].

"Lysine can be produced in different ways, including chemical synthesis, extracting from protein hydrolyzate, enzymatic method, fermentation method, protoplast fusion technique and recombinant DNA technology" [6,7]. "Among these methods, fermentation is the most economical and practical means of producing lysine, as in this method, low temperature, low pressure and low-cost carbon sources are used and a biological form of lysine (L-lysine) is produced" [8]. L-lysine commercial form requires different downstream processing to achieve the degree of purity.

L-Lysine is being produced on industrial scale using *Corynebacterium glutamicum*, species of *Arthrobacter, Bacillus* and *Brevibacterium* as fermenting agent [9,10]. High yielding strains have also been developed from *Bacillus subtilis* and *Escherichia coli* [11].

Due to increasing market demand and price competition, extensive research has been made in order to improve the fermentation process, not only from the point of lowering production costs, but also of increasing productivity. The lysine industry has shown consistency in developing efficient and better microbial strains either through classical mutagenesis or by applying modern biotechnology tools.These approaches are expected to improve the economics of fermentation process [12].

"A great variety of microorganisms, including auxotrophic as well as regulatory mutants, has been reported to over-produce lysine" [13]. "The selection of such mutants has led to isolation of high producers, which are used for industrial production of lysine, glutamic acid, threonine and a variety of other amino acids" [14].

We had isolated three *Bacillus* species (which included *Bacillus subtilis* PR13, *B. subtilis* PR9, and *B. pumilus* SS16) from Nigerian soil, which produced various yields of L-lysine [15]. In another study, the *Bacillus* species were used for L-lysine production using carbohydrates as carbon and seed meals as nitrogen sources respectively [16]. The present study was aimed at enhancing L-lysine production by UV irradiated and AEC derived mutants of *Bacillus* species using agricultural products as carbon and nitrogen s ources.

2. MATERIALS AND METHODS

2.1 Microorganisms

B. subtilis PR13, *B. subtilis* PR9 and *B. pumilus* SS16 isolated from different soil in Awka town, were used in the study. They were purified and Identified as *B. subtilis* PR13, *B. subtilis* PR9 and *B. pumilus* SS16 using cultural and biochemical characteristics. The *Bacillus* species were grown on nutrient agar slants for 24 h at 37 ^{o}C. Thereafter, the cultures were then preserved at 4^{o}C and transferred to new slants after 30 days in order to keep them viable for use in L-lysine production.

2.2 Inoculum Preparation

Two (2) loopfuls of *B. subtilis* PR13, *B. subtilis* PR9 and *B. pumilus* SS16 were inoculated in Erlenmeyer flasks containing sterile 50 ml of seed medium. The seed medium consisted of peptone, 10.0g; yeast extract, 10.0 g; NaCl, 5.0 g; water, 1litre; pH adjusted to 7.2. The inoculated flasks were incubated for 24 h on a rotary shaker at 120 rpm and 30 ^{o}C. Duplicate flasks were used.

2.3 Preparation of Fermentation Media

The submerged production of L-lysine by *B. subtilis* PR13, *B. subtilis* PR9 and *B. pumilus* SS16 was conducted in three different fermentation media, namely fermentation medium 1,2 and 3 (FM1, FM2 and FM3 respectively). For *Bacillus subtilis* PR 13, L-lysine production was carried out in 100 ml Erlenmeyer flasks, containing FM1. The medium, was composed of KH_2PO_4, 0.5g; K_2HPO_4, 0.5g; $MgSO_4.7H_2O$, 0.001g; $MnSO_4.H_2O$, 0.001g; $FeSO_4.7HO$, 0.001g; $CaCO_3$, 50g, the carbon source (glucose) was replaced with millet starch hydrolysate 60g; the nitrogen source (ammonium sulphate) was replaced by soyabean meal 40g; water, 1 litre; pH adjusted to 7.2. For *B. subtilis* PR 9 and *B. pumilus* SS16, the FM2 and FM3 media were used for L-lysine production respectively. FM2 and FM3 were similar to FM1, except that for FM2, the carbon source was replaced with sorghum starch hydrolysates 60g, the nitrogen source was replaced by deffated peanut meal 40g, while for FM3 the carbon source was replaced with sorghum hydrolysates 60g, the nitrogen source was replaced by deffated soyabean meal 20g. The carbon source substrates were prepared in the laboratory using the method of Umerie et al. [17].

2.4 UV Irradiation and Mutant Selection

Two (2) loopfuls of a 24 h culture of *B. subtilis* PR13, *B. subtilis* PR9 and *B.* pumilus SS16 were used to inoculate a 250ml Erlenmeyer flask containing 50ml of nutrient broth medium. The flask was incubated for 24 h on a rotary shaker at 120 rpm and 30 ^{0}C. One milliliter of the broth culture was suspended aseptically in 4 ml of phosphate buffer(pH 7.2) contained in sterile glass petri dishes of 100 mm diameter. The UV light exposure was carried out in a cabinet fitted with UV lamp (253.7nm). The exposure was carried out at a distance of 20 cm and the exposure times were 0, 2, 4, 6, 8,10 and 12 mins. Each UV exposed cell suspension was stored in dark for 10 mins to avoid photo-reactivation (getting revertants), then 0.1ml of the irradiated cells was inoculated on Dextrose Peptone agar (DPA) plates for growth and colonies which appeared after 24 h incubation at 30^{0}C, were subsequently plated out on minimal agar medium. Mutant strains which showed no growth on the minimal medium were selected and stored on Nutrient agar slants at 4^{0}C.The mutant strains were screened for lysine production.

2.5 Screening of UV Irradiated Mutant Strains for Lysine Production

Mutants derived after UV mutagenesis were screened for lysine accumulation. Fermentation was carried out in 100 ml Erlenmeyer flasks containing the various fermentation media (FM1, FM2 and FM3). The preparation of the media and inocula were as was previously described. Two (2) milliliters volume of 24 h cultures of *B. subtilis* PR13, *B. subtilis* PR9 and *B.* pumilus SS16 were inoculated into the fermentation media. Uninoculated flasks served as control. The flasks were placed on a rotary shaker (at

160 rpm) and incubated at 30^{o}C for 72 h. Following the termination of fermentation, the fermentation medium were subjected to centrifugation at 5,000 rpm for 15 min to obtain the cell free supernatant which is the crude L-lysine. The cell free supernatant was used for the determination of lysine. The experiments were conducted in triplicate.

2.6 Derivation of S-2-amino Ethyl Cysteine (AEC) Resistant Mutants

The method of Tosaka et al. [18] was used for the derivation of S-2-aminoethyl cysteine mutants.Two loopfuls of 24 h culture of *B. subtilis* PR13, *B. subtilis* PR9 and *B. pumilus* SS16 were grown in an Erlenmeyer flasks containing 20 ml of the seed medium which consisted of peptone, 10.0g; yeast extract, 10.0 g; NaCl, 5.0 g; water, 1litre; pH 7.2.The flasks were incubated at 30 ^{0}C on a rotary shaker at 120 rpm for 24 h. A 5 ml portion of the broth culture was centrifuged and the cells washed with 0.5 ml of 0.1M sodium phosphate buffer, before resuspending in equal volume of the buffer. A loopful of the cell suspension was inoculated on minimal agar plates containing various concentrations (0.5, 1.0, 1.5, 2.0 mg/ml) of S-2-aminoethylcysteine. S-2-aminoethylcysteine resistant (AEC^{R}) colonies that appeared after 3 to 4 days of incubation at 30^{0}C were screened for lysine production.

2.7 Screening of S-2-amino-ethyl-cysteine Resistant Mutant for Lysine Production

Mutants were screened for lysine accumulation. Fermentation was carried out in 100 ml Erlenmeyer flasks containing the various fermentation media (FM1, FM2 and FM3). The preparation of the media and inocula were as was previously described. Two (2) milliliters volume of a 24 h cultures of *B. subtilis* PR13, *B. subtilis* PR9 and *B. pumilus* SS16 were inoculated into the fermentation media. Uninoculated flasks served as control. The flasks were placed on a rotary shaker (at 160 rpm) and incubated at 30 ^{o}C for 72 h. Following the termination of fermentation, the fermentation medium were subjected to centrifugation at 5,000 rpm for 15 min to obtain the cell free supernatant which is the crude L- lysine. The cell free supernatant was used for the determination of lysine. The experiments were conducted in triplicate.

3. RESULTS

3.1 L- lysine Production by UV Irradiated Mutants

Result of L-lysine production by UV irradiated mutants derived from *B. subtilis* PR13, *B. subtilis* PR9 and *B. pumilus* SS16 is shown in Table 1. Four UV irradiated mutants, were derived from the wild type of *B. subtilis* PR13, one was derived from *B. subtilis* PR9 and three was obtained from *B. pumilus* SS16 (Table 1). Mutants PRMU-8 and PRMU-14 derived from *Bacillus subtilis* PR13 accumulated higher lysine yield of 2.47 and 2.55 mg/ml compared to the wild type (used as control), which recorded 2.13 mg/ml. The mutants PRMU-2 and PRMU-15 recorded reduced lysine yield compared to the wild type. Mutant MUPR- 4 derived from *Bacillus subtilis* PR9 recorded the highest lysine yield of 1.77 mg/ml compared to the wild type which recorded 1.28 mg/ml. Enhanced lysine accumulation of 1.96 and 2.34 mg/ml was observed in mutants SSMU-4 and SSMU-9 derived from *Bacillus pumilus* SS16, compared to the wild type which recorded 1.75mg/ml. Mutant SSMU-11 recorded reduced lysine yield of 1.27mg/ml more than the wild type.

3.2 L- lysine Production by S-2-Amino-ethyl-L-cysteine Resistant (AEC^{R}) Mutants

Table 2 shows the result of L- lysine production by S-2-Amino-ethyl-L-cysteine resistant (AEC^{R}) mutants derived from *B. subtilis* PR13, *B. subtilis* PR9 and *B. pumilus* SS16**.** One AEC^{R} mutants were derived from the wild type of *B. subtilis* PR13, three were derived from *B. subtilis* PR9 and one was *B. pumilus* SS16 respectively. AEC^{R} mutant PRT-2 derived from *Bacillus subtilis* PR13, accumulated higher lysine yield of 2.35 mg/ml compared to the wild type which recorded 2.13mg/ml. Enhanced lysine production of 1.47 and 1.32 mg/ml was observed in mutants ART-5 and ART-8 derived from *Bacillus subtilis* PR9 compared to the wild type which recorded 1.28mg/ml. Mutant SRT-4 derived from *Bacillus pumilus* SS16 recorded enhanced lysine accumulation of 2.18 mg/ml compared to the wild type which recorded 1.75 mg/ml.

Table 1. L- lysine production by UV irradiated mutants derived from *Bacillus subtilis* and *B. pumilus*

Wild type	UV irradiated mutant	Treatment time (min)	Lysine (mg/ml)
Bacillus subtilis PR13	PRMU – 2	10	1.83
	PRMU – 8	10	2.47
	PRMU – 14	12	2.55
	PRMU – 15	12	1.74
	Control (control)		2.13
Bacillus subtilis PR9	MUPR-4	8	1.77
	Control		1.28
Bacillus pumilus SSI6	SSMU-4	10	1.96
	SSMU-9	10	2.34
	SSMU-11	8	1.27
	Control		1.75

Table 2. L- lysine production by S-2-Amino-ethyl-L-cysteine resistant (AEC^R) mutants derived from *Bacillus subtilis* and *B. pumilus*

Wild type	AEC^R mutant	Lysine (mg/ml)
Bacillus subtilis PR 13	PRT– 2	2.35
	Control(wild type)	2.13
Bacillus subtilis PR9	ART-5	1.47
	ART-7	0.91
	ART-8	1.32
	Control	1.28
Bacillus pumilus SS16	SRT-4	2.18
	Control	1.75

4. DISCUSSION

Mutant strains of the organisms derived after UV light treatment were found to produce lysine in higher concentrations than the wild type (parent strain). This is supported by the findings of other researchers. Shah et al. [4] reported an improved lysine production from mutants developed after UV treatment of *Corynebacterium glutamicum,* with the most potent mutants producing 38, 33 and 28.5g/l lysine, in the media containing glucose, molasses and starch hydrolysate. Ekwealor and Obeta [19], in a study of "the effects of UV irradiation on lysine production by *Bacillus megaterium*, observed that mutant MR-10, and MR-25 derived from *Bacillus megaterium* SP 76 and MS-3 and MS-5 derived from *Bacillus megaterium* SP 86 produced higher lysine levels than the parent strains". Sharma [20], reported a methionine concentration of 4g/l using UV mutants.

Strain improvement by classical mutagenesis techniques is well established and widely used to isolate amino acid over producers [21,20].The mutation approach has become the most extensively used tool for industrial organisms [22,23]. Mutagenesis is a phenomenon by which changes can be introduced in the metabolic process. The most direct and general method for over production is the genetic removal of the feed back control [4]. Different mutagens can be used for the desirable changes in the genetics of the strains of interest [24]. UV light has been recommended as mutagen of the first choice. The ratio of mutation is usually very high and UV light is a relatively safe mutagen for the experimenters [25].

The result from the study showed increased lysine production from some AEC^R mutants. Ekwealor and Obeta [19], observed that AEC resistant mutants derived from *B. megaterium* SP14 and *B. megaterium* sp 76 , were found to accumulate higher lysine yields. Odunfa et al. [26], reported the lysine production by AEC resistant *lactobacillus* and yeast isolates. The result showed that 42.5% of *Lactobacillus* and 83.3% of the yeast isolates tested, were capable of lysine production. Siripoke et al. [27], reported the selection of AEC^R *Bacillus* SWU41, a *Bacillus* resistant to a toxic lysine analogue (AEC), which produced 3.68g/l of lysine (about 6 times of the prototroph) in the medium containing 5% glucose. Nadeem et al. [28], observed lysine production of 0.1-0.5g/l by mutant of *Escherichia*

coli, which showed resistance against AEC a lysine analogue. Though they noted that resistance to AEC does not necessarily mean the overproduction of lysine as seen in their results. Sano and Shiio [29], developed AEC resistant mutant of *Bacillus subtilis, Brevibacterium flavum* and *Escherichia coli*. Among them, *B. flavum* mutant resistant to the growth inhibition of AEC plus threonine was the best, producing 32g L-lysine for 100g glucose. Hilliger and Prauser [30], reported that AEC resistant mutants of *Oerskonia* accumulated up to 10g/l of lysine. Yakoto and Shiio [31], described 40g/l of L-lysine production in 10% glucose medium by threonine negative mutant of AEC resistant *Brevibacterium flavum.* [32] noted that "some mutants resistant to amino acid analogues are suitable as amino acid producers. The enhanced lysine production by AEC resistant mutants is likely due to the mechanism of control operative in the biosynthesis of lysine in the parent strains being interfered with in the mutants, thus making them over producers". This view is supported by the findings of [33-35]. These researchers working with *Arthrobacter globiformis* and *Bacillus stearothermophilus*, noted the improved lysine yields of AEC resistant mutants of these organisms. Analogue-resistant-mutant isolation is developed for the purpose of overproduction of amino acid and it has yielded higher production of arginine, lysine, tryptophan threonine etc utilizing different bacterial strains [36]. Amino acid analogues can effectively function as true feed-back inhibitors without participating in functions in the cell [37,38]. Mutants resistant to lysine analogues have altered and deregulated enzymes that are not sensitive to feedback inhibition and repression [39] however suggested that apart from feed-back inhibition and side reaction metabolic interlocks regulate the synthesis of amino acid. He opined that by the release of this regulation the productivity of amino acid increases.

5. CONCLUSION

In the study, it was observed that some UV irradiated mutants stimulated enhanced lysine production, with mutant PRMU-8 accumulating the highest lysine yield of 2.47 mg/ml. Also, some of the AEC^R mutants produced enhanced yield of L-lysine, with mutant PRT– 2 accumulating the highest yield of 2.35mg/ml . The study showed that UV irradiated and AEC^R mutants can produce enhanced lysine yield. This development indicates that large scale L-lysine production is feasible in Nigeria and it will help to meet present-day needs in its industrial sector.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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