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Optimization of Process Parameters for Erythromycin Production Under Solid State Fermentation by *Saccharopolyspora erythraea* NCIMB 12462

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Solid-state fermentation (SSF) was conducted on laboratory scale in 250 ml Erlenmeyer flask at 30°C for erythromycin production by *Saccharopolyspora erythraea* NCIMB 12462. Several agro-industrial wastes were selected and screened in order to determine the most suitable one for the production of erythromycin by the above mentioned microorganism. Of all the substrates evaluated, sugarcane bagasse was found above mentioned to be the best. Water was the best eluent for the extraction of the antibiotic from the solid-state culture compared with other organic solvents yielded about 197.91±3.11 µg/g solid .The cultural conditions were optimized such as inoculum size of 4 ml vegetative culture supported a maximum erythromycin production of 197.97±15.52 µg erythromycin /g solid, moisture content of 85% culture resulted in a maximum production of 308.±48.2 µg/g . Furthermore , the weight of sugarcane bagasse of 2.5 g gave 414±32.5 µg/g solid. After medium optimization the production was enhanced to 416.41±6.55 µg /g solid. Through the incubation time, erythromycin detection started on the 3rd day and attained its maximum level of 600.65±47.1 µg/g solid on the 10th day.

Key words: Solid state fermentation, Erythromycin, Sugarcane bagasse, *Saccharopolyspora erythraea* NCIMB 12462.

Erythromycin is a 14-carbon macrolide antibiotic produced by a soil inhabiting actinomycete *Saccharopolyspora erythraea* (formerly *Streptomyces erythreus*), which was used for treatment of many infectious diseases caused by some bacteria . Erythromycin has also been recommended as an alternative in patients who are allergic to penicillin or in cases of penicillin failure. In turn, erythromycin is the starting material for second and third generation semi-synthetic derivatives. Recently, erythromycin and its semi-synthetic derivatives are widely used in medicine[1]. Many investigators have attempted to optimize submerged cultures for erythromycin production from *Saccharopolyspora erythraea* [2] .The production of antibiotics by solid state fermentation (SSF) has gained much attention in biotechnology studies for production of cephamycin, oxytetracycline, iturin, neomycin, cephalosporin C , penicillin , and rifamycins [3] . Since culture conditions affect the kind and quantity of antibiotic production[4, 5], It is possible to increase antibiotic production through nutrient optimization using (SSF) [6]. Optimization of solid fermentation media is generally done at a two-step

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level, where initially the effects of the nutrients on product formation are screened and a few parameters are selected and optimized [7]. SSF compared to SmF is more simple, requires lower capital, has superior productivity, reduced energy requirement, simpler fermentation media and absence of rigorous control of fermentation parameters, uses less water and produces lower wastewater, has easier control of bacterial contamination, and requires low cost for downstream processing [8] . Some of the common substrates used in solid state fermentation are wheat bran, rice and rice straw, hay, fruit and vegetable waste, paper pulp, bagasse, coconut coir, and synthetic media [9]. In order to curtail the production cost, one should use inexpensive substrates and follow an efficient fermentation process as solid state fermentation (SSF), which features by higher productivity with better exploitation of agro residues as substrates to achieve the economic viability of these otherwise waste resources as well as safeguard the environment [10]. There are very few reports on the production of erythromycin by SSF. The objective of the present study was to investigate the effect of process parameters on erythromycin production in an impregnated support SSF, growing *Saccharopolyspora erythraea* NCIMB 12462 on sugarcane bagasse.

MATERIALS AND METHODS

Culture media and fermentation conditions

Saccharopolyspora erythraea NCIMB 12462 provided by the National Collection of Industrial and Marine Bacteria, Limited, Aberdeen, Scotland, UK **,** was used in the present study and it was maintained on slopes containing starch-nitrate agar medium composed of (g/l): starch, 20; $NaNO_3$, 2; K_2HPO_4, 0.5; $MgSO_4.7H_2O$, 0.5 and agar 20 [11] The pH was adjusted to 7.2 and sterilized at 121°C for 20 min. Subculturing was carried out once in 2 weeks and the cultures slants were stored at 4°C. The spore suspension was prepared from a 10 days old culture by adding 10 ml of sterile saline solution. This spore suspension (containing 1×10^8 spores/ml) was used as an inoculum for submerged fermentation as well as for solid state fermentation. Starch-nitrate broth contained the same ingredients was used for vegetative inoculum culture by transferring a loopful of 10 days old seed culture into 50 ml medium in a 250 ml Erlenmeyer flask and incubated on a rotary shaker at 200 rpm for 48 hours at 30°C.

Substrates

Sugarcane bagasse was obtained from a local sugar cane juice company in Cairo, Egypt. It was dried in an oven at 60°C and ground in a hammer mill without any pretreatment. Corn bran, wheat bran, corn flour and wheat germ were obtained from local market. Traditional agro-industrial residues such as potato, sweet potato, banana peel, colcasia, carrot, tangerine residues and date seed were obtained from local shops.

Solid state fermentation

Five grams of each solid substrate were moistened with 10 mL of fermentation media (70% moisture level) into a 250-mL Erlenmeyer flask containing medium composed of (g/l): glucose 25; corn steep liquor 4; $(NH_4)_2SO_4$, 3; $CaCO_3$, 5 and NaCl 1. They were mixed and autoclaved at 121°C for 30 min. The cooled medium was inoculated with 2ml of 48 h old vegetative culture mixed thoroughly with a sterile glass rod for uniform distribution of *Saccharopolyspora erythraea* cells in the medium and then incubated at 30 °C for 10 days. Samples as whole flask in duplicate were withdrawn each 24 h for erythromycin estimation.

Optimization of cultural conditions for erythromycin production in SSF

Factors like selection of different solid substrates, solvent type, inoculum type and size, initial moisture content, optimization of medium contents, solid support weight and incubation period (2 to 20 days) were investigated.

Antibiotic extraction

After incubation of each fermentation process, 20 ml of each solvent system (distilled water; dimethylsulphoxide (DMSO); ethanol; methanol; acetone and distilled water mixed with equal volume of each organic solvent (v/v) were added to each flask and mixed vigorously by shaking in an orbital shaker 150 rpm at room temperature for 1 h. At the end of the extraction time, the resulting extract was centrifuged at 4000 rpm for 10 min to get a clear supernatant which was used as the antibiotic source.

Antibiotic assay

The resulting clear filtrate was used to determine its antibacterial activity by the agar diffusion method [12]. 100µl filtrate fill the agar hole (0.9 mm diameter) punched in the nutrient agar

plates freshly seeded with 0.1 ml of *Bacillus subtilis* NRRL B-543 strain as the test organism. The inhibition zone diameter was measured in cm after incubation of plates at 30°C for 24 h and concentrations were calculated through standard erythromycin (Sigma Aldrich) calibration curve. All experiments were conducted in duplicate and the mean of the three is represented as micrograms of erythromycin produced per gram solid.

RESULTS AND DISCUSSION

Selection of solid substrates

SSF offers a number of advantages over conventional submerged fermentation for antibiotic production [13]. The production medium is often simple, using agro-industrial by-products like wheat bran, rice bran or wheat straw as substrate [14]. Among the factors important and most crucial for microbial growth and activity in a solid-state culture are substrate type, particle size, and moisture level/water activity. Generally, smaller substrate particles would provide a larger surface area for microbial attack. However, too small substrate particles may interfere with microbial respiration and thus result in poor growth [15]. At the same time, larger particles provide better respiration/aeration efficiency because of increased space between particles but provide limited surface for microbial attack. The results presented in (Figure 1) showed that erythromycin production varied with type of the substrate. All the substrates supported erythromycin production with different ratios depending on the nature of these materials. However, among the agricultural substrates sugarcane bagasse supported a maximum yield of 101.82 µg/ g solid. While the other solid materials (rice husk, potato residue, corn bran, corn flour, wheat germ) produced appreciable amount of the antibiotic (60, 59, 49,49 and 37.48 µg/g solid, respectively). Sweet potato residue, date seed and carrot residue produced the lowest erythromycin yield (10, 5 and 1 µg/g solid, respectively).

The observed reduction in erythromycin production with corn flour, sweet potato residue, tangerine residue, carrot residue, banana peel and colcasia residue may be due to the fact that in low moisture content they become agglomerated and it might be possible that at these lower moisture levels, transport of nutrients or supply during idiophase becomes difficult [16]. Moreover, this could be also attributed to the fact that solid materials dual role-supply of nutrients and anchorage to the growing microbial culture which influence the microbial growth and subsequent metabolite production. Penicillin was produced by using *Penicillum chrysogenum* with substrates such as wheat bran of high moisture content (70 %) and sugarcane bagasse. In other report, wheat raw supplemented with cottonseed- de-oiled cake and sunflower cake were used for production of cephamycin C using SSF. Also, wheat raw supplemented with raspberry proved to be optimum for production of neomycin by SSF [17]. On the other hand, wheat bran was the better solid support

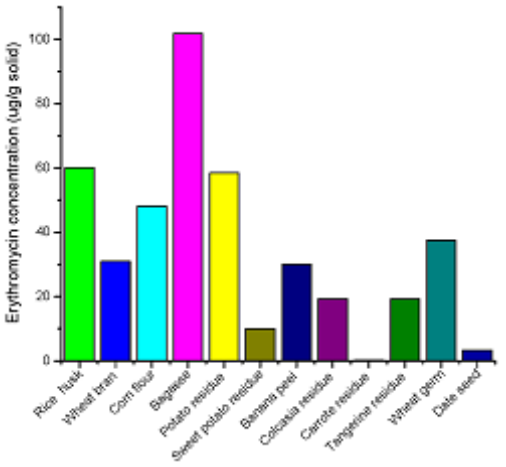

Fig. 1. Effect of different solid substrates on erythromycin production by SSF

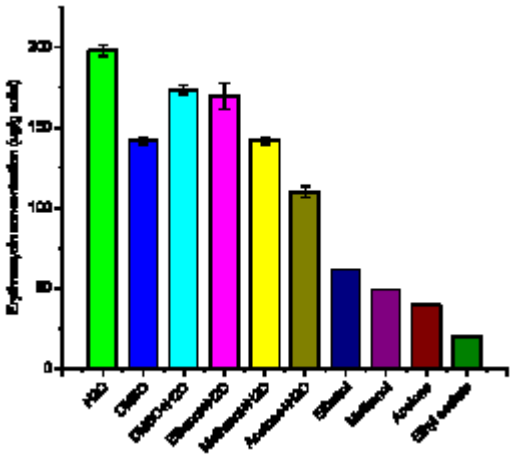

Fig. 2. Effect of different solvent systems on erythromycin extraction from SSF

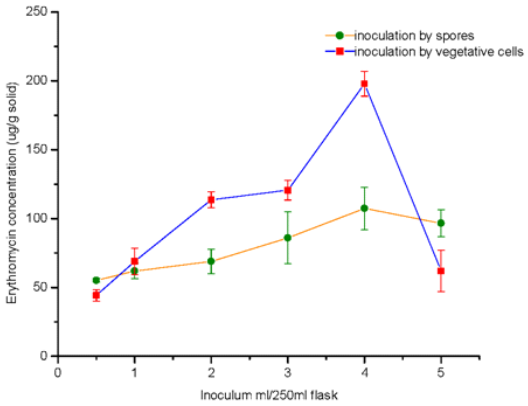

Fig. 3. Effect of different inoculum types and sizes on erythromycin production under SSF

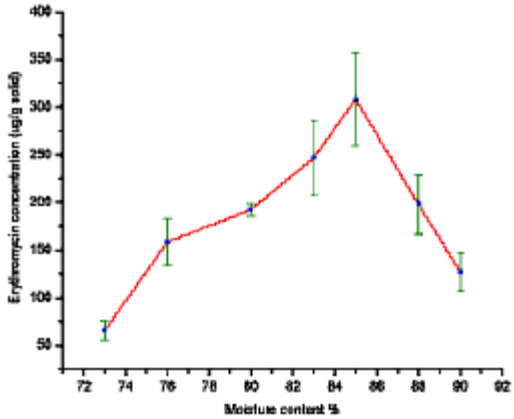

Fig. 4. Effect of different moisture contents on erythromycin production under SSF

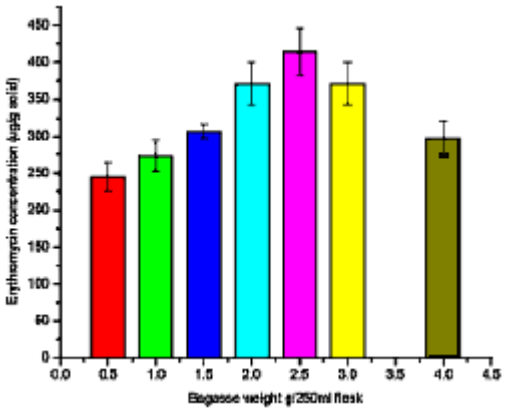

Fig. 5. Effect of different bagase weights on erythromycin production under SSF

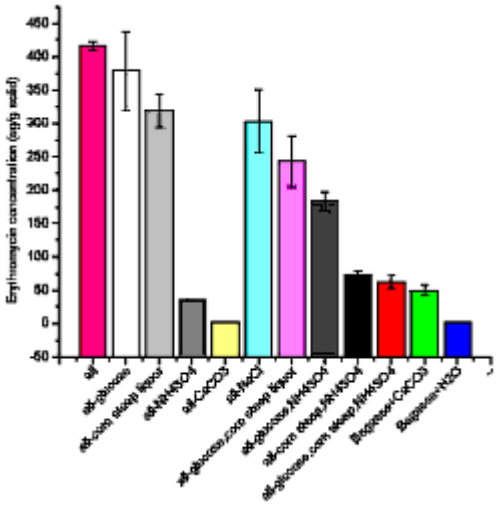

Fig. 6. Effect of media content on erythromycin production

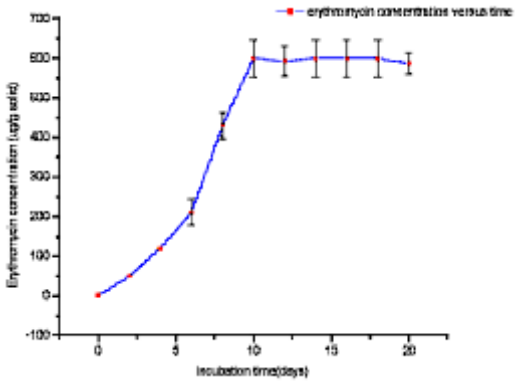

Fig. 7. Effect of different incubation time on erythromycin production

material for the production of neomycin with *Streptomyces marinensis* NUV 5 under SSF [11]. Such substrate dependent microbial product yield variations were also reported in literature [18].

Effect of different solvent systems on erythromycin extraction

The findings obtained from extraction of erythromycin SSF cultures revealed that there was maximum efficiency in extraction of erythromycin using water as an excessive polar inorganic solvent followed by DMSO as organic polar solvent, DMSO: water; ethanol: water; methanol: water and acetone: water (50:50 v/v). Extraction efficiency from SSF is directly influenced by the nature of the

solvent and the nature of erythromycin (Figure 2). Since erythromycin A is both hydrophobic and hydrophilic, and the presence of some inorganic salts in the fermentation medium (NaCl) increase the ionic strength of water (the cheapest solvent of all the tested) which facilitate the extraction of erythromycin from fermentation broth. Erythromycin recovery increased when distilled water was added to all organic solvents used. The low recovery obtained with pure DMSO, ethanol, methanol and acetone could be due to the irreversible retention of erythromycin A to the solid sorbents [19]. It was suggested that water alone works significantly more cheaply, likewise reducing the requirement for solvent and the risk of infection and also reduces waste water pollution and the volume of waste water produced.

Effect of different inoculum types and sizes on erythromycin production

Provision of optimum level of inoculum is also very critical in SSF. It is important to provide an optimum inoculum level in fermentation process [20, 21]. The type of inoculum (spores and vegetative cells) affects the production of secondary metabolites [22] and therefore, this parameter should also be given a proper consideration. The effect of inoculum size on the production of erythromycin was estimated using 2, 3, 4 and 5 ml of vegetative cells / 250ml flask in the production medium. The highest level of erythromycin was produced when the inoculum was in the form of vegetative cells in which the microorganism was able to produce higher erythromycin yield than spore inoculum. By the increment in inoculum size, augmentation in biomass formation was attained hence the antibiotic yield was increased. However, the yield was reduced at lower inoculum size and was found to be inadequate for antibiotic production. Maximum yield of erythromycin (197.97±15.52 µg/g solid) was achieved when 4ml vegetative inoculum was used compared to 4ml spore inoculum which gave 113.66±8.9 µg /g solid (Figure 3).

Our experiments proved an agreement with those reported by Grag and Neelakantan (1981)[23] who proved that the size of inoculum may be an important factor in microbial fermentations. A low inoculum density may give insufficient biomass causing reduced product formation where as higher inoculum than optimum may produce too much biomass and may deplete the nutrients necessary for secondary metabolite production[24]. Adequate inoculum can initiate fast mycelial growth and product formation thereby reducing the growth of contaminants [25]. A decrease in antibiotic production was observed when the inoculums size was increased beyond the optimum level. Antibiotic production attains its peak when sufficient nutrients are available to the biomass. Conditions with a misbalance between nutrients and proliferating biomass result in decreased antibiotic synthesis [20].

Effect of different moisture contents on erythromycin production

The importance of substrate moisture level in SSF for the production and secretion of secondary metabolites has been well established[26]. The moisture content of the medium changes during fermentation as a result of evaporation and metabolic activities and thus optimum moisture level of the substrate is therefore most important[27,28].

The influence of moisture contents on erythromycin was studied by adjusting moisture contents range between 73 -90 %.The results obtained in (Figure 4) clearly revealed that maximum yield of 308.48±48.2 µg/g solid was obtained by 85% moisture after 10 days. Below this value, a gradual decrease in erythromycin yield occur confirmed the fact that low moisture levels decrease the solubility and availability of nutrients, minimize heat exchange and oxygen transfer rates thus lowering the activity of microbial cultures and resulting in reduced productivity [29]. On the other hand, above the optimum value of moisture percent, similar decrease in the yield was obtained. These results were related to the fact that higher substrate moisture in SSF resulted in less productivity because of reduced mass transfer process such as diffusion of solutes and gases to the cells during fermentation process and also reduces the porosity of substrate [30]. At very high moisture level, air present in the void volume is replaced by water, thereby decreasing the available oxygen [31].

Effect of different bagasse weights on erythromycin production

Surface-to-mass ratio of solid substrate was one of the important factors in SSF, as it was directly related to the surface area available for the

growth of cells [32]. From the results obtained it was evident that 2.5 g bagase/250 ml flask gave highest yield of erythromycin 414.4±32.5 µg/g solid (Figure 5). With the higher weights, decreasing of the yield was observed which indicated that too much amount of substrate in a fixed container produced a thicker substrate bed which finally reduced the substrate pore size and reduced the transferring of oxygen in between the substrate particles. It also interferes with the oxygen diffusion in substrate, especially at the basement part of the flask where the nutrients was not fully fermented or utilized. With smaller weights, the surface area for growth was greater but the inter-particle porosity was less. Decreasing in lower and higher weight are two opposing factors probably interacted to give the value corresponding to optimum growth and product formation [33] . Therefore, a weight of 2.5 bagasse / 250ml flask was used for the production of erythromycin by *Saccharopolyspora erythraea* NCIMB 12462.

Effect of different media content on erythromycin production

Nutritional parameters affect highly the production yield and cost [34]. For optimizing erythromycin production, experiments with the removal and supplementation of nutrients based on single–dimension optimization were carried out. As shown in (Figure 6), it was found out the presence of glucose, ammonium sulfate, corn steep and $CaCO_3$ yielded the highest erythromycin production (416.41±6.55 µg/g solid). Elimination of glucose or corn steep liquor from the fermentation media where the other media components were kept constant revealed a mild decrease in erythromycin yield reached 379.12±59.2 and 319.5±25 µg/g solid respectively. Although, corn steep liquor usually included as a nitrogen source, it does contain lactic acid, small amounts of reducing sugars and complex polysaccharides, the absence of glucose could not fulfill the needs of organism to grow and then to produce maximum erythromycin . Increasing the productivity of rifamycin in the presence of glucose as a source of carbon was confirmed in previous report [35].

On the other hand, the elimination of corn steep liquor influence on the quantitative nature of the antibiotic produced [36, 37]. In our study, a high decrease in the yield reached 35.26±55 µg/g solid was observed by the elimination of ammonium sulfate from the fermentation media. It was stated that inorganic ammonium controls the synthesis of the precursors of the antibiotics [38]. Results from previous study pointed out that the inorganic nitrogen sources compared to organic nitrogen sources at the given concentrations of nitrogen, promoted higher antibiotic yield. Nitrogen source as ammonium sulfate was the best with respect to the formation of rapamycin and supported cell growth comparable to the organic nitrogen sources used in the control chemically defined medium [3]. Tetracycline production by different *Streptomyces* strains in SSF was supported by the presence of various inorganic nitrogen sources (1% w/w) such as ammonium chloride, ammonium sulphate, ammonium nitrate [5]. It was also observed that the absence of $CaCO_3$ from the fermentation media inhibit erythromycin production. Earlier studies showed that calcium carbonate can control pH of the fermentation media and considered as the most common buffering agent used to avoid acidic conditions in the fermentation medium [39]. Calcium carbonate has been used as a source of Ca^{+2}. Also it compensates lowering of the pH by consumption of carbon sources and maintained the pH of broth at optimum level for production of erythromycin [40]. Use of calcium carbonate in the antibiotic production media has been reported by many workers. It was also used in the production medium of aristostatins A and B from a *Micromonospora* sp. Similarly luminacins by *Streptomyces* sp., dihydroniphimycin by *S. hygroscopicus* and vinylamycin from a *Streptomyces* sp. was produced when 0.3% $CaCO_3$ was added to the medium [41]. It was seen that, the same inhibition of erythromycin production was detected when sugarcane bagasse and water existed only in the fermentation culture which attribute to the fact that *Saccharopolyspora erythraea* NCIMB 12462 used in the present study may not utilized sugarcane bagasse so it is considered as inert solid support material for the production of erythromycin under SSF. These results was in accordance with some studies which concluded that most of the bioactive compounds are produced by SSF using agro-industrial wastes as substrate, such as sugarcane bagasse or agar were utilized as inert solid supports [33]. Wheat bran was the better solid support material for the production of neomycin and not utilized by *Streptomyces marinensis* NUV 5 under SSF [11].

Similar results reported that wheat bran supported least production of neomycin produced by *Streptomyces fradiae* NCIM 2418 [3].

Effect of different incubation times on erythromycin production

Different incubation periods with optimized parameter (85% substrate moisture level) were employed to study their effect on erythromycin production. Significant variation in erythromycin production was observed during different fermentation periods. Detectable erythromycin yield was attained on day 4 and gradual increased with incubation time passed through a maximum between day 8 and 10 of the experiments reached 600.65±47.1 µg/g solid and later a gradual decrease in antibiotic production was noticed after 12 day. This decrease in the yield may be due to accumulation of end product which hampers erythromycin production or may be due to accumulation of toxic metabolites secreted during fermentation [35].

CONCLUSION

The results of the present study pointed to the feasibility of SSF and agro-industrial residues for erythromycin production. Works on using statistical methods to further improve the yields of erythromycin through SSF are in progress.

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