The present study includes the isolation and identification of some mycoflora from 40 soil samples in six places (Remnants of fat-born, parks, edges of the river, animal wastes, sewage and rubbish) during October 2015–January 2016 in Nasseriya City, Iraq. According to different environmental factors, the isolated genera were Aspergillus, Penicillium, Mucor, Rhizopus, Cladosporium, Sepedonium chrysospermum, Alternaria chlamydospora, Bipolaris, Chrysosporium, Candida albicans, Rhododendron flavum, Humicola, Geotrichum candidum, Fusarium and Acremonium. Three isolation methods were used. Dilution method, direct plate method, alcohol and heat treatment technique using the cultural media viz. Potato Dextrose Agar (PDA), Sabouraud Dextrose Agar (SDA) and Potato Carrots Agar (PCA). Aspergillus represented the highest fungal isolates which represent 62 (37.12%). followed by Penicillium with 47 (28.14%), Mucor 22 (13.16%), Rhizopus 15 (8.98%), Cladosporium 6 (3.59%), Sepedonium chrysospermum and Alternaria chlamydospora, 3 (1.80%), Biopolaris, Chrysosporium and Candida albicans with 2 (1.20%), and finally Rhododendron flavum, Humicola, Acremonium, Fusarium and Geotrichum candidum recorded the lowest fungal isolation with one isolate for each (0.60%). The study was aimed to isolation of Penicillium from soil and assay its antibacterial activity. The results showed that dilution method gave a best fungal growth in comparison with direct plate method and alcohol and heat treatment technique in 25 ºC and pH = 6. PDA appeared as an optimum medium for isolation in comparison with other media such as SDA and PCA. The preliminary results showed that Penicillium sp. exerted antibacterial activity against Gram positive and Gram negative bacteria; Therefore, this fungus was used for production 7 of antibiotic on Potato Dextrose Broth (PDB) medium. The optimum conditions were obtained at pH = 6, incubation temperature of 25 ºC and shaking rate of 180 rpm for 7 days to fermentation. Ethyl acetate was a good organic solvent to extraction of antibiotic which produced (3g/L) as white to creamy crystals. The characterization of the antibiotic product after extraction and purified by chemical methods included Thin Layer Chromatography (TLC) test, Nuclear Magnetic Resonance (NMR) Spectra and Mass Spectra. The Minimum Inhibitory Concentration (MIC) for clinical bacterial species was 1-10mg/ml. The PCR method used in this study utilized Internal transcribed spacer (ITS1-4) as a primer for identification of isolates. Two PCR products of the targeted gene of Penicillium chrysogenum isolates which locally named as (Penicillium chrysogenum F1 and Penicillium chrysogenum F2) were selected and subjected to partial DNA sequencing for the ITS gene to follow up the possible molecular relationship between these local isolates and what recorded globally in Genbank.